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OXALATE FORMATION FROM GLYOXAL IN ERYTHROCYTES

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Abstract

OBJECTIVES—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.

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.

CONCLUSIONS—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.

Keywords

urolithiasis; oxalate synthesis; glyoxal; erythrocytes

INTRODUCTION

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

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Conflict of Interest The authors declare that there are no conflicts of interest.

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stones and end-stage renal disease in the rare genetic diseases, Primary Hyperoxaluria types 1–3^{3,4}. Furthermore, it 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 two carbon dialdehyde, glyoxal, may be an important source⁷. Glyoxal in part is derived from lipid peroxidation and protein glycation⁸. 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 formation¹¹ and urinary oxalate excretion is higher in stone formers¹², suggesting that increased glyoxal and oxalate synthesis may be a link between these two diseases.

Glyoxylate is recognized as the major precursor of oxalate in mammalian metabolism¹. 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 model¹³.

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 (90mM) and disulfiram (20mM) stocks were prepared in ethanol and DMSO, respectively.

Whole blood was obtained from normal healthy human adult volunteers (n=3) with IRB approval and the informed consent of participants. After removing plasma and the buffy coat, red blood cells (RBC) were washed twice in ice-cold 0.9% saline and subjected to centrifugation at 1500 g for 5min at 4°C. Incubations were performed in triplicate with cells resuspended at 10% hematocrit in Hyclone Hanks 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.

Glycolate and oxalate were measured in the incubation media following removal of erythrocytes by centrifugation at 1500g for 5min at 4°C. Glycolate was determined using ion chromatography (IC) coupled with mass spectroscopy (Thermo Fisher Scientific Inc.). The IC 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 min at a flow rate of 0.3 ml min⁻¹ was used to separate sample anions. The mass spectrometer (MSQ-PLUS) was operated in ESI negative mode, needle voltage 1.5V, cone voltage 30V, 450°C, and column eluent was mixed with 50% acetonitrile at 0.30 ml/min using a zero

dead volume mixing tee prior to entry into the MSQ. Samples were diluted in the same amount of $^{13}\text{C}_2$ - glycolate. Selected-ion monitoring (SIM) at the following mass/charge ratios, $^{12}\text{C}_2$ - glycolate (SIM75) and $^{13}\text{C}_2$ - 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 30mM sodium tetraborate eluent at a flow rate of 0.30 ml min^{-1} 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 $1500g$ 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 volume) were mixed with Milli-Q water (1 volume) to lyse the cells, and then 1 volume 1.5 M PCA was added. The PCA extract was obtained following centrifugation of the sample at $20,000g$ for 10 minutes at 4°C to remove precipitated material.

Glyoxylate in extracts was determined by reversed phase HPLC following derivatization with phenylhydrazine, as previously described ¹⁴. A Kinetex 100×4.6 mm, 2.6 micron, C18, 100A column (Phenomenex Inc, Torrance, CA) was used at a flow rate of 0.4 ml/min , 20°C , with UV detection at 320nm to separate and measure the phenylhydrazone products. The mobile phase contained 0.1M ammonium acetate, 4% acetonitrile and 4% methanol. Assays were incubated at room temperature in the dark for 15 minutes before injection. Glyoxal and methylglyoxal were determined by reversed phase HPLC following derivatization with o-phenylenediamine, as previously described ¹⁵. PCA extracts were incubated at 65°C for 1 hour for determination of glyoxal and 4 hours at room temperature for determination of methylglyoxal. Assays contained the internal standards 2,3-hexanedione and 5-methylquinoxaline. A Kinetex $100 \times 4.6\text{mm}$, 2.6micron, C18, 100A column (Phenomenex Inc) was used at a flow rate of 0.7ml/min , 25°C , with UV detection at 316nm. A linear gradient program of 40mM 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 metabolism of glyoxal to glycolate and oxalate synthesis was analyzed by the Student's *t*-test. Data are expressed as mean $\pm$ SD. The criterion for statistical significance was $P < 0.05$.

