J. steroid Biochem. Vol. 35, No. 6, pp. 705–710, 1990
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0022-4731/90 $3.00 + 0.00
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PEROXIDASE-CATALYZED DISPLACEMENT OF TRITIUM FROM REGIOSPECIFICALLY LABELED ESTRADIOL AND 2-HYDROXYESTRADIOL

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(*Received* 4 *October* 1989)

Summary—Estradiol and 2-hydroxyestradiol labeled with ^3H at different positions in rings A, B or D were incubated with lactoperoxidase without added H_2O_2 and their oxidative transformation was followed by transfer of ^3H into ^3H_2O. With estradiol, ^3H loss from different positions in the aromatic ring was almost equal and also occurred to a lesser extent from the alicyclic portion of the molecule. Glutathione had less effect on the formation of ^3H_2O from the aromatic ring of estradiol than from that of the catechol estrogen where it increased the yield 6-fold. The rate of ^3H loss was also very much greater from tritiated 2-hydroxyestradiol than from estradiol and NADPH was inhibitory with both steroids. Conditions for the release of ^3H from estradiol and 2-hydroxyestradiol by peroxidase as well as the effect of some biochemical inhibitors were also investigated. The possible contribution of peroxidative formation of ^3H_2O during the radiometric assay for catechol estrogen biosynthesis by tissue monooxygenases is discussed.

INTRODUCTION

In recent years, considerable evidence has been obtained for peroxidative metabolism of estrogens with the formation of reactive intermediates capable of binding covalently to glutathione (GSH) and protein. Enzymes which have been shown to catalyze a stepwise one electron oxidation of estrone or estradiol to a phenoxy radical or to a quinone include horseradish peroxidase [1–4], lactoperoxidase [5], uterine peroxidase [6–8], prostaglandin H synthase [9–11], breast tumor and placental enzymes [12, 13] and pseudoperoxidases [14]. The formation of catechol estrogens by direct isolation has been shown in some of these systems [9, 12, 13], while in other cases, binding to macromolecules [1–4] or formation of ^3H_2O from the ^3H-labeled steroid was measured [8, 11, 14]. The further oxidation and covalent binding of the catechol estrogen by rat liver microsomes has also been studied [15, 16] and evidence provided for the generation of superoxide anion and estrogen semiquinone by electron abstraction mediated by cytochrome *P*-450. This heme enzyme has also been shown to act peroxidatively [17, 18].

In this paper, we compared the relative displacement of ^3H from estradiol and its catechol derivative labeled in different positions of the steroid nucleus, using lactoperoxidase as a model system. We examine the specificity and optimum conditions for this reaction and the role of glutathione which can act as an acceptor for the activated steroid [14] and may also generate H_2O_2 [1]. This information should help to assess the contribution of reactions other than the classical monooxygenase step catalyzed by cytochrome *P*-450 in the radiometric assay for estrogen-2/4-hydroxylation in different tissues [19, 20].

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MATERIALS AND METHODS

Materials

[2-^3H]E_2 (25.3 Ci/mmol), [6,7-^3H]E_2 (52 Ci/mmol) and [16α-^3H]E_2 (15–30 Ci/mmol), were purchased from New England Nuclear (Boston, Mass); the other ^3H-labeled estrogens or catechol estrogens were prepared and purified as described previously [28]. ^3H-labeled 2-OHE_2 was generally re-purified by TLC on silica gel (cyclohexane–ethyl acetate–ethanol 10:9:1) before use. Lactoperoxidase (LP; 80–100 units/mg protein), catalase (17,500 units/mg protein) from bovine liver, glutathione (GSH) and the coenzymes were obtained from Sigma Chemical Co. (St Louis, Mo.). Superoxide dismutase (5000 units/mg lyophilisate) from bovine erythrocytes was purchased from Boehringer Mannheim (Montreal, PQ). All other chemicals were the purest available commercially.

Conditions of incubation

The ^3H-labeled steroids were incubated for 20 min with constant shaking at 37°C with lactoperoxidase (12.5 μg/ml) in the presence and absence of NADPH (0.3 mM) or GSH (2 mM) in 4 ml of Tris–HCl

Table 1. Effect of NADPH and glutathione on the loss of ^3H or formation of water-soluble products from regiospecifically-labeled estradiol by lactoperoxidase

