Oxalate Formation From Glyoxal in Erythrocytes

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OBJECTIVE To determine whether glyoxal can be converted to oxalate in human erythrocytes. Glyoxal synthesis is elevated in diabetes, cardiovascular disease, and other diseases with significant oxidative stress. Erythrocytes are a good model system for such studies as they lack intracellular organelles and have a simplified metabolism.

MATERIALS AND METHODS Erythrocytes were isolated from healthy volunteers and incubated with varying concentrations of glyoxal for different amounts of time. Metabolic inhibitors were used to help characterize metabolic steps. The conversion of glyoxal to glycolate and oxalate in the incubation medium was determined by chromatographic techniques.

RESULTS The bulk of the glyoxal was converted to glycolate, but ~1% was converted to oxalate. Inclusion of the pro-oxidant, menadione, in the medium increased oxalate synthesis, and the inclusion of disulfiram, an inhibitor of aldehyde dehydrogenase activity, decreased oxalate synthesis.

CONCLUSION The glyoxalase system, which utilizes glutathione as a cofactor, converts the majority of the glyoxal taken up by erythrocytes to glycolate, but a small portion is converted to oxalate. A reduction in intracellular glutathione increases oxalate synthesis and a decrease in aldehyde dehydrogenase activity lowers oxalate synthesis and suggests that glyoxylate is an intermediate. Thus, oxidative stress in tissues could potentially increase oxalate synthesis. UROLOGY 88: 226.e11–226.e15, 2016.

Endogenous oxalate synthesis contributes approximately half of the oxalate excreted in urine and occurs primarily in the liver.[1,2] An increased oxalate synthesis is associated with kidney stones and end-stage renal disease in the rare genetic diseases, primary hyperoxaluria types 1-3.[3,4] Furthermore, an increased oxalate synthesis may be associated with the development of idiopathic calcium oxalate stone disease, but its contribution has not been well defined.[1,2] We have shown that sources of oxalate in humans include hydroxyproline, glycine, and phenylalanine.[5,6] Studies with cultured human hepatocytes have also suggested that the 2-carbon dialdehyde, glyoxal, may be an important source.[7] Glyoxal in part is derived from lipid peroxidation and protein glycation.[8] Of note, blood levels of glyoxal and urinary oxalate excretion are both higher in diabetics.[9,10] Furthermore, diabetes is a risk factor for kidney stone formation[11] and urinary oxalate excretion is higher in stone formers,[12] suggesting that increased glyoxal and oxalate synthesis may be a link between these 2 diseases.

Glyoxylate is recognized as the major precursor of oxalate in mammalian metabolism.[1] We have previously hypothesized that the oxidation of glyoxal to glyoxylate may contribute to endogenous oxalate synthesis.[13] Erythrocytes lack intracellular organelles and have a simplified metabolism, making them a useful model system to examine the pathways involved in the conversion of glyoxal to oxalate. Through the use of inhibitors and the analysis of metabolites, we provide support for our previously proposed pathway for the conversion of glyoxal to oxalate using a human erythrocyte model.[13]

MATERIALS AND METHODS

Reagent grade chemicals were obtained from either Sigma-Aldrich Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Glyoxal, methylglyoxal, menadione, and disulfiram were purchased from Sigma-Aldrich. Menadione (90 mM) and disulfiram (20 mM) stocks were prepared in ethanol and DMSO, respectively.

Whole blood was obtained from normal healthy human adult volunteers (n = 3) with institutional review board approval and the informed consent of participants. After removing plasma and the buffy coat, red blood cells were washed twice in ice-cold 0.9% saline and subjected to centrifugation at 1500 *g* for 5 minutes at 4°C. Incubations were performed in triplicate with cells resuspended at 10% hematocrit in HyClone Hank's Buffered Salt Solution containing calcium, magnesium, and 5.6 mM glucose (catalog # 14025-076; Life Technologies, Carlsbad, CA). Aliquots in TPP polystyrene, flat-bottom, 24-well tissue culture plates were rocked gently (ORBI-Shaker JR, model BT300, 80 rpm) at 37°C. When examining the impact of menadione or disulfiram, appropriate ethanol and DMSO control incubations were also performed.

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Glycolate and oxalate were measured in the incubation media following removal of erythrocytes by centrifugation at 1500 *g* for 5 minutes at 4°C. Glycolate was determined using ion chromatography coupled with mass spectroscopy (Thermo Fisher Scientific Inc.). The ion chromatography equipment consisted of a Dionex ICS-5000 system with an AS15, 2 × 150 mm, anion-exchange column using a controlled temperature of 30°C and a Dionex ERS 500 anion electrolytically regenerated suppressor. A gradient of KOH from 3 to 80 mM over 30 minutes at a flow rate of 0.3 mL/minute was used to separate sample anions. The mass spectrometer (MSQ-PLUS, San Jose, CA) was operated in electrospray ionization negative mode, needle voltage 1.5 V and cone voltage 30 V, 450°C, and the column eluent was mixed with 50% acetonitrile at 0.30 mL/minute using a 0 dead volume mixing tee before entry into the MSQ. Samples were diluted in the same amount of $^{13}C_2$-glycolate. Selected-ion monitoring (SIM) at the following mass-to-charge ratios, $^{12}C_2$-glycolate (SIM75) and $^{13}C_2$-glycolate (SIM77), was undertaken. This was used to quantify glycolate. Oxalate was determined using an AS22, 2 × 150 mm, anion-exchange column at a controlled temperature of 30°C. A 30 mM sodium tetraborate eluent at a flow rate of 0.30 mL/minute was used to separate oxalate from other anions.

Changes in intracellular glyoxylate and glyoxal were measured over time in erythrocytes incubated with 1 mM glyoxal. Samples were centrifuged at 1500 *g* for 5 minutes at 4°C through 1-bromodecane to achieve rapid separation of erythrocytes from media. Intracellular glyoxylate and glyoxal were measured in erythrocytes following extraction with perchloric acid (PCA). Erythrocytes (1 vol) were mixed with Milli-Q water (1 vol) to lyse the cells, and then 1 vol 1.5 M PCA was added. The PCA extract was obtained following centrifugation of the sample at 20,000 *g* for 10 minutes at 4°C to remove precipitated material.

Glyoxylate in extracts was determined by reversed-phase high-performance liquid chromatography following derivatization with phenylhydrazine, as previously described.[14] A Kinetex 100 × 4.6 mm, 2.6 micron, C18, 100 A column (Phenomenex Inc, Torrance, CA) was used at a flow rate of 0.4 mL/minute, 20°C, with ultraviolet detection at 320 nm to separate and measure the phenylhydrazone products. The mobile phase contained 0.1 M ammonium acetate, 4% acetonitrile, and 4% methanol. Assays were incubated at room temperature in the dark for 15 minutes before injection. Glyoxal and methyglyoxal were determined by reversed-phase high-performance liquid chromatography following derivatization with o-phenylenediamine, as previously described.[15] PCA extracts were incubated at 65°C for 1 hour for determination of glyoxal and for 4 hours at room temperature for determination of methylglyoxal. Assays contained the internal standards 2,3-hexanedione and 5-methylquinoxaline. A Kinetex 100 × 4.6 mm, 2.6 micron, C18, 100 A column (Phenomenex Inc) was used at a flow rate of 0.7 mL/minute, 25°C, with ultraviolet detection at 316 nm. A linear gradient program of 40 mM ammonium acetate-acetic acid buffer, pH 4.5 (eluent A) and 85% acetonitrile (eluent B) was utilized to separate the quinoxaline and methylquinoxaline products.

The effect of menadione and disulfiram on the metabolism of glyoxal to glycolate and oxalate synthesis was analyzed by Student's *t* test. Data are expressed as mean ± standard deviation. The criterion for statistical significance was $P < .05$.

RESULTS

Freshly isolated erythrocytes contained low levels of glyoxal, methylglyoxal, oxalate, glyoxylate, and glycolate as shown in Table 1. While the glycolate and oxalate levels were similar to those reported previously in plasma (11), the levels of glyoxal, methylglyoxal and glyoxylate were higher than their levels in plasma, which were reported to be below 300 nM using LC-MS (20).

Table 1. Metabolite levels in freshly isolated erythrocytes

Metabolite	Concentration (μmol/L Hemolysate)
Glyoxal	1.4 ± 0.6
Methylglyoxal	0.7 ± 0.4
Oxalate	2.9 ± 1.1
Glyoxylate	2.3 ± 0.1
Glycolate	8.1 ± 2.6

Erythrocytes effectively metabolized exogenous glyoxal to glycolate, as shown in Figures 1 and 2. This conversion was dependent on glyoxal concentration (Fig. 1A) and time of incubation (Fig. 1B). This metabolism is consistent with the activity of the glyoxalase system (GLO), which consists of 2 enzymes, glyoxalase-1 and glyoxalase-2, using glutathione as a cofactor.[16] With 1 mM glyoxal in the incubation medium, approximately 90% of glyoxal was converted to glycolate in 3 hours (Fig. 1B), suggesting that glyoxal freely permeated into the cell. Small amounts of the glyoxal, ~1.0%, were converted to oxalate.

As glyoxylate is the major precursor of oxalate, the levels of glyoxylate within cells were also examined. The time-dependent increase in intracellular glyoxal and glyoxylate is shown in Figure 2. After 20 minutes' incubation with 1 mM glyoxal, intracellular glyoxal and glyoxylate concentrations increased ~5-fold and ~2-fold, respectively. The level of glyoxal within cells peaked at 10 minutes and glyoxylate at 20 minutes.

To provide support for the pathway, we previously proposed to account for the conversion of glyoxal to oxalate in cells[7]; we incubated cells with menadione, a pro-oxidant that decreases the glutathione concentration in erythrocytes[17] and as a result will decrease glyoxalase-1 activity. The results in Figure 3 show that menadione decreased the amount of glycolate formed by 75% and doubled the amount of oxalate produced ($P < 0.05$). Disulfiram treatment, which inhibits aldehyde dehydrogenase (ALDH) activity[18] and therefore the oxidation of glyoxal to glyoxylate,[19] did not alter glycolate synthesis ($P = .30$) but reduced oxalate synthesis by ~60% (Fig. 3).

COMMENT

Our previous experiments with HepG2 cells indicated that they were capable of converting the dialdehyde, glyoxal, to the dicarboxylic acid, oxalate.[7] The pathway accounting for this synthesis was not elucidated. Erythrocytes, lacking intracellular organelles, have a much simpler metabolism than other cells and are attractive models for analyzing metabolic pathways. They have also been reported to contain glyoxal, which increases in the plasma of individuals with diabetes and other disorders associated with