Glutathione-Dependent Reduction of Arsenate in Human Erythrocytes—a Process Independent of Purine Nucleoside Phosphorylase

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Reduction of arsenate (AsV) to the more toxic arsenite (AsIII) is toxicologically important, yet its mechanism is unknown. To clarify this, AsV reduction was investigated in human red blood cells (RBC), as they possess a simple metabolism. RBC were incubated with AsV in gluconate buffer, and the formed AsIII was quantified by high performance liquid chromatography–hydride generation–atomic fluorescence spectrometry (HPLC-HG-AFS). The observations are compatible with the following conclusions. (1) Human RBC reduce AsV intracellularly, because 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS, inhibitor of the chloride-bicarbonate exchanger, which also mediates phosphate and AsV uptake), as well as chloride and phosphate, countered AsIII formation. (2) Purine nucleoside phosphorylase (PNP), whose AsV reductase activity has been directly demonstrated, cannot be a physiologically relevant AsV reductase, because its inhibitor (BCX-1777) failed to decrease the basal erythrocytic AsV reduction, although it prevented the increase in AsIII formation caused by artificial activation of PNP with inosine and dithiothreitol. (3) The basal (PNP-independent) AsV reduction requires glutathione (GSH), because the GSH depletor diethylmaleate strongly diminished AsIII formation. (4) The erythrocytic AsV reduction apparently depends on NAD(P) supply, because oxidants of NAD(P)H (i.e., pyruvate, ferricyanide, methylene blue, nitrite, tert-butyldihydroperoxide, dehydroascorbate, 4-dimethylaminophenol) enhanced AsIII formation from AsV. The oxidant-stimulated AsV reduction is PNP-independent, because BCX-1777 failed to affect it, but is GSH-dependent, because diethylmaleate impaired it. (5) Pyruvate-induced glucose depletion, which causes NAD enrichment in the erythrocytes at the expense of NADH, enhanced AsV reduction. This suggests that the erythrocytic AsV reduction requires both NAD supply and operation of the lower part of the glycolytic pathway starting from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that, unlike the upper part, remains fed with substrates originating from the degradation of 2,3-bisphosphoglycerate in RBC depleted of glucose by pyruvate. (6) Fluoride, which arrests glycolysis at enolase and thus prevents NAD formation, inhibited AsV reduction in glucose-sufficient RBC, but increased it in glucose-deficient (NAD-enriched) cells, suggesting that the section of glycolysis coupled to AsV reduction lies between GAPDH and enolase. In conclusion, besides the artificial PNP-dependent AsV reduction, human RBC contain a PNP-independent AsV-reducing mechanism. This appears to require the supply of GSH, NAD, and substrate to one or more of the glycolytic enzymes localized between GAPDH and enolase.

Key Words: arsenate; arsenite; purine nucleoside phosphorylase; reduction; glutathione; erythrocyte.

Arsenic is a long-known poison of environmental and industrial origin. Prolonged exposure is associated with vascular disease, skin lesions, and cancer (Goering et al., 1999; Hindmarsh, 2000; Hughes, 2002; Rossman, 2003). The predominant form of arsenic in the nature is the pentavalent arsenate (AsV), which enters the body mainly via contaminated drinking water. Owing to its structural similarity to inorganic phosphate (P_i), AsV may be taken up by the cells through their P_i transporters (Csankay and Gregus, 2001; Ginsburg and Lotspeich, 1963), and then it may interfere with cellular metabolism by replacing P_i in enzymatic reactions and transport processes (Dixon, 1997). Alternatively, AsV may be reduced by hitherto unidentified cellular enzymes to the trivalent arsenite (AsIII) (Thomas et al., 2001), which has much greater toxic potential than AsV because of its thiol-reactivity (Knowles and Benson, 1983). Further metabolism of arsenic yields mono- and dimethylated metabolites, among which the trivalent ones are even more toxic, whereas the pentavalent ones are relatively atoxic (Petrick et al., 2001; Rossman, 2003; Thomas et al., 2001). Therefore, the first step of AsV metabolism, its reduction to AsIII, is not only a toxification step, but may also primarily determine the fate, toxicity, and carcinogenicity of arsenic.

Recent papers have demonstrated that liver mitochondria (Németi and Gregus, 2002a) and cytosol (Németi and Gregus, 2002b; Radabaugh and Aposhian, 2000) reduce AsV to AsIII. Attempts to identify the mitochondrial AsV reductase have failed; nevertheless, a cytosolic enzyme capable of AsV reduction has been identified as purine nucleoside phosphorylase (PNP) (Gregus and Németi, 2002; Radabaugh et al., 2002). PNP effectively catalyzes the reduction of AsV in vitro during the course of the arsenolytic cleavage of inosine or guanosine,
provided an appropriate thiol, such as dithiothreitol (DTT) is present. Despite these promising findings, it was later demonstrated that PNP failed to contribute significantly to the reduction of AsV either in intact human erythrocytes or in rats in vivo (Néméti et al., 2003). Complete inhibition of PNP in rats by administration of BCX-1777, a highly potent inhibitor of the enzyme, did not delay the elimination of AsV and the formation of AsV metabolites. In addition, intact human red blood cells (RBC) retained most of their AsV-reducing activity even in the presence of high concentrations of BCX-1777. The observations that the AsV-reducing activity of PNP is not supported by glutathione (GSH) (Gregus and Néméti, 2002), whereas reduction of AsV is apparently GSH-dependent in cells (Bertolero et al., 1987) and rats (Csanaly and Gregus, in press; Gyurasics et al., 1991) also question the role of PNP as an in vivo relevant AsV reductase.