Additions to LP	No. of experiments	^3H-transfer into ^3H_2O (%)	Water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
[1-^3H]E_2				
—	5	12.2 ± 2.6	17.4 ± 3.3	5.2
NADPH	5	0.6 ± 0.4	2.1 ± 0.4	1.5
GSH	5	8.0 ± 0.8	38.6 ± 3.4	30.6
NADPH + GSH	5	6.2 ± 0.8	23.9 ± 1.9	17.7
[2-^3H]E_2				
—	23	12.9 ± 1.3	17.2 ± 1.7	4.3
NADPH	8	0.8 ± 0.3	3.9 ± 1.2	3.1
GSH	25	14.4 ± 1.7	42.2 ± 2.6	27.8
NADPH + GSH	11	12.8 ± 0.8	32.0 ± 1.1	19.2
[4-^3H]E_2				
—	8	8.0 ± 1.6	11.2 ± 2.0	3.2
NADPH	4	0.4 ± 0.2	2.5 ± 0.2	2.1
GSH	8	10.4 ± 1.2	37.9 ± 4.4	27.5
NADPH + GSH	6	8.6 ± 0.9	27.0 ± 2.0	18.4
[6,7-^3H]E_2				
—	10	6.7 ± 1.0	12.5 ± 1.4	5.8
NADPH	5	0.3 ± 0.1	3.2 ± 0.6	2.9
GSH	9	1.5 ± 0.5	39.4 ± 5.0	37.9
NADPH + GSH	5	0.9 ± 0.5	25.0 ± 3.2	24.1
[16α-^3H]E_2				
—	5	5.1 ± 1.4	8.4 ± 2.4	3.1
NADPH	4	0.1 ± 0.1	1.2 ± 0.6	1.1
GSH	5	0.6 ± 0.6	53.2 ± 3.3	52.6
NADPH + GSH	4	0.7 ± 0.4	30.9 ± 2.4	30.2

^3H-labeled E_2 (9.2 μM) was incubated for 20 min at 37°C in 4 ml of Tris–HCl (0.1 M), pH 7.4 with lactoperoxidase (LP, 12.5 μg/ml) in the presence and absence of NADPH (0.3 mM) or GSH (2 mM). The amount of ^3H_2O formed during the reaction and the amount of ^3H radioactivity remaining in the ether-extracted aqueous fraction was determined as described in the text. Data are presented as means ± SE. ^3H-transfer into ^3H_2O or water-soluble radioactivity in the absence of LP: <0.5% or 0.5–2.2% respectively.

(0.1 M), pH 7.4. After extraction three times with equal volumes of diethyl ether, the radioactivity in a portion (0.1 ml) of the aqueous fraction was determined and also the amount of ^3H_2O formed was measured as described below.

Determination of ^3H release into ^3H_2O

This was determined by the radiometric assay of Fishman *et al.*[19]. Aliquots (0.5 ml) of the ether-extracted aqueous fraction were allowed to evaporate to dryness in a fumehood at room temperature, and ^3H_2O formation was determined from the difference in ^3H radioactivity in the original sample and that in the dry residue redissolved in the same volume of H_2O. This assay gave results identical to those obtained by lyophilization [28].

RESULTS

The results in Table 1 show that lactoperoxidase catalyzes a significant loss of ^3H from not only the aromatic but also rings B or D of estradiol and that glutathione (GSH) at 2 mM enhanced this process in ring A but inhibited ^3H_2O formation from C-6,7 and C-16. In all cases NADPH was inhibitory while GSH increased the yield of ^3H-labeled aqueous metabolites (water-soluble radioactivity minus ^3H_2O). The higher yield of these metabolites from E_2 labeled at C-6,7 or C-16 can be explained if conjugation with GSH occurs primarily in the aromatic ring of the steroid with little preference for positions C-1, C-2 or C-4. With each regiospecifically-labeled steroid only 2 of the 3 possible water-soluble conjugates would still retain the radioactive label, the third one losing ^3H as ^3H_2O during the reaction with GSH. Some ^3H on C-6 might be displaced when conjugation occurs at C-4, and is supported by the results of the experiments with 2-hydroxyestradiol (Table 2) but such a mechanism would not apply to ^3H loss from ring D. Very little ^3H_2O (< 0.5%) or water-soluble radioactivity (0.5–2.2%) was observed in the absence of the enzyme or with a boiled solution of peroxidase. With regiospecifically-labeled 2-hydroxyestradiol, lactoperoxidase caused a large transfer of ^3H into ^3H_2O from either the C-1 or C-4 labeled catechol estrogen when GSH was present but had little effect when ^3H was localized in rings B or D (Table 2). However, some release of radioactivity from C-6,7 of the catechol estrogen was observed. The rate of reaction catalyzed by lactoperoxidase in the presence of GSH was much greater with 2-hydroxyestradiol than with estradiol as substrate reaching its maximum within 2 min (Fig. 1). The marked differential loss of ^3H from various carbon atoms in ring A of either E_2 or 2-OHE_2 observed previously with liver monooxygenase or tyrosine [16, 27] was not produced by lactoperoxidase and, in contrast to those experiments, was lower when ^3H was located on C-4 of E_2. It should also be noted that no H_2O_2 or other hydroperoxide was added to the system and it was found that the loss