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).

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 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 it 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 Fig.2. After 20 minutes incubation with 1mM 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, which decreases the GSH concentration in erythrocytes ¹⁷ and as a result will decrease glyoxalase-1 activity. The results in Fig. 3 show that menadione decreased the amount of glycolate formed by 75% and doubled the amount of oxalate produced. (p values) Disulfiram treatment, which inhibits aldehyde dehydrogenase activity ¹⁸ and therefore the oxidation of glyoxal to glyoxylate ¹⁹, did not alter glycolate synthesis (P = 0.30), 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 metabolic stress ^{9,10}. The results reported here with intact human erythrocytes suggest that glyoxal is metabolized in these cells by the pathways previously postulated ¹³. These pathways consisted of the glyoxalase system (GLO), which converts the bulk of the glyoxal to glycolate, and a minor pathway involving aldehyde dehydrogenase (ALDH) that converted some glyoxal to glyoxylate which could then be converted to glycolate by glyoxylate reductase (GR) or to oxalate by lactate dehydrogenase (LDH).

The importance of the glyoxalase system in erythrocytes to detoxify glyoxal and methylglyoxal has been recognized for some time. Attention has focused on the known production of methylglyoxal during glycolysis, the major source of energy in the erythrocyte, as a side reaction of the activity of triose phosphate isomerase ²⁰. The formation of glyoxal, which may be derived from lipid peroxidation or glucose autooxidation ²¹, has attracted less attention. Erythrocytes have been reported to contain lipid peroxides suggesting that the oxidation of unsaturated fatty acids could be a glyoxal source in these cells.

Erythrocytes have been reported to contain aldehyde dehydrogenase activity but the isoforms present has not been determined ^{18,22}. ALDH1A1 is the only isoform identified by

proteomics²³, but it has been reported to have a low affinity for short chain aldehydes²⁴. Glyoxylate reductase appears to be ubiquitously expressed in cells and it has been confirmed as part of the erythrocyte proteome²⁵. We have further confirmed its presence in erythrocytes by Western blotting and the analysis of its enzymatic activity (unpublished results). This enzyme, which is NADPH dependent, has a higher affinity for glyoxylate than LDH and will preferentially convert the glyoxylate formed from glyoxal to glycolate²⁶. LDH is still capable of converting a small portion of the glyoxylate to oxalate, however. We have observed with a number of substrates that are metabolized to glyoxylate when fed to HepG2 cells, including glycine and hydroxyproline, that 5 –10% of the glyoxylate formed is converted to oxalate and the rest to glycolate. A similar equilibrium may occur in erythrocytes.

The pathway we have proposed for the conversion of glyoxal to oxalate¹³ is supported by the response of the metabolism to inhibitors. Oxalate synthesis increased when the conversion of glyoxal to glycolate through the glyoxalase system was reduced by menadione which is known to lower glutathione concentrations. The potential role of aldehyde dehydrogenase is supported by the inhibition of oxalate synthesis by disulfiram, an inhibitor of aldehyde dehydrogenase activity. The formation of oxalate via this pathway in healthy individuals may be limited but could be enhanced in individuals with diabetes and possibly other metabolic diseases including Primary Hyperoxaluria and calcium oxalate stone disease. Chronic oxidative stress due to hyperglycemia, hyperoxaluria or calcium oxalate crystalluria could deplete antioxidant defenses within cells, including GSH, which could potentially increase the production of both glyoxal and oxalate in susceptible cells. It could directly contribute to the increased incidence of calcium oxalate stone disease observed in diabetic individuals¹¹. Individuals with Primary Hyperoxaluria Type 1 could be especially affected due to glycolate cycling in hepatocytes as glycolate is oxidized in peroxisomes to glyoxylate by glycolate oxidase which in turn is converted back to glyoxylate in the cytosol by glyoxylate reductase. The hydrogen peroxide generated with this cycling could exacerbate the oxidative stress.