The goal of the present work has been to initiate a search for enzymes that do play a significant role in AsV reduction, more specifically, to characterize the AsV-reducing activity of intact human erythrocytes that is independent of PNP. For this purpose, we tested the role of GSH and glucose metabolism in the erythrocytic AsV reduction by determining the effects of the chemically induced GSH depletion and glutathione reductase (GR) inhibition, as well as the effects of various NAD(P)H oxidants known to facilitate glucose metabolism in RBC, and of pyruvate-induced glucose depletion. These studies were carried out in human RBC, because these cells readily take up AsV (Kenney and Kaplan, 1988), are capable of reducing AsV to AsIII (Néméti et al., 2003), and importantly, are devoid of mitochondria, which precludes the contribution of these organelles to the reduction of AsV.

MATERIALS AND METHODS

**Chemicals.** BCX-1777 (also called Immucillin-H) was a generous gift from BioCryst Pharmaceuticals (Birmingham, AL). D-glucic acid, 4,4'-diisothiocyanoterstilbene-2,2'-disulfonic acid (DIDS), and N,4-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were from Sigma. Diethylmaleate (DEM) was from Koch-Light Laboratories (England). The sources of arsenic compounds, and chemicals used in arsenic speciation have been given elsewhere (Csanaly et al., 2003; Néméti and Gregus, 2002a). All other chemicals were of the highest purity commercially available.

**RBC preparation.** This research was approved by the Regional Scientific Research Ethics Committee of the University of Pécs, Center for Medical and Health Sciences. Blood (approximately 5 ml) was collected from healthy human volunteers after informed consent into heparinized Vacutainer tubes. The blood was immediately centrifuged at 1,000 g and 4°C for 10 min, and the plasma and buffy coat were discarded. In order to maximize the AsV-reducing activity of RBC, erythrocyte suspension was prepared and assayed in a buffer free of chloride and phosphate, because these ions antagonize the uptake and/or reduction of AsV, as demonstrated in this paper. The pelleted RBC were resuspended in an equal volume of ice-cold buffer containing 150 mM sodium glutonate, 10 mM HEPES, and 5 mM glucose, pH 7.4. This RBC suspension was then centrifuged under the same conditions as previously, followed by removal of the supernatant. This washing procedure was repeated once more. After the final centrifugation, the pellet was measured gravimetrically, and then resuspended in an equal volume of ice-cold buffer resulting in a 50% RBC suspension. The RBC suspension was kept in ice until use for assaying AsV reduction within 3 h.

**RBC incubations.** In order to determine AsV-reducing activity of intact RBC, the RBC were incubated (50 μl packed cells/ml) with 50 μM AsV at 37°C in a total volume of 300 μl of buffer used for washing. The duration of incubation was 30 min, except in the combined presence of the PNP substrate inosine and the diethyl DTT, when it lasted for 10 min. The incubations were started by adding RBC (30 μl from 50% suspension); however, when the RBC suspension was preincubated with some test compounds, the incubations were started by adding AsV. Specific conditions of preincubations are given in the figure legends. The incubations were stopped by successive addition of 100 μl of 25 mM CdSO4 solution containing 1% Triton X-100, and 100 μl of 1.5 M perchloric acid solution containing 25 mM HgCl2. Pilot experiments clarified that Hg2⁺ ions effectively displaced thiol-bound AsIII even in strongly acidic environment. Nevertheless, Hg2⁺ ions oxidized the formed AsIII when applied at neutral pH, but not in acid. Therefore, we added Cd2⁺ first, which binds to thiol groups at neutral but not at acidic pH (Fuhr and Rabenstein, 1973), and which displaced thiol-bound AsIII, but did not oxidize the released AsIII. The incubates were stored at −80°C until analysis. Before analysis, the incubates were centrifuged at 10,000 × g and 4°C for 10 min, and the resultant supernatant was applied to high performance liquid chromatography–hydride generation–atomic fluorescence spectrometry (HPLC-HG-AFS) to separate and quantify AsIII and AsV. AsV reductase activity was expressed as nmol formed AsIII per minute and ml packed RBC.

**Analyses.** AsIII and AsV in the deproteinized RBC incubates were separated and quantified by HPLC–hydride generation–atomic fluorescence spectrometry (HPLC-HG-AFS) according to Gomez-Ariza et al. (1998), as described in detail by Gregus et al. (2000). However, after ascertaining that the incubates contained no other AsV metabolites besides AsIII, we used isotopic rather than gradient elution routinely with 60 mM sodium phosphate buffer (pH 5.75) as an eluent at 1.1 ml/min flow rate.