Table 2. Effect of NADPH and glutathione on the loss of ^3H or formation of water-soluble products from regiospecifically-labeled 2-hydroxyestradiol by lactoperoxidase

Additions to LP	^3H-transfer into ^3H_2O (%)	Water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
[1-^{3}H]2-OHE$_2$			
—	8.9 (4.7)	27.0 (14.2)	18.1 (9.5)
NADPH	3.2 (0)	7.5 (3.6)	4.3 (3.6)
GSH	52.6 (1.9)	92.2 (6.3)	39.6 (4.4)
NADPH + GSH	42.9 (1.1)	96.2 (6.7)	53.3 (5.6)
[4-^{3}H]2-OHE$_2$			
—	11.3 ± 1.6 (7.1 ± 2.0)	22.1 ± 4.6 (15.8 ± 4.5)	10.8 (8.7)
NADPH	2.5 ± 0.5 (1.4 ± 0.5)	5.1 ± 0.7 (3.4 ± 1.3)	2.6 (2.0)
GSH	68.4 ± 1.8 (3.8 ± 1.6)	91.6 ± 1.9 (6.7 ± 2.4)	23.2 (2.9)
NADPH + GSH	64.5 ± 1.1 (4.1 ± 1.7)	93.9 ± 1.7 (7.8 ± 3.2)	29.4 (3.9)
[6,7-^{3}H]2-OHE$_2$			
—	2.5 (0.7)	15.7 (15.6)	12.2 (14.9)
NADPH	0 (0)	5.7 (4.7)	5.7 (4.7)
GSH	4.9 (0)	93.7 (7.0)	88.8 (7.0)
NADPH + GSH	5.5 (0)	91.8 (6.2)	86.3 (6.2)
[16α-^{3}H]2-OHE$_2$			
—	0.6 (0.3)	21.8 (10.0)	21.1 (10.0)
NADPH	0.3 (0)	5.1 (6.1)	4.7 (6.1)
GSH	2.2	88.7 (10.2)	86.5 (10.2)
NADPH + GSH	3.0	89.2 (8.3)	86.2 (8.0)

^{3}H-labeled 2-OHE$_2$ (9.2 μM) was incubated with lactoperoxidase in the presence or absence of NADPH or GSH as in Table 1. Values in parentheses were obtained in the absence of enzyme. The results are expressed as a means of two experiments or of 3–5 experiments (±SE).

of ^3H_2O from [2-^{3}H]E$_2$ was insensitive to pH change between 6 and 8 (not shown). GSH behaved in a biphasic manner being inhibitory at low and also high concentrations with an optimum at 3 mM (Fig. 2). H_2O_2 as expected, enhanced the displacement of ^3H_2O from [2-^{3}H]E$_2$ reaching a maximum at 20 μM (Fig. 3).

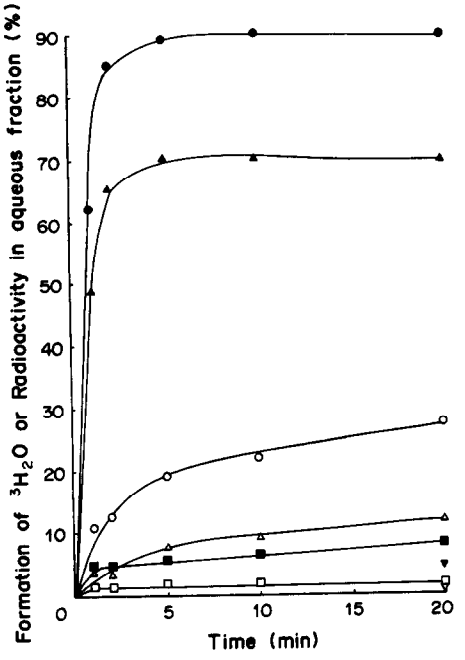

Fig. 1. Rates of formation of ^3H_2O or water-soluble products from ^{3}H-labeled estradiol and 2-hydroxyestradiol catalyzed by lactoperoxidase. [2-^{3}H]E$_2$ or [4-^{3}H]2-OHE$_2$ (9.2 μM) was incubated for various time periods at 37°C with lactoperoxidase (LP) (12.5 μg/ml) and GSH (2 mM) in 4 ml of Tris–HCl (0.1 M), pH 7.4. The amount of ^3H_2O formed during the reaction and the amount of ^{3}H remaining in the ether-extracted aqueous fraction was determined as described in the text. Open symbols: [2-^{3}H]E$_2$, closed symbols: [4-^{3}H]2-OHE$_2$. ○, ● water-soluble ^{3}H-radioactivity; △, ▲ ^3H_2O; □, ■ water-soluble ^{3}H-radioactivity, no LP; ▽, ▼ ^3H_2O, no LP.

The effect of various inhibitors on the lactoperoxidase catalyzed formation of ^3H_2O and aqueous metabolites is shown in Table 3. In these experiments, GSH was also added to optimize conjugation and to determine whether this reaction was entirely dependent on the previous activation of the steroid. Some adduct formation can also occur to lactoperoxidase itself and other proteins in the enzyme preparation. Ascorbate and the reduced coenzymes NADH and NADPH were effective inhibitors of the system but catalase and superoxide dismutase, even at high concentration, did not inhibit completely the lactoperoxidase-catalyzed release of ^3H_2O from [2-^{3}H]E$_2$. Glutathione prevented the inhibitory action of NADH and NADPH but was less effective against catalase and did not reverse the inhibition produced by ascorbate.

DISCUSSION

The catechol estrogens formed by 2- or 4-hydroxylation of the parent estrogen have been shown to modulate a number of important endocrine functions when administered in pharmacological doses [21, 22]. Several tissues can convert estrogens to their catechol metabolites [21, 22] and therefore it is important to have a sensitive and reliable assay to follow this reaction. In the liver, where 2-hydroxylation by microsomal NADPH-dependent cytochrome *P*-450 predominates, the radiometric assay [20] has been found to agree relatively well with product isolation [23]. However, in the brain and other tissues where yields are low, some loss of ^{3}H may be due to

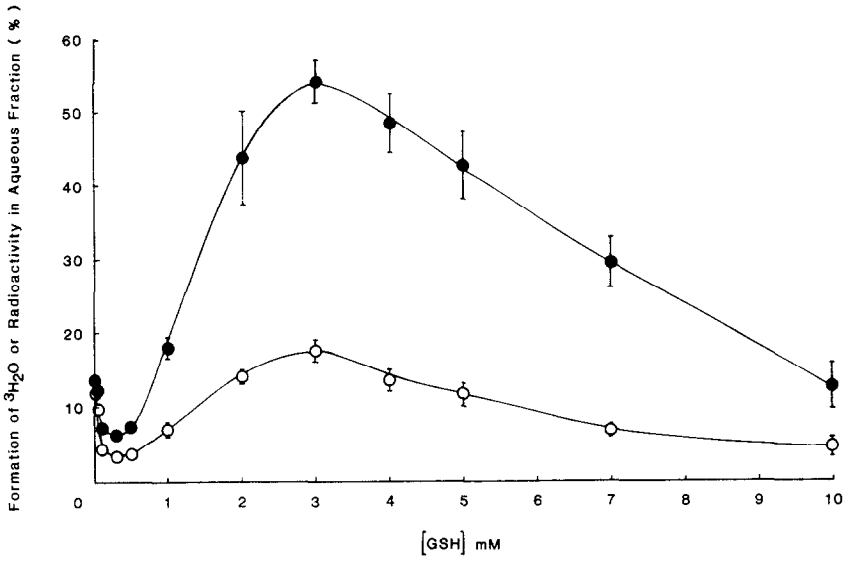

Fig. 2. Effect of glutathione on the formation of ^3H_2O or water-soluble products from [2-^{3}H]estradiol by lactoperoxidase. [2-^{3}H]E_2 (9.2 μM) was incubated for 20 min at 37°C with lactoperoxidase (12.5 μg/ml) and various concentrations of glutathione (GSH). The amount of ^3H_2O formed ○ and ^{3}H remaining in the ether-extracted aqueous medium ● was determined as described in the text. Values are the means of 3 or 4 experiments ± SE.

non-enzymatic or peroxidative reactions [12, 13, 20] and give rise to erroneous results. In addition, phenoxy radicals able to react with macromolecules with the formation of ^3H_2O as well as catechol estrogens which can also bind irreversibly to a variety of cell constituents may be formed by such mechanisms.

Weisz and co-workers [12, 13, 25] have used cumene hydroperoxide to assess the peroxidative component of catechol estrogen formation in a number of tissues and found the enzyme responsible for it to have low affinity and high capacity for estradiol, a different pH optimum and similar 2- and 4-hydroxylase activities. In our current studies, we have avoided adding hydroperoxides and used concentrations of GSH in the physiological range within cells [26] at pH 7.4 in a model system with lactoperoxidase. It has been shown previously [14] that GSH increases the conversion of estradiol to water-soluble products and forms glutathionyl conjugates with the steroid. We have also looked at the effect of NADPH, which is an essential component of the microsomal monooxