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Conjugation of the linoleic acid oxidation product, 13-oxooctadeca-9, 11-dienoic acid, a bioactive endogenous substrate for mammalian glutathione transferase

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Abstract

The oxidation of linoleic acid leads to the generation of several products with biological activity, including 13-oxooctadeca-9,11-dienoic acid (13-OXO), a bioactive 2,4-dienone that has been linked to cell differentiation. In the current work, the conjugation of 13-OXO by human glutathione transferases (GSTs) of the alpha (A1–1, A4–4), mu (M1–1, M2–2) and pi (the allelic variants P1–1/ile, and P1–1/val) classes, and a rat theta (rT2–2) class enzyme has been evaluated. The kinetics and stereoselectivity of the production of the 13-OXO-glutathione conjugate (13-OXO-SG) have been examined. In contrast to many xenobiotic substrates, the endogenous substrate 13-OXO does not exhibit an appreciable non-enzymatic rate of conjugation under physiological conditions. Therefore, the GST-catalyzed conjugation takes on greater significance as it provides the only realistic means for formation of 13-OXO-SG in most biological systems. Alpha class enzymes are most efficient at catalyzing the formation of 13-OXO-SG with k_{cat}/K_m values of 8.9 $mM^{-1}\,s^{-1}$ for GST A1–1 and 2.14 $mM^{-1}\,s^{-1}$ for GST A4–4. In comparison, enzymes from the mu and pi classes exhibit specificity constants from 0.4 to 0.8 $mM^{-1}\,s^{-1}$. Conjugation of 13-OXO with glutathione at C-9 of the substrate can yield a pair of diastereomers that can be resolved by chiral HPLC. GSTs from the mu and pi classes are the most stereoselective enzymes and there is no apparent relationship between catalytic efficiency and stereoselectivity. The role of GST in the metabolic disposition of the bioactive oxidation products of linoleic acid has implications for the regulation of normal cellular functions by these versatile enzymes.

Keywords: Glutathione transferase; 13-oxooctadecadienoic acid; Lipid oxidation; Linoleic acid oxidation; Glutathione conjugation

1. Introduction

The enzymatic oxidation of linoleic acid produces a number of products with biological activity (Fig. 1). Among these are hydroxy fatty acids, such as 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE), and the 2,4-dienone 13-oxooctadeca-9,11-dienoic acid (13-OXO). The production of these compounds in mammalian systems has been linked to both normal and pathologic processes. Thus, their metabolic disposition is likely to play an important role in the ultimate biological responses resulting from linoleate oxygenation [1–5]. For example, 13-HODE is a contributor to the EGF signal transduction cascade, while the enzyme that produces 13-OXO has been implicated in cellular differentiation [6–9]. Thus, the formation and persistence of these products can determine the physiological consequences of their generation.

An important metabolic fate of 13-OXO is conjugation with glutathione and the subsequent energy-dependent export of the 13-OXO-glutathione conjugate (13-OXO-SG) out of the cell [10,11]. This metabolic process is likely to silence the signals produced by the oxidative metabolism of linoleic acid. Given that 13-OXO represents one of the endogenous substrates for glutathione transferases (GST), it is important to characterize the contribution of individual GSTs to the production of 13-OXO-SG [12,13].

The present studies have investigated the specificity of a variety of human and one rat GST enzymes for conjugation of 13-OXO. The kinetics of the process and the stereoselectivity of product formation have been determined for GSTs of the alpha, pi, mu, and theta classes. In addition to

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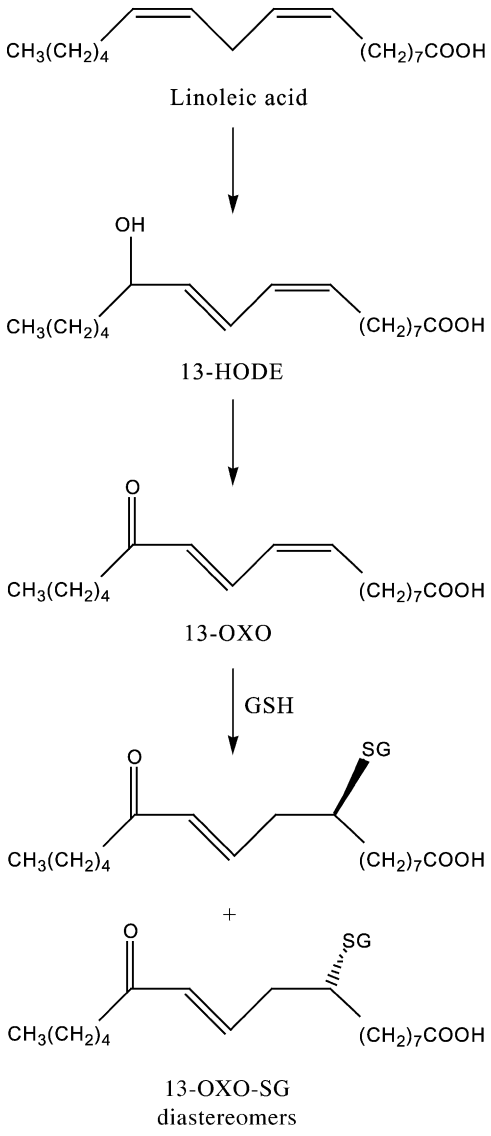