It will be possible to confirm the enzymatic steps proposed in this pathway by examining glyoxal metabolism in cultured cells and genetically modified mice where the expression of the proposed enzymes can be manipulated by various techniques. The process by which glyoxal enters cells is not known and warrants further investigation. In a fully hydrated form it is possible that it shares structural similarities with glycerol and is transported by an aquaglyceroporin²⁷. The use of these model systems may also lead to novel therapies that suppress glyoxal formation and reduce this source of oxalate.

The origin of the relevant acids and aldehydes identified within erythrocytes in Table 1 is of interest. The oxalate and glycolate concentrations are similar to plasma concentrations and may represent equilibrium values between extracellular and intracellular concentrations. Glyoxylate, glyoxal and methylglyoxal are higher within these cells suggesting they may be derived from red cell metabolism. Methylglyoxal may be formed primarily by the secondary activity of triose phosphate isomerase during glycolysis and levels in the range of 100 – 400 nM have been reported^{28,29}. The concentration of glyoxal in Table 1 is higher but lower

than that of malondialdehyde, a dialdehyde also formed by lipid peroxidation, which has been reported to be 13.2 μM in erythrocytes³⁰.

The carbon source for glyoxal formation is not known with any certainty. The peroxidation of polyunsaturated fatty acids, the autooxidation of glucose, and the metabolism of glycated amino acid residues in proteins are believed to be potential sources. Without this knowledge it is difficult to discern how much glyoxal is produced in the body each day. It is very possible that a conversion rate of 1% of the glyoxal formed to oxalate is quite significant.

This study has limitations. The reactions were observed in vitro and may not reflect what occurs under in vivo conditions with physiological concentrations of glyoxal. The metabolism was studied in erythrocytes from a small number of normal individuals and more variable results may be observed in a larger and diseased populations. Some carbonylation of lysine residues in proteins by glyoxal may have occurred and could have diminished the metabolism that was observed. Whether this pathway in erythrocytes reflects metabolism in other cells and tissues remains to be resolved.

CONCLUSIONS

Erythrocytes contain low levels of glyoxal, methylglyoxal, oxalate glycolate and glyoxylate. They take up exogenous glyoxal and convert it primarily to glycolate. Approximately 1% is converted to oxalate. Inhibition of oxalate synthesis by disulfiram suggests that glyoxylate is an intermediate and that aldehyde dehydrogenase converts a small fraction of the glyoxal to oxalate. Depleting intracellular glutathione decreases glycolate formation from glyoxal and increases the amount of oxalate formed. This pathway for the formation of oxalate may be enhanced in diabetes and other disease with increased oxidative stress.

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Fig. 1. Relationship of glyoxal concentration (**A**) and time (**B**) to the conversion of glyoxal to glycolate and oxalate in erythrocytes. Media concentrations of oxalate (□) and glycolate (●) were determined by IC/MS after incubation with erythrocytes (10% hematocrit).

Fig. 2.
Time dependence of the changes in the intracellular levels of glyoxal and glyoxylate. PCA extracts were obtained at varying time intervals after the incubation of erythrocytes (10% hematocrit) with 1mM glyoxal to measure glyoxal (■) and glyoxylate (△) by HPLC.

Fig. 3.

Impact of menadione and disulfiram on metabolism of glyoxal to glycolate and oxalate in erythrocytes. Erythrocytes (10% hematocrit) were incubated for 1 hour with 1mM glyoxal and inhibitor, and media harvested for metabolite measurements. Media harvested from erythrocytes without inhibitor and with inhibitor are represented by the black bars, (■), and open bars, (□), respectively. * indicates a significant difference ($P < 0.05$).

Table 1

Metabolite levels in freshly isolated erythrocytes.

Metabolite	Concentration ($\mu\text{mol/L}$ 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