PNP activity of human RBC hemolysate was assayed according to the method of Kalkkar (1947), as described in detail by Gregus and Néméti (2002). This assay measures the formation of uric acid in the presence of excess Pi and xanthine oxidase.

After pyruvate-induced glucose depletion, RBC glucose concentration was measured according to the method of Trinder (1969), which uses glucose oxidase coupled with horseradish peroxidase and measures the formation of a color product at 515 nm.

**Statistics.** SPSS 10.0 for Windows (SPSS Inc.) was used for statistical analysis. Data were analyzed using one-way ANOVA followed by Duncan’s test and Students’ t-test with p < 0.05 as the level of significance.

RESULTS

**Effect of Compounds Interfering with the Erythrocytic AsV Uptake on AsV Reduction by RBC**

Washed intact human erythrocytes reduced AsV to AsIII at a rate of 1.48 ± 0.08 nmol per minute and ml packed RBC (Fig. 1). It was of interest to determine whether the observed AsV reduction took place intra- or extracellularly. Since RBC take up AsV via their chloride-bicarbonate exchanger (also called Band III protein) (Kenney and Kaplan, 1988), we investigated if the physiological substrates of this transporter (i.e., chloride and phosphate) and the irreversible inhibitor DIDS were able to diminish AsIII formation. Figure 1 demonstrates that chloride inhibited AsV reduction in a concentration-dependent manner (left), causing more than 90% inhibition at the physiological 100 mM concentration. Phosphate also exhibited concentration-dependent
inhibition in AsV reduction, decreasing its rate by more than 80% at 1 mM (Fig. 1, middle), whereas DIDS abolished AsIII formation at a concentration as low as 10 µM (Fig. 1, right). Importantly, neither chloride nor DIDS influenced AsV reduction by hemolyzed RBC (data not shown). These findings prompted us to use a buffer free of chloride and phosphate for preparing and incubating RBC in order to maximize the reducing activity of erythrocytes.

**Effect of BCX-1777 on the Basal and the DTT-, Inosine-, and DTT-Plus-Inosine-Stimulated AsV Reduction by RBC**

PNP is capable of reducing AsV, provided its nucleoside substrate (e.g., inosine) and a thiol (e.g., DTT) are present simultaneously. Since PNP is abundant in RBC, we assessed to what degree this enzyme contributed to the erythrocytic AsV reduction under various conditions. Figure 2 depicts that 0.5 mM DTT barely affected AsIII formation, whereas exogenous inosine (1 mM) strongly enhanced it. The combination of these two agents resulted in a 5.5-fold increase in AsV reduction. BCX-1777, a transition state analogue of PNP substrates, is a potent inhibitor of PNP. At 20 µM, a concentration that exceeds the one causing complete PNP inhibition 40-fold, it effectively prevented the stimulatory action exhibited by DTT, inosine, or the combination of both (Fig. 2). However, a basal AsV-reducing activity of 1.0–1.4 nmol/min/ml packed RBC remained even though PNP activity fell from 960.5 U/ml packed RBC of control erythrocyte suspension to 0.1 ± 0.1 U/ml packed RBC (mean ± SEM, n = 4) in erythrocyte suspension incubated with 20 µM BCX-1777. Importantly, BCX-1777 failed to decrease the erythrocytic AsV reduction significantly in the absence of exogenous PNP-activating agents (i.e., DTT and inosine) (Fig. 2, bars labeled none).

**Effect of GSH Depletion and GR Inhibition on AsV Reduction by RBC**

In order to characterize the basal (i.e., PNP-independent) AsV-reducing activity of RBC, we investigated the effects of the glutathione-depletor DEM and the GR inhibitor carmustine (BCNU). We observed that after 15 min preincubation with as low as 0.25 mM DEM, the AsIII formation by RBC decreased by approximately one-half when neither exogenous inosine nor DTT were present (Fig. 3, left, curve labeled none), and higher concentrations of DEM resulted in more pronounced diminution in AsV reduction. When either DTT or inosine was present alone, significant decrease in AsIII formation was found only at 0.5 mM DEM and above. If both PNP stimulants (i.e., inosine and DTT) were present, even 1 mM DEM failed to diminish AsV reduction significantly (Fig. 3, left). The GR inhibitor BCNU caused in a slight, nonsignificant decrease in AsIII formation (Fig. 3, right).

**Effect of Compounds Promoting Oxidation of Cellular NAD(P)H on AsV Reduction by RBC**

Erythrocytes possess a relatively simple metabolic capacity, which is strictly controlled by several factors, one of which is the ratio of the oxidized and the reduced pyridine nucleotides.

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**FIG. 1.** Effect of inorganic anions and DIDS that interfere with erythrocytic AsV uptake on AsV reduction by intact RBC. Erythrocytes (50 µl packed cells/ml) were incubated at 37°C in 150 mM sodium gluconate solution containing 10 mM HEPES and 5 mM glucose (pH 7.4) for 30 min with AsV (50 µM) in the presence of chloride, phosphate, or DIDS at concentrations indicated, and formation of AsIII was quantified. Symbols and bars represent means ± SEM of three experiments with erythrocytes prepared from different individuals.

**FIG. 2.** Effect of BCX-1777 on the basal as well as on inosine-, DTT-, and inosine-plus-DTT-stimulated AsV reduction by intact RBC. Erythrocytes (50 µl packed cells/ml) were preincubated at 37°C in gluconate-HEPES-glucose buffer for 5 min either in the absence or presence of 20 µM BCX-1777 that completely inhibits PNP. Thereafter, incubation buffer (none), or DTT (0.5 mM), or inosine (1 mM), or DTT plus inosine was added followed immediately by AsV (50 µM) at the start of incubation, which lasted for 30 min, but only for 10 min when DTT and inosine were present simultaneously. Bars represent means ± SEM of three experiments with erythrocytes prepared from different individuals. Asterisks indicate significant difference (p < 0.05) from the respective AsIII formation rates obtained in the absence of BCX-1777.
NADH oxidation (i.e., increased availability of NAD) enhances the flux through the glycolytic pathway, whereas NADPH oxidation (i.e., increased NADPH supply) facilitates the flux through the pentose phosphate pathway. Therefore, it was of interest to know whether oxidants of the pyridine nucleotide system (i.e., NAD/NADH and NADP/NADPH) influenced AsIII formation by RBC. Pyruvate strongly enhanced AsV reduction (Fig. 4, top left); even at a concentration as low as 10 μM, it increased AsIII formation more than two-fold and exerted its maximal, almost four-fold stimulatory effect at 25 μM. Further increase in pyruvate concentration did not improve its stimulant action. The other three NAD(P)H oxidants, whose effects are depicted in Figure 4, also enhanced AsV reduction by RBC, and exhibited a qualitatively similar concentration-effect relationship with a plateau at high concentrations. The membrane impermeant oxidant ferricyanide, the redox cyclers methylene blue, and the methemoglobin forming nitrite increased AsV reduction up to 3, 1.9, and 1.6-fold, respectively, and reached their maximal stimulatory concentration at 250 μM, 10 μM, and 1 mM, respectively (Fig. 4).

The response of the erythrocytic AsV reduction to some other oxidants differed profoundly. Tert-butylhydroperoxide (tBOOH) increased AsIII formation at low concentrations (Fig. 5, left), exhibiting maximal stimulation at 50 μM; however, at higher concentrations, its effect reversed, and it abolished AsV reduction almost completely at 250 μM. Dehydroascorbic acid (DHA) (Fig. 5, middle), and 4-dimethylaminophenol (4-DMAP) (Fig. 5, right) also enhanced AsIII formation at low concentrations, but inhibited it at higher concentrations.

In order to clarify if PNP was involved in the AsV reduction stimulated by the NAD(P)H oxidants, RBC were incubated with AsV in the presence of BCX-1777 (20 μM) and one of the NAD(P)H oxidants applied at maximal stimulatory concentration. Figure 6 demonstrates that the potent PNP inhibitor failed to influence the oxidant-enhanced AsIII formation by intact erythrocytes.

The Chemical Responsiveness of the Ferricyanide-Stimulated AsV Reduction by RBC

Despite being a nonpermeant anion, ferricyanide greatly facilitates the erythrocytic AsIII formation (Fig. 4). Therefore, it was of interest to explore the influence of additional supply of intracellular NAD and deficiency of GSH in the ferricyanide-stimulated AsV reduction. The NAD-producing pyruvate that readily permeates the red cell membrane and strongly enhances the basal AsV reduction (Fig. 4, top left), failed to further enhance the ferricyanide-stimulated AsIII formation (Table 1). Table 1 also demonstrates that, while the ferricyanide-stimulated AsV reduction is unresponsive to the PNP inhibitor BCX-1777, it is severely diminished by the GSH-depleting DEM.
The Effect of Pyruvate-Induced Glucose Depletion on AsV Reduction by RBC

