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GLUCOSE METABOLISM OF OXIDATIVELY STRESSED HUMAN RED BLOOD CELLS INCUBATED IN PLASMA OR MEDIUM CONTAINING PHYSIOLOGIC CONCENTRATIONS OF LACTATE, PYRUVATE AND ASCORBATE

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Abstract—Red cells suspended in either defined medium or buffered plasma were oxidatively stressed by incubation in the presence of 1,4-naphthoquinone-2-sulfonate at concentrations which caused less than 50% methemoglobin accumulation, stimulation of the hexose monophosphate shunt to less than 15% of capacity, and about a 30% increase in flux through glycolysis. Normal plasma concentrations of lactate and pyruvate in either defined medium or buffered plasma allowed increased contribution of reducing equivalents from glycolysis in response to oxidative stress. Increased utilization of reducing equivalents by the red cell was observed as increased accumulation of pyruvate, whereas accumulation of lactate represented storage of reducing equivalents. Exogenous lactate or pyruvate did not serve as a net electron source or sink since the total content in red cell suspensions of both lactate and pyruvate was increased during exposure to oxidative stress. If exogenous lactate had been used as a net source of reducing equivalents, the lactate concentration would have decreased during incubation of red cell suspensions. Plasma ascorbate or other constituents did not alter the qualitative response of glycolysis to oxidative stress (decreased lactate accumulation, increased pyruvate accumulation, and increased total flux through glycolysis), but plasma constituents did raise significantly the dose of oxidant agent required to elicit a given quantitative response. At levels of oxidative stress likely to be encountered *in vivo*, glycolysis and the hexose monophosphate shunt may be equal in importance as aerobic/antioxidant pathways.

Studies on responses of the red cell to oxidative stress have usually been carried out in defined medium containing a physiologic concentration of glucose. Experimental protocols have often involved exposure of red cells to levels of oxidative stress that overwhelmed cell defenses, resulting in hemoglobin degradation, membrane lipid peroxidation, and cell lysis. Such experiments have led to the view that the hexose monophosphate shunt is the aerobic and/or antioxidant pathway of the red cell. Glycolysis is often viewed as the anaerobic pathway maintaining cellular ATP and NADH, the latter used for the less acute cellular function of methemoglobin reduction.

In a previous paper, we demonstrated that both glycolysis and the hexose monophosphate shunt contributed reducing equivalents to oxygen or other electron sinks in response to mild oxidative stress produced by incubation of red cells with a variety of oxidative agents at concentrations which resulted in moderate methemoglobin accumulation (less than 50% without irreversible loss of functional hemoglobin) and stimulation of the hexose monophosphate shunt (less than 10% of capacity) [1]. The addition to the incubation medium of physiologic (plasma) concentrations of lactate and pyruvate greatly increased the capacity of glycolysis to contribute reducing equivalents in response to oxidative stress. 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 was increased during exposure to oxidative stress. In the presence of oxidative stress, exogenous lactate and pyruvate allowed a shift from accumulation of newly formed lactate to accumulation of newly formed pyruvate with minimal increase in total glycolytic flux (accumulation of lactate plus pyruvate). Increased flow of reducing equivalents through NADH (accumulation of pyruvate) was partially uncoupled from increased turnover of ATP and/or 2,3-diphosphoglycerate (total flux through glycolysis). The addition to the incubation medium of physiologic (plasma) concentrations of ascorbate increased the capacity of the hexose monophosphate shunt to contribute reducing equivalents in response to oxidative stress [1]. The effects of lactate plus pyruvate and ascorbate were superimposable without any apparent inhibition or synergism.

From results of experiments using defined medium we hypothesized that concentrations of lactate, pyruvate and ascorbate present in blood allow increased flow of reducing equivalents from glycolysis in response to oxidative stress coupled to relatively smaller increases in turnover of ATP and/or 2,3-diphosphoglycerate. We further hypothesized that, during mild oxidative stress, glycolysis and the hexose monophosphate shunt are equally important as sources of reducing equivalents in the red cell in

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its physiologic environment. In this paper we present direct tests of these hypotheses using red cells suspended in plasma buffered with glycylglycine. Mild oxidative stress was provided by incubation of red cell suspensions in the presence of 1,4-naphthoquinone-2-sulfonate. 1,4-Naphthoquinone-2-sulfonate is an oxidizing agent which reacts with reducing agents to yield its semiquinone and quinol derivatives and with oxyhemoglobin to generate superoxide, H_2O_2 and methemoglobin [1–3].

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 0.9% NaCl. Particulate material was removed from plasma by centrifugation. Red cell suspensions (25%, v/v) were prepared in either defined medium (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, 5 mM D-glucose, 2 mM L-lactic acid, 0.1 mM sodium pyruvate, 0.1 mM sodium L-ascorbate) or buffered plasma (92% (v/v) plasma, 50 mM glycylglycine). The pH of defined medium or buffered plasma was adjusted with 4 M NaOH so that the final red cell suspension had a pH of 7.35. Stock solutions of ascorbate, lactate and pyruvate were prepared daily and brought to pH 7.4 before addition to defined medium.

Incubation conditions. Incubations were carried out in 10-ml Erlenmeyer flasks containing 1 ml of 25% (v/v) red cell suspensions with or without 1,4-naphthoquinone-2-sulfonate (10^{-4} M or 5×10^{-4} M inclusive of red cell and medium compartments). Flasks were closed with rubber stoppers and 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. In all experiments, the pH of red cell suspensions was unchanged after incubation for 1 hr at 37°.

Assays. Flux through the hexose monophosphate shunt was measured by following $^{14}CO_2$ formation from [U-^{14}C] glucose as described by Trotta *et al.* [4]. U-^{14}C-labeled glucose was used instead of 1-^{14}C-labeled glucose so that the entire flux was measured, including recycling of pentoses through the hexose monophosphate shunt.

Pyruvate and lactate in medium and red cell compartments were measured by techniques described by Beutler [5]. At zero time or after incubation for 1 hr at 37°, 1 ml of 25% (v/v) red cell suspension was thoroughly mixed with 0.75 ml of cold 4% $HClO_4$. The precipitate was removed by centrifugation and the supernatant fraction was kept at 5°. This supernatant fraction contained pyruvate and lactate from both medium and red cell compartments; 0.75 ml of it was mixed with 0.25 ml of 0.4 M K_2CO_3, and the resulting precipitate was allowed to settle at 5°. Pyruvate and lactate were measured using a Cary 14 spectrophotometer to follow the reaction with NADH or NAD catalyzed by lactate dehydrogenase. For the measurement of pyruvate, 0.3 ml of the final supernatant fraction was mixed with 0.7 ml of 0.3 mM NADH in 1 M K_2HPO_4/KH_2PO_4, pH 8.0, in the spectrophotometric cuvette. Lactate dehydrogenase (50 μl, 5 units, Sigma) was added to the sample and reference cuvettes, and the decrease in A_{340} was followed at 25°. For the measurement of lactate, 0.15 ml of the final supernatant fraction was mixed with 0.85 ml of 1.4 mM NAD, 0.73 M NaOH, 0.71 M glycine, 0.29 M hydrazine sulfate, and 3.8 mM disodium EDTA in the spectrophotometric cuvette. Lactate dehydrogenase (50 μl, 50 units, Sigma) was added to the sample and reference cuvettes, and the increase in A_{340} was followed at 25°. The magnitude of ΔA_{340} was proportional to the pyruvate or lactate concentration at zero time or at 1 hr. The rate of accumulation of newly formed lactate or pyruvate was calculated from the change in concentration in 1 hr.

Glucose was measured using Sigma diagnostic kit No. 15–10 (Sigma, St. Louis. MO). Total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus diketogulonate) in red cell suspensions (medium plus red cell compartments) was measured as the 2,4-dinitrophenylhydrazone derivative by the method of Bessey *et al.* [6]. Oxyhemoglobin, methemoglobin and intact hemoglobin (defined as the sum, oxyhemoglobin plus methemoglobin) were measured by a modification of the technique of Harley and Mauer [7] as previously described [8]. No increase of intact hemoglobin was observed in any experiment.

RESULTS

Table 1 shows the concentrations of glucose, lactate, pyruvate and ascorbate in red cell suspensions prepared in defined medium or buffered plasma. These concentrations are the average metabolite concentration over the red cell and the medium compartments. Concentrations of all four metab-

Table 1. Metabolite concentrations and pH in red cell suspensions before experimental incubations*

Red cell suspension (25%, v/v) prepared in:	Glucose (mM)	Lactate (mM)	Pyruvate (mM)	Total ascorbate† (mM)	Methemoglobin (%)	pH
Defined medium	4.49 (2)	1.99 (4)	0.15 (4)	0.09 (2)	1 (3)	7.35
Buffered plasma	3.51 (2)	1.35 (4)	0.11 (4)	0.06 (2)	1 (3)	7.35

* Concentrations of metabolic intermediates inclusive of red cell and medium compartments were measured in 25% (v/v) red suspensions prepared in defined medium or buffered plasma as described in Materials and Methods. Data are reported as the mean with the number of experiments in parentheses.

† Total ascorbate is defined as reduced ascorbate plus dehydroascorbate plus diketogulonate.

Table 2. Effects of 1,4-naphthoquinone-2-sulfonate on methemoglobin formation and on flux through the hexose monophosphate shunt*

Red cell suspension (25%, v/v) prepared in:	Addition to incubation medium	% Methemoblobin after 1 hr at 37°	[μmoles $CO_2 \cdot hr^{-1} \cdot$ (ml red cell)$^{-1}$]
Defined medium	None	1 (3)	0.072 ± 0.011 (4)
Defined medium	1,4-Naphthoquinone-2-sulfonate (10^{-4} M)	33 ± 2 (3)	0.481 ± 0.019 (4)
Buffered plasma	None	1 (3)	0.052 ± 0.005 (4)
Buffered plasma	1,4-Naphthoquinone-2-sulfonate (5×10^{-4} M)	39 ± 3 (3)	0.825 ± 0.120 (4)

* Red cell suspensions (25%, v/v) were incubated for 1 hr at 37° in various incubation media. Hemoglobin analyses were carried out at 1 hr as described in Materials and Methods. The formation of CO_2 in 1 hr was determined as described in Materials and Methods. Data are reported as mean ± S.E. with the number of experiments in parentheses.

olites were slightly lower in suspensions in buffered plasma than in defined medium. In both types of suspension methemoglobin content was between zero and 1%, and the pH was 7.35.

Table 2 shows the effects of 1,4-naphthoquinone-2-sulfonate on methemoglobin formation and flux through the hexose monophosphate shunt. Concentrations of 1,4-naphthoquinone-2-sulfonate were chosen which resulted in moderate methemoglobin accumulation (30–40%) and acceleration of the hexose monophosphate shunt (5–15% of capacity) without irreversible loss of functional hemoglobin. Approximately 5-fold the concentration of 1,4-naphthoquinone-2-sulfonate was required to produce a comparable oxidative stress in red cells suspended in buffered plamsa (10^{-4} M, 2.5×10^{-4}M and 5×10^{-4} M 1,4-naphthoquinone-2-sulfonate resulted in 0, 20 and 39% methemoglobin formation, respectively) as compared to red cells suspended in defined medium (10^{-4} M 1,4-naphthoquinone-2-sulfonate, 33% methemoglobin formation).

The effects of 1,4-naphthoquinone-2-sulfonate on accumulation of products of glycolysis are shown in Table 3. The total flux of glucose through glycolysis (accumulation of newly formed pyruvate plus lactate) was increased about 30% by 1,4-naphthoquinone-2-sulfonate in both defined medium and buffered plasma. In defined medium this increase was caused by a 5-fold increase in the accumulation of newly formed pyruvate coupled with a small decrease in lactate accumulation. In buffered plasma in the absence of oxidative drug total flux of glucose through glycolysis was reflected in lactate accumulation with either no accumulation of pyruvate or reduction of pyruvate (present at zero time) to lactate (shown as a negative accumulation of pyruvate in Table 3). In buffered plasma, the presence of 1,4-naphthoquinone-2-sulfonate resulted in pyruvate accumulation that accounted for about 40% of glycolytic flux coupled with a significant decrease in lactate accumulation.

The relative contributions of cellular sources of reducing equivalents during incubation with 1,4-naphthoquinone-2-sulfonate are shown in Table 4. After incubation for 1 hr at 37°, the total oxidative effect was similar in the two model systems with about 40% greater total flux of reducing equivalents in the buffered plasma system. In both defined medium and buffered plamsa, net heme oxidation accounted for more than 50% of the flux of reducing equivalents. The flux of reducing equivalents from glucose metabolism was split between glycolysis and the hexose monophosphate shunt with a 50% and 36% greater contribution from glycolysis in defined medium and buffered plasma, respectively.

DISCUSSION

Theoretical considerations. 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 turnover 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 [9, 10]. 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. When red cells were exposed to mild oxidative stress produced by incubation with a variety of oxidative agents, it was observed that increased total glycolytic flux with maintained or increased formation of lactate was coupled to methemoglobin reductase activity and accumulation of pyruvate [1]. 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) [1]. 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 was increased by 1 hr at 37°. The import-

Table 3. Effects of 1,4-naphthoquinone-2-sulfonate on flux through glycolysis*

Red cell suspension (25%, v/v) prepared in:	Addition to incubation medium	[μmoles Pyruvate · hr^{-1} · (ml red cell)$^{-1}$]	[μmoles Lactate · hr^{-1} · (ml red cell)$^{-1}$]	[μmoles Pyruvate + lactate · hr^{-1} · (ml red cell)$^{-1}$]
Defined medium	None	0.30 ± 0.06 (4)	2.62 ± 0.31 (4)	2.92 ± 0.35 (4)
Defined medium	1,4-Naphthoquinone-2-sulfonate (10^{-4} M)	1.53 ± 0.11† (4)	2.14 ± 0.50‡ (4)	3.67 ± 0.55§ (4)
Buffered plasma	None	−0.09 ± 0.11 (4)	3.94 ± 0.86 (4)	3.85 ± 0.75 (4)
Buffered plasma	1,4-Naphthoquinone-2-sulfonate (5×10^{-4} M)	2.01 ± 0.15‖ (4)	3.03 ± 0.99‖ (4)	5.04 ± 0.98§ (4)

* Red cell suspensions (25%, v/v) were incubated for 1 hr at 37° in various incubation media, and formation of pyruvate and lactate 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. Statistical comparisons were made between paired incubations with and without 1,4-naphthoquinone-2-sulfonate. The differences observed were highly significant in spite of the large S.E. since most of the variance was between different paired experiments, while the effect of 1,4-naphthoquinone-2-sulfonate was consistent within each paired experiment.

† $P < 0.001$. § $P < 0.05$.

‡ $P > 0.05$, not significant. ‖ $P < 0.01$.

ance 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. Metabolic pathways allowing increased turnover might not be available or might be deleterious to the red cell. Exogenous lactate and pyruvate allow for increased functioning of the methemoglobin reductase system with minimal increases 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.

Confusion concerning the role of red cell glycolysis as an aerobic/antioxidant pathway has resulted from two misconceptions. First, the effect of an oxidative agent on glycolysis is often described in terms of increased glucose consumption or, worse, lactate production. Total flux through glycolysis reflects the rate of formation of ATP and/or 2,3-diphosphoglycerate whereas reducing equivalents are usually stored as lactate. The normally small percentage of glycolytic flux which reflects aerobic/antioxidant activity should be measured as pyruvate accumulation. Second, when increases in total glycolytic flux are linked to pyruvate accumulation, increases are often belittled by expressing the results as percent increases of total glycolytic flux. However, a 40% increase in flux of glucose through glycolysis is equivalent to a 500% increase in flux of glucose through the hexose monophosphate shunt.

Specific conclusions. Measurements of metabolites in red cell suspensions demonstrated that the defined medium was a good approximation of plasma in regard to concentrations of glucose, lactate, pyruvate and ascorbate. Five-fold concentrations of 1,4-naphthoquinone-2-sulfonate were required to produce a similar level of oxidative stress in red cells suspended in plasma compared to defined medium. The distribution of oxidative stress was similar in the two types of suspension with respect to methemoglobin, CO_2 and pyruvate formation. In the absence of oxidative stress, plasma was more efficient than defined medium in storage of reducing equivalents from glycolysis as lactate. In defined medium 10% of glycolytic products appeared as pyruvate whereas in plasma part of the pyruvate pool (present at zero time) was actually reduced to lactate in a 1-hr incubation. Plasma and defined medium responded similarly to oxidative stress with partial uncoupling of increased pyruvate accumulation from ATP and/or 2,3-diphosphoglycerate turnover (total glycolytic flux). This was accomplished by decreasing lactate accumulation. In defined medium an increase of 1.23 μmoles pyruvate · hr^{-1} · (ml red cell)$^{-1}$ was coupled to an increase of 0.75 μmoles · hr^{-1} · (ml red cell)$^{-1}$ of lactate plus pyruvate (or ATP plus 2,3-diphosphoglycerate). In plasma, an increase of 2.10 μmoles pyruvate · hr^{-1} · (ml red cell)$^{-1}$ was coupled to an increase of 1.19 μmoles lactate plus pyruvate · hr^{-1} · (ml red cell)$^{-1}$. These results are in contrast to previously reported results [1] with medium not containing physiologic levels of lactate, pyruvate and ascorbate where little uncoupling of pyruvate accumulation from total glycolytic flux was observed: 0.55 μmole pyruvate · hr^{-1} · (ml red

Table 4. Metabolic sources of reducing equivalents in red cells incubated with 1,4-naphthoquinone-2-sulfonate*

	[μmoles Reducing equivalents · hr^{-1} · (ml red cell)$^{-1}$]	
Metabolic source	Defined medium	Buffered plasma
Heme	6.60	7.83
Glycolysis	2.46	4.20
Hexose monophosphate shunt	1.64	3.09
Total	10.70	15.12

* 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 oxidized 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) × 0.206; μmoles of reducing equivalents from glycolysis = (pyruvate accumulated in the presence of drug − pyruvate accumulated in the absence of drug) × 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) × 4.

cell)$^{-1}$ versus 0.48 μmole lactate plus pyruvate · hr^{-1} · (ml red cell)$^{-1}$. Uncoupling of the two processes allowed a greater contribution of reducing equivalents from glycolysis: 2.46 μmoles · hr^{-1} (ml red cell)$^{-1}$ in the presence of physiologic lactate, pyruvate and ascorbate versus 1.10 μmoles · hr^{-1} · (ml red cell)$^{-1}$ in their absence. The results support the conclusion that normal plasma concentrations of lactate and pyruvate are the main agents controlling the glycolytic response to oxidative stress. Ascorbate or other plasma constituents do not alter the qualitative response of glycolysis to oxidative stress although interactions with plasma protein or other plasma constituents may raise the dose of oxidant agent required to elicit a given quantitative response. The results also suggest that, at levels of oxidative stress that are likely to be encountered *in vivo*, glycolysis and the hexose monophosphate shunt may be equal in importance as aerobic/antioxidant pathways.

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