Fig. 1. Oxidative metabolism of linoleic acid to bioactive products. The final reaction is conjugation of 13-OXO with glutathione to produce a pair of diastereomers.

identifying the most active GSTs for conjugation of 13-OXO, the results show that catalytic efficiency and stereoselectivity are not tightly coupled.

2. Methods

The substrate 13-OXO was prepared by acetyl chloride-mediated dehydration of 13-hydroperoxyoctadecadienoic acid [14]. The 13-hydroperoxy precursor was prepared by soybean lipoxygenase-catalyzed oxygenation of linoleic acid [15]. Silicic acid chromatography was used to isolate 13-OXO and the purity was analyzed by HPLC [2,10].

Human GSTs A1–1, A4–4, M2–2, the allelic variants P1–1/ile105 and, P1–1/val105, as well as the rat enzyme rGST T2–2 were prepared by heterologous expression in *Escherichia coli* and purified by affinity chromatography as previously described [16–18]. GST M1–1 was purchased from PanVera Corp., Madison, WI.

2.1. Enzyme assay

The conjugation of 13-OXO catalyzed by GST was determined by measuring the decrease in absorbance at 285 nm in 0.1 M sodium phosphate at pH 6.5 and 30 °C. The concentration of GSH was 1 mM for all experiments, and for determination of k_{cat}/K_m the concentration of 13-OXO was varied between 2.5 and 60 μM using at least six different concentrations. In an attempt to generate complete saturation curves, concentrations of 13-OXO up to 180 μM were assayed, and for some, but not all enzymes, saturation was observed. A minimum of two incubations at each concentration were performed. Due to the high molar absorptivity of 13-OXO, 180 μM was the highest concentration at which reaction rates could be reliably determined. A molar absorptivity of 28.0 mM^{-1} cm^{-1} was used for 13-OXO to convert the change in absorbance to reaction rates. The rate of the non-enzymatic reaction between 13-OXO and GSH at pH 6.5 was measured periodically and found to be negligible. The kinetic constant k_{cat}/K_m was obtained by nonlinear regression analysis using GraphPad PRISM version 2.0 (GraphPad Software Inc., San Diego, CA).

The activity of GST was also measured using 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig et al. [19]. The non-enzymatic rate of this reaction was measured and subtracted prior to calculating CDNB-based enzyme activity. To determine the effect of 13-OXO on the conjugation of CDNB by GST, the enzyme was incubated with 2.5, 5, 10, or 20 μM 13-OXO for 5 min prior initiating the reaction by the addition of GSH and CDNB.

2.2. Chromatography

Chiral HPLC was performed to resolve the stereoisomers of 13-OXO-SG produced by the various GSTs [10]. A 4.0 ml reaction volume containing 200 μM 13-OXO, 5 mM GSH, and 12.5 μg/ml GST in 20 mM phosphate pH 6.5 was incubated at 37 °C for 1 h. The reaction mixture was acidified to pH 4.0 then applied to a 1 ml ODS solid phase extraction (SPE) column that had been conditioned with 5 ml of methanol and 5 ml of phosphate buffer prior to sample application. The SPE column was washed with 10 ml of water and 5 ml of 20% (v/v) acetonitrile in water. Then the 13-OXO-SG conjugate was obtained by elution with 5 ml of 40% acetonitrile in water. The volume of the 40% acetonitrile fraction was reduced to about 100 μl by rotary evaporation then 300 μl of HPLC solvent was added. The HPLC solvent was 54 mM ammonium acetate in 10% acetonitrile pH 4.5. Analysis was performed using a Chirobiotic V column (Advanced Separation Technologies Inc., Whippany, NJ). The conjugates were eluted isocratically at 1.0 ml/min while monitoring the absorbance at 235 nm. The

elution time of the two diastereomers of 13-OXO-SG was verified by co-injection of a racemic mixture prepared non-enzymatically. Data for each diastereomer are presented as the mean percent ± S.D. of total 13-OXO-SG from a minimum of three incubations.

3. Results

The enzymes examined include human GSTs A1–1, A4–4, M1–1, M2–2, the allelic variants P1–1/ile105 and P1–1/val105, and rat T2–2. All the enzymes tested catalyze the conjugation of 13-OXO with GSH. The specific activities of the various GSTs with 60 μM 13-OXO and 1 mM GSH are presented in Table 1. Under these conditions, the highest specific activity is seen in the alpha class GST A4–4. However, the specific activity of the alpha, mu, and pi classes are all within a factor of five of one another. Also, there is no marked difference between the allelic GST P1–1 variants. The rat theta class enzyme is a comparatively poor catalyst for conjugation of 13-OXO, but does catalyze the reaction. It is important to note that the non-enzymatic rate of the reaction between 13-OXO and GSH was measured repeatedly over the course of these experiments and is spectrophotometrically undetectable under the conditions used to determine the specific activities. A non-enzymatic rate of reaction was only detectable when the GSH concentration was at least 5 mM in the presence of 100 μM 13-OXO.

The catalytic efficiency (k_{cat}/K_m) of the various GSTs is also presented in Table 1. The alpha class enzymes clearly have the highest efficiency using 13-OXO as a substrate, with the theta class enzyme being least efficient. While determining the saturation curves for the various enzymes, GST A4–4 showed evidence of substrate inhibition at higher concentrations of 13-OXO. A typical saturation curve for GST A4–4 is shown in Fig. 2. The highest concentration that could be examined was 180 μM and at this concentration of 13-OXO, the activity of GST A4–4 is barely detectable. None of the other enzymes showed any evidence of substrate inhibition under similar conditions, although in some cases saturation was observed.

Table 1
Specific activity and specificity constants (k_{cat}/K_m) for various GSTs using 13-OXO as substrate

GST	Specific activity (μmol min^{-1} mg^{-1})[a]	k_{cat}/K_m (mM^{-1} sec^{-1})[b]
A1–1	0.23	8.9
A4–4	0.60	2.1
M1–1	0.32	0.40
M2–2	0.20	0.66
P1–1/ile105	0.13	0.80
P1–1/val105	0.11	0.43
rT2–2	0.003	0.021

[a] The reaction system contained 60 μM 13-OXO and 1 mM GSH.
[b] Values determined by non-linear regression analysis of kinetic data obtained with 0–60 μM 13-OXO and 1 mM GSH with a minimum of duplicate incubations at each concentration tested.

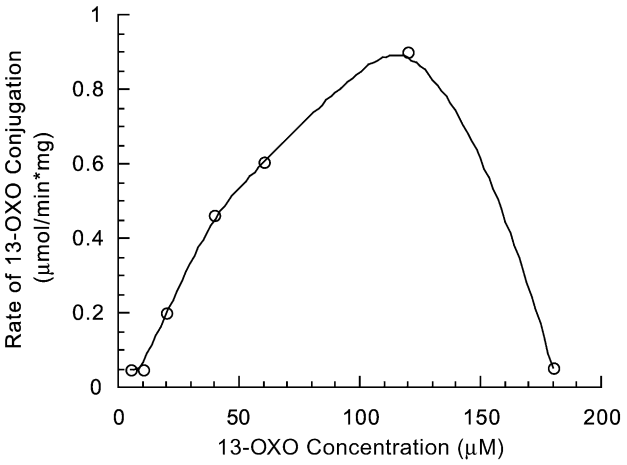

Fig. 2. Saturation curve for GST A4–4 with 13-OXO as substrate in the presence of 1 mM GSH. Each data point shown is the mean of at least duplicate incubations. The low activity at the highest 13-OXO concentration is unique to GST A4–4.

To further examine the nature of the substrate inhibition of GST A4–4, the effect of 0–20 μM 13-OXO on the GST-catalyzed substitution reaction of CDNB was determined and the results are presented in Fig. 3. The GST was incubated with 13-OXO for 5 min prior to measuring CDNB conjugation. At concentrations of 13-OXO below 20 μM the conjugation of CDNB is not affected. However, at 20 μM 13-OXO, noncompetitive inhibition is evident.

The conjugation of 13-OXO by addition of GSH to C-9 yields two diastereomeric products depending on which face of the 2,4-dienone system is attacked. For example, rat GST A4–4 and the GST from HT-29 cells produce different

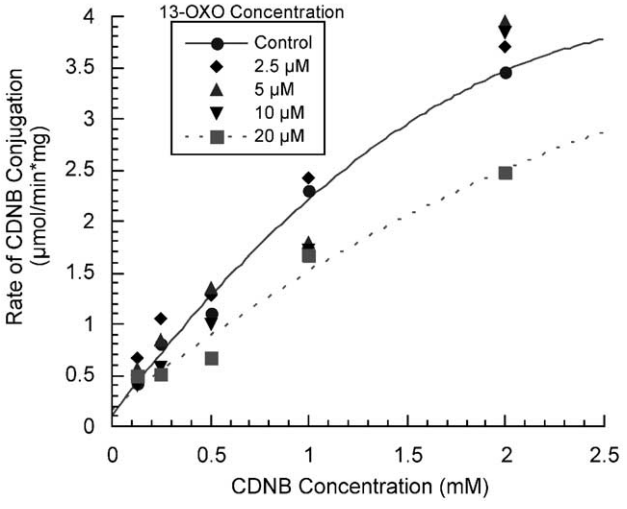

Fig. 3. Effect of low concentrations of 13-OXO on the conjugation of CDNB by GST A4–4. The enzyme was incubated with 13-OXO for 5 min prior to measuring the rate of CDNB conjugation. Each data point is the mean of at least three determinations. The inhibition of CDNB conjugation by low concentrations of 13-OXO is not simple substrate competition.

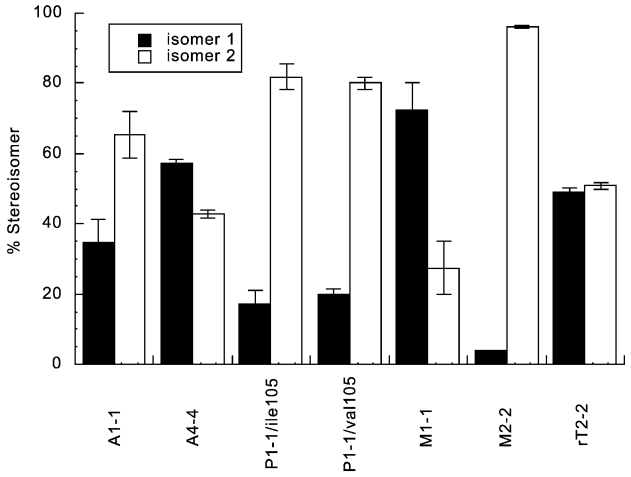

Fig. 4. Stereoselectivity of 13-OXO-SG formation by various GSTs. Diastereomer production was determined by chiral phase HPLC each column represents the mean ± S.D. of triplicate incubations.

diastereomeric products [10]. Therefore, to characterize the stereoselectivity of the GST enzymes under study, reaction mixtures were purified by SPE, then analyzed by chiral phase HPLC [10]. The absolute stereochemistry of the two diastereomers is not yet known, thus, the compounds are identified as isomer 1 and isomer 2 based on the elution time. The results are presented in Fig. 4. From these experiments, it is clear that the various GSTs display different stereoselectivity and that the specificity is a characteristic of the individual enzyme and not the class of GST. For example, the two mu class enzymes show distinctly different stereoselectivity as do the two alpha class enzymes, although to a lesser extent than the mu class. There does not appear to be any relationship between enzymatic activity as measured by catalytic constants and the stereoselectivity of product formation.

4. Discussion

The GSTs catalyze a variety of reactions utilizing a diverse array of substrates [13,20,21]. A vast majority of these substrates are xenobiotics or, alternatively, compounds formed via non-enzymatic processes such as those that occur under conditions of oxidative stress. In the present report, we have examined the conjugation of 13-OXO an endogenous substrate for GSTs derived from the oxidative metabolism of linoleic acid (Fig. 1). It is important to characterize the enzymology of the reaction between 13-OXO and GSH as this could provide insight into tissue-specific responses to linoleate oxygenation.

The alpha class GSTs have the largest specificity constants for 13-OXO. This is the physiologically most relevant parameter, since the specificity constant is a measure of catalytic efficiency and governs the activity at the low substrate concentrations likely to occur in biological systems. However, with the exception of the rT2-2 enzyme, the differences between the various enzymes are not dramatic. Thus, any tissue expressing GST from the alpha, mu, or pi class should be able to readily conjugate 13-OXO. The high levels of 13-HODE dehydrogenase in the liver and colon are accompanied by similarly high levels of the alpha and pi class GSTs in these tissues [22,23]. For example, GST A1–1 is a major component in hepatocytes, representing 2–3% of the cytosolic protein and GST P1–1 is present in the colon as well as in most other tissues (except liver) [23]. Such correlations raise the possibility that GST from the alpha and pi classes are especially important for oxidized linoleate metabolism in liver and colon.

The conjugation of 13-OXO with glutathione is one of several Michael addition reactions of physiological significance. In comparison to other substrates undergoing Michael addition, 13-OXO is a relatively sluggish substrate [16,24,25]. For example, GST A4–4, when using the lipid peroxidation product 4-hydroxynonenal as a substrate, exhibits a k_{cat}/K_m of 3100 mM^{-1} s^{-1} [16]. In comparison, with 13-OXO as a substrate, GST A4–4 exhibits a k_{cat}/K_m of 2.14 mM^{-1} s^{-1}. On the other hand, for many GSTs, the Michael addition of GSH to ortho-quinones and base propenals is comparable to the efficiency with which 13-OXO is conjugated [24,25]. An exception is the mu class of GST, which are most efficient at conjugating ortho-quinones, but unexceptional when using 13-OXO as a substrate. These results suggest that either 13-OXO is a relatively inefficient substrate, or the most efficient GST for catalyzing 13-OXO conjugation has not yet been identified. However, there may be a biological advantage to relatively slow conjugation of 13-OXO. In particular, the modification of specific protein targets by 13-OXO has been proposed as one mechanism by which the compound exerts biological activity [26]. Therefore, the modification reactions are favored if the reactive 13-OXO is not removed too rapidly.

There are other important differences between the conjugation of exogenous compounds and conjugation of the endogenous substrate 13-OXO. For example, most xenobiotic substrates for GST have a significant rate of reaction with GSH even in the absence of the enzyme. This is not the case with 13-OXO since under physiologic conditions, the rate of non-enzymatic conjugation is undetectable. Thus, in biological systems, conjugation of 13-OXO must be enzyme-catalyzed and could provide an important mechanism for termination of the linoleate-mediated signals.

The interaction of 13-OXO with GST A4–4 is notably different than interactions with other GST enzymes. In particular, substrate inhibition of GST A4–4 is observed at high concentrations of 13-OXO although the origin of this effect is not clear. Given the high concentrations of 13-OXO required, it is doubtful whether the phenomenon has a direct effect upon the metabolic disposition of linoleate oxidation products. On the other hand, relatively low concentrations of 13-OXO perturb the metabolism of xenobiotic substrates

catalyzed by GST A4–4. For example, at 20 μM 13-OXO the rate of CDNB conjugation is reduced. The data presented in Fig. 3 suggest a mode of inhibition more complex than simple alternative substrate competition, since the noncompetitive effect shows that both CDNB and 13-OXO can bind simultaneously to the enzyme. Thus, while it is conceivable there is more than one binding site for 13-OXO further experiments will be required to definitively answer this question [21].

In an effort to more fully characterize the conjugation of 13-OXO by GST, the stereoselectivity of product formation was determined. It is not yet known if the stereochemistry of this conjugation reaction is biologically significant. Nonetheless, distinct differences in stereoselectivity of 13-OXO-SG production are observed between the various GSTs and the stereochemistry is not class-specific. For example, the two alpha class enzymes produce different ratios of diastereomers, as do the two mu class enzymes. The highest stereoselectivity is demonstrated by the pi and mu classes, while the theta class enzyme is least specific. Bogaards et al. [27] reported that the stereochemical outcome of GST-catalyzed reactions is substrate as well as enzyme-dependent. Their results are supported by the present experiments as the stereoselectivity of a particular class of GST toward 13-OXO is not the same as the stereoselectivity with other unsaturated carbonyl compounds. We have also observed that the stereoselectivity of a particular GST is not related to catalytic efficiency. For example, GST A4–4 is a relatively efficient catalyst of conjugation, however, this enzyme is only slightly more stereoselective than the inefficient rGST T2–2 enzyme.

The experiments in the present report have examined the specificity of several forms of GST toward an enzyme-generated, bioactive polyunsaturated fatty acid oxidation product. The endogenous substrate, 13-OXO, is relatively sluggish compared to numerous xenobiotic and non-enzymatic lipid peroxidation products. However, in contrast to these compounds, the endogenous substrate does not react with GSH in the absence of GST. The relatively low rate of conjugation of 13-OXO may be beneficial in that it increases the probability of alternate reaction paths that are presumably important for the biological activity of 13-OXO [26].

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