Since the metabolism of RBC strictly depends on the availability of glucose, we tested what effects the pyruvate-induced glucose depletion had on the reduction of AsV by erythrocytes. The applied method (see in the note of Table 2) provided...
virtually complete depletion of glucose in erythrocytes (data not shown). Surprisingly, the AsIII formation by RBC subjected to pyruvate-induced glucose depletion was significantly higher (2.34 ± 0.27 nmol/min/ml packed RBC) than the AsIII formation in glucose-sufficient RBC (1.48 ± 0.08 nmol/min/ml packed RBC). Table 2, which presents the changes in erythrocytic AsV reduction relative to these control rates, demonstrates that the glucose-sufficient and glucose-deficient RBC responded to various chemical agents in qualitatively different ways. While addition of an extra 1 mM glucose to the glucose-sufficient RBC (suspended and incubated in the presence of 5 mM glucose) is not expected to influence AsV reduction, supplementation with 1 mM glucose enhanced AsV reduction by glucose-depleted RBC 2.5-fold. Furthermore, the NAD(P)H oxidants found to facilitate AsV reduction by glucose-sufficient RBC failed to increase it (e.g., pyruvate, ferricyanide, dehydroascorbic acid), or even inhibited it (e.g., methylene blue, tert-butylhydroperoxide) in glucose-deficient erythrocytes. In contrast, fluoride, or even inhibited it (e.g., methylene blue, tert-butylhydroperoxide) in glucose-deficient erythrocytes. Inosine markedly stimulated erythrocytic reduction of AsV to AsIII, especially in the presence of the dithiol DTT. Inosine is a substrate for PNP, which cleaves this nucleoside into hypoxanthine and ribose-1-phosphate or ribose-1-arsenate using phosphate or AsV, respectively. It has been demonstrated that purified PNP can reduce AsV to AsIII in this process, provided an appropriate thiol, such as DTT, is present (Gregus and Németi, 2002), and that BCX-1777, a potent inhibitor of the enzyme, inhibits PNP-catalyzed AsV reduction (Gregus and Németi, 2002). Therefore, the finding that the PNP inhibitor abolished the increment in AsV reduction brought about by inosine plus DTT indicates that this increase in AsV reduction is dependent on the activity of PNP and suggests that, under those conditions, PNP may be involved in conversion of AsV to AsIII in the RBC. It is important to realize, however, that, while abolishing the inosine- and DTT-induced increase in erythrocytic AsV reduction, BCX-1777 failed to diminish the basal AsV-reducing activity of RBC and failed to lower AsV-reducing activity of erythrocytes not supplemented with inosine and DTT (Fig. 2). These data collectively suggest that RBC possess not only a PNP-dependent AsV reduction, which can be triggered by inosine plus DTT supplementation, but a PNP-independent AsV reduction as well, which is actually the prevailing mechanism under basal conditions.

Different responsiveness of the erythrocytic AsV reduction to GSH depletion under PNP-stimulated (i.e., inosine- and/or DTT-supplemented) and basal conditions also reveals the existence of fundamentally different mechanisms for reduction of AsV in RBC. While AsV reduction by purified PNP was well supported by the physiologically irrelevant DTT, it was not supported by GSH, the physiologically most important nonprotein thiol compound (Gregus and Németi, 2002). Accordingly, when both inosine and DTT were present, and thus the RBC carried out the reduction of AsV in a PNP-dependent fashion (i.e., inhibitable by BCX-1777), the AsIII formation from AsV was barely affected by GSH depletion (Fig. 3, left). However, a significant role has long been attributed to GSH in AsV reduction in vivo (Vahter, 2002). For example, the well-known GSH-depleting agent DEM (Plummer et al., 1981) compromised AsV reduction by mouse embryo cells (Bertolero et al., 1987) and abolished the biliary excretion of trivalent arsenicals in AsV-dosed rats (Gyurasics et al., 1991), whereas buthionine-S,R-sulfoximine-induced GSH depletion has been directly shown to impair the reduction of AsV in rats (Csanaky and Gregus, in press). May et al. (2001) reported (and we confirmed in a pilot experiment) that DEM added to the suspension of human erythrocytes decreased GSH levels in the RBC in a concentration-dependent manner, causing a 75% decrease at 1 mM DEM in 30 min, but it did not cause oxidative injury. On carrying out the incubation under virtually identical conditions with suspended human RBC not supplemented with inosine and/or DTT, we found that 15 min preincubation with 1 mM DEM followed by 30 min incubation with AsV (with DEM being still present) diminished AsIII formation by 78% (Fig. 3, left). This decrease indicates that the basal (i.e., PNP-independent) erythrocytic AsV reduction is highly dependent on the availability of GSH. It is important to note that, although GSH can reduce AsV at extremely high concentrations (300 mM) (Delnomdedieu et al., 1994), we found that, at a concentration it occurs in the RBC (2 mM), the direct reduction of AsV by GSH was negligible, indicating that GSH-dependent erythrocytic reduction of AsV is an enzymatic process.

The observation that the GR inhibitor carmustine (BCNU) impaired AsV reduction by RBC only minimally (Fig. 3, right), even at 100 μM concentration that causes a rapid and complete

**DISCUSSION**

This work demonstrates that intact human red blood cells can reduce AsV to AsIII, provided AsV can enter erythrocytes. AsV is taken up into RBC via the chloride-bicarbonate exchanger (Kenney and Kaplan, 1988), which also mediates the uptake of phosphate. The observation that both the natural substrate and an irreversible inhibitor of the transporter (i.e., chloride and DIDS, respectively) inhibited AsV reduction by intact, but not by lysed, RBC indicates that the reduction took place inside the erythrocytes. However, inorganic phosphate might diminish reduction of AsV not only by interfering with its transport, but also with its enzymatic reduction, due to its structural similarity to AsV.

This paper also clearly demonstrates that there are at least two mechanisms in the human erythrocyte for reduction of AsV. Inosine markedly stimulated erythrocytic reduction of AsV to AsIII, especially in the presence of the dithiol DTT. Inosine is a substrate for PNP, which cleaves this nucleoside into hypoxanthine and ribose-1-phosphate or ribose-1-arsenate using phosphate or AsV, respectively. It has been demonstrated that purified PNP can reduce AsV to AsIII in this process, provided an appropriate thiol, such as DTT, is present (Gregus and Németi, 2002), and that BCX-1777, a potent inhibitor of the enzyme, inhibits PNP-catalyzed AsV reduction (Gregus and Németi, 2002). Therefore, the finding that the PNP inhibitor abolished the increment in AsV reduction brought about by inosine plus
inactivation of GR (Zhang et al., 1988), apparently contradicts to the importance of GSH in reduction of AsV. However, the quantity of GSH in RBC greatly exceeds that utilized for AsV reduction, as 1 ml packed RBC contains approximately 1400 nmol GSH (calculated from the 2 mM GSH concentration in these cells; May et al., 2001) and formed only 45 nmol AsIII in 30 min. Thus, the large excess of reduced GSH present in the erythrocytes should generously meet the GSH demand for AsV reduction at that rate for 30 min even with inactivated GR.

The metabolism of RBC is relatively simple compared to other cell types. Erythrocytes possess two major metabolic pathways, namely glycolysis, which is the only route to produce ATP, and pentose phosphate shunt, which is the only route to produce NADPH (Fig. 7). The glycolysis in RBC is regulated primarily by the NAD-to-NADH ratio (Tilton et al., 1991), whereas pentose phosphate shunt is regulated primarily by the NADP-to-NADPH ratio (Afolayan and Luzzatto, 1971). The oxidized pyridine nucleotides stimulate the respective metabolic pathway, while the reduced ones slow it. In washed human RBC, which were suspended and incubated as in our studies, the concentrations of NAD, NADH, NADP, and NADPH were 24, 50, 1, and 36 μM, respectively (May et al., 2004).

We tested the effect on erythrocytic AsV reduction of a number of chemicals known to shift these ratios to favor oxidized pyridine nucleotides, NAD or NADP. Based on their effects, these chemicals fall into two major categories. The first includes pyruvate, methylene blue, nitrite, and ferricyanide. Pyruvate readily crosses RBC membrane, and then it is reduced to L-lactate by lactate dehydrogenase (LDH) while consuming NADH (Tilton et al., 1991). The reaction catalyzed by LDH is reversible, but under steady state, production of lactate and NAD is strongly favored (Nelson and Cox, 2000). Therefore, pyruvate increases the levels of NAD at the expense of NADH, which in turn enhances the flux through the glycolytic pathway (Fig. 7). Apparently on the outer surface of the cell membrane (May et al., 2004) methylene blue is reduced to leukomethylene blue by human RBC (Metz et al., 1976) with concomitant oxidation of erythrocytic NADH and NADPH. The reduced dye is then taken up by the cells, where it may be reoxidized by electron acceptors, such as methemoglobin. At a concentration of 10–20 μM, methylene blue added to washed human RBC suspension lowered the levels of NADH and NADPH to approximately 5 μM, while elevating the levels of NAD and NADP to 50 and 35 μM, respectively (May et al., 2004). Nitrite induces oxidation of hemoglobin to methemoglobin (Chiodi and Mohler, 1987), which is reduced by methemoglobin reductase at the expense of NADH, forming NAD, resulting in increased glycolytic flux. Ferricyanide is a nonpermeant anion, which is rapidly reduced extracellularly by the trans-plasmamembrane oxidoreductase system (Schipfer et al., 1985). This enzyme is supported by ascorbic acid (May, 1999) or NADH as electron source; thus ferricyanide increases cellular NAD concentration (Schipfer et al., 1985). All these agents enhanced AsIII formation by intact erythrocytes in a similar concentration-dependent fashion: at a certain concentration (methylene blue, 10 μM; pyruvate, 25 μM; ferricyanide 250 μM; nitrite, 1 mM) they exerted maximal stimulatory action, which was maintained with further increase of their concentrations (Fig. 4). It is most likely, and this is clearly demonstrated with methylene blue (May et al., 2004), that the concentrations, at which these oxidants maximally stimulate AsV reduction, correspond to their levels at which they maximally oxidize NAD(P)H into NAD(P), and this effect could not be enhanced by further increases in their concentrations. Nevertheless, their NAD(P)H-oxidizing power may differ, which could explain that their stimulating effect on AsIII formation varied in strength.

The second category of oxidants, which include tBOOH, DHA, and 4-DMAP, differed fundamentally in their

FIG. 7. A simplified scheme of glucose metabolism in erythrocytes. Most agents used in this study may promote glycolysis by increasing NAD supply for GAPDH. These include pyruvate, ferricyanide, and dehydroascorbic acid, which are reduced enzymatically at the expense of NADH, as well as chemicals (methylene blue, nitrite, t-butylhydroperoxide, and 4-dimethylaminophenol) that cause formation of methemoglobin, which in turn is also reduced at the expense of NADH by methemoglobin reductase. Fluoride inhibits enolase. Experimental data support the hypothesis that the section of glycolysis coupled to AsV reduction lies between GAPDH and enolase (see Discussion).

Gluc-6-P, glucose-6-phosphate; GA-3-P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 2,3-BPG, 2,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PP-pathway, pentose phosphate pathway; HK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, monophosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.
concentration-dependent effect on AsV reduction by RBC, causing stimulation at low and inhibition at high concentrations (Fig. 5). A common property of these compounds is that they consume GSH during their metabolism, in addition to oxidizing NAD(P)H to NAD(P). tBOOH is substrate for glutathione peroxidase (Kurata et al., 2000), and its reduction is concomitant with oxidation of GSH to oxidized glutathione (GSSG), which in turn is reduced to GSH by GR, using NADPH (Kosower et al., 1967). In addition, tBOOH can promote methemoglobin formation (Murakami and Mawatari, 2003), thereby enhancing NAD production at the expense of NADH by methemoglobin reductase. DHA can be reduced extracellularly to ascorbate by the trans-plasmamembrane oxidoreductase system that consumes intraerythrocytic NADH and produces NAD (Himmelreich et al., 1998). Upon entering RBC, DHA can also be reduced intracellularly (May et al., 2001). In RBC this reduction may be carried out by glutathione peroxidase (Washburn and Wells, 1999), GSH-dependent DHA reductase (Xu et al., 1996), and glutaredoxins (Wells et al., 1990), each consuming GSH and producing GSSG, the reduction of which causes NADPH oxidation. 4-DMAP is a potent cyanide antidote, which forms methemoglobin. During this process, 4-DMAP forms of phenoxyl radicals (Ludwig and Eyer, 1995a), which may then disproportionate to yield 4-DAMP and its quinone imine derivative. Being an electrophile, this latter compound spontaneously conjugates with GSH. The conjugation products (e.g., 2-GS-DMAP; 2,6-bis-GS-DMAP) are still reactive and can redox cycle, resulting in increased oxidative stress, GSH oxidation (Ludwig and Eyer, 1995b), and in turn NADPH oxidation. In addition, methemoglobin thus formed is reduced, causing NADH oxidation. In conclusion, these compounds at low concentrations enhanced AsV reduction, probably because they increased the cellular NAD(P)/NAD(P)H ratio and, subsequently, the fluxes through the glycolytic and/or the pentose phosphate pathways. However, at higher concentrations of these oxidants, the consumption of GSH by oxidation into GSSG (and also by conjugation in 4-DMAP-exposed RBC) exceeded its regeneration by GR, resulting in a net decrease in availability of GSH. Since GSH is apparently required for the PNP-independent AsV reduction by RBC (Fig. 3, left), the shortage in GSH should diminish AsIII formation at higher concentrations of these chemicals.

The findings of studies with the oxidizing agents collectively suggest that these chemicals enhance AsV reduction by the RBC because they increase NAD(P)/NAD(P)H ratio and/or the flux through the glycolytic or pentose phosphate pathways. The observation that inhibition of PNP failed to diminish AsIII formation stimulated by any of these oxidants (Fig. 6) clearly indicates that PNP does not contribute to the reduction of AsV under these conditions.

Further circumstantial evidence for the role of NAD supply in erythrocytic AsV reduction was obtained in the experiments carried out with RBC depleted of glucose by pyruvate (Table 2). The pyruvate-induced glucose depletion causes substantial changes in RBC metabolism. Since pyruvate is rapidly reduced to lactate by LDH, the NAD/NADH ratio is markedly elevated, which in turn strongly stimulates the glycolysis at glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 7). Concomitantly, the cellular ATP pool is loaded, while P; is consumed. It must be emphasized, however, that these glucose-depleted erythrocytes will not become devoid of all glycolytic substrates. The reason for this is that RBC, unlike other cells, contain huge amounts of 2,3-bisphosphoglycerate (2,3-BPG) (up to 7 mM; Mulquiney et al., 1999) that is channeled into the glycolysis after being converted into 3-phosphoglycerate. This in turn may move “downwards,” ultimately giving rise to pyruvate, or “upwards,” forming 1,3-bisphosphoglycerate (1,3-BPG), the substrate for GAPDH (Fig. 7). In pyruvate-pretreated cells, with virtually all NADH converted into NAD, 1,3-BPG cannot move any “higher” in the glycolytic pathway, because the lack of NADH prevents GAPDH from reducing 1,3-BPG into glyceraldehyde-3-phosphate. Therefore, in the RBC depleted of glucose with pyruvate, the enzymes of the lower glycolytic pathway starting from GAPDH are supplied with substrates, owing to the abundance of 2,3-BPG in erythrocytes. In contrast, enzymes in the glycolytic pathway “above” GAPDH, as well as the enzymes of the pentose phosphate shunt, are devoid of substrates under this experimental condition. Thus, the observation that under pyruvate-induced glucose depletion the RBC possessed increased capacity to reduce AsV to AsIII points to the possible roles of NAD as well as the lower glycolytic enzymes starting from GAPDH in AsV reduction, while it calls into question such a role of the upper glycolytic enzymes and the pentose phosphate pathway.

If we accept the hypothesis that NAD supply facilitates erythrocytic reduction of AsV, it becomes readily apparent why the NAD(P)H oxidants facilitated AsV reduction in glucose-sufficient RBC, but failed to do so in the cells depleted of glucose with pyruvate (Table 2). It is most likely that in the latter cells pyruvate had maximally elevated the NAD levels, having converted virtually all NADH into NAD, and thus the NAD(P)H oxidants could not increase the NAD levels any further. Moreover, except for pyruvate, they tended to diminish or significantly diminished AsV reduction after pyruvate-induced glucose depletion. This may be accounted for by the susceptibility of glucose-deficient cells to GSH depletion. Indeed, methylene blue, DHA, and tBOOH induced GSH depletion in RBC at relatively low concentrations in the absence of glucose (May et al., 2001, 2004; Kurata et al., 2000, respectively). As pointed out earlier, in these glucose-depleted cells the pentose phosphate shunt remains idle, and NADPH is not generated (Fig. 7); therefore regeneration of GSH from GSSG by GR becomes compromised, causing decline in GSH levels.

The experiments with fluoride are also revealing with respect to the further delimitation of the step(s) of glucose metabolism that is/are important in the PNP-independent AsV reduction in erythrocytes. By inhibiting enolase (Wang and Himoe, 1974; Warburg and Christian, 1941–1942), fluoride causes accumulation of the glycolytic substrates preceding enolase as well as
of NADH, because pyruvate formation is prevented; hence NADH produced by GAPDH cannot be reoxidized to NAD by LDH (Fig. 7). AsV reduction by glucose-sufficient RBC markedly decreased in response to fluoride (Table 2), probably because fluoride diminished the flux through critical steps of the glycolysis and decreased the NAD/NADH ratio. Unexpectedly, the pyruvate-induced glucose depletion reversed this response: instead of inhibiting AsIII formation, fluoride enhanced it in the pyruvate-pretreated cells. It may be speculated that in these cells the pyruvate-induced elevation in NAD/NADH ratio was large enough to offset the effect of fluoride to decrease this ratio and, thereby, to decrease AsV reduction. In addition, the blockade of enolase by fluoride could cause the substrates originating from 2,3-BPG to accumulate on the enzymes between GAPDH and enolase. It remains to be determined whether or not this could account for the fluoride-induced increase in AsV reduction by erythrocytes after pyruvate-induced glucose depletion.

Since the findings discussed so far indicate that increased availability of NAD facilitates reduction of AsV in RBC, it was of interest to determine whether compounds that have been reported to increase NAD supply would slow AsIII formation from AsV. To this end, the effects of lactate and formaldehyde were tested. Oxidation of lactate into pyruvate by LDH parallels reduction of NAD into NADH (Fig. 7). However, this is a slow process, partly because the reverse of this reaction is strongly favored, and partly because in glucose-sufficient RBC the level of NAD is low compared to that of NADH (May et al., 2004; see above). In contrast, after pyruvate-induced glucose depletion the level of NAD should greatly exceed that of NADH; this would facilitate oxidation of lactate with concomitant production of NADH. These considerations may explain why lactate diminished AsV reduction insignificantly in glucose-sufficient erythrocytes, but significantly after the RBC had been depleted of glucose by pyruvate, as in the latter cells lactate could produce more NADH than in the former ones. Formaldehyde is substrate for aldehyde dehydrogenase present in erythrocytes (Inoue et al., 1978) that oxidizes formaldehyde to formic acid, while reducing NAD to NADH. The inhibitory effect of formaldehyde on AsV reduction was significant in glucose-sufficient cells, but it was weak in glucose-depleted RBC, for not readily apparent reasons.

Finally, it must be emphasized that human erythrocytes are unlikely to play a significant role in reduction of AsV to AsIII in vivo, because chloride and P i at physiological plasma concentrations (94–108 mM, and 1.0–1.5 mM, respectively) very strongly inhibit AsV uptake and/or reduction by intact erythrocytes. Indeed, addition of plasma to washed erythrocytes markedly inhibited their AsV-reducing activity (observation not shown). Nevertheless, RBC represent an extremely valuable research tool in identification of AsV reductase(s) residing in the cytosol.

In summary, intact human erythrocytes possess at least two AsV-reducing activities: a PNP-dependent activity, which is artificial (i.e., appears only after addition of inosine and DTT) and GSH-independent, as well as a PNP-independent activity, which is physiological (i.e., present in the erythrocytes under basal conditions) and GSH-dependent. The physiological AsV reductase activity is facilitated by supply of cellular NAD(P) and/or an increased glycolytic flux and inhibited by supply of NAD(P)H and/or decreased glycolytic flux. Circumstantial evidence indicates that one or more steps of the glycolytic path stretching from GAPDH to enolase may have important role in reduction of AsV to the more toxic AsIII. Studies are in progress to identify the in vivo relevant enzyme(s) responsible for reduction of AsV in erythrocytes and other cells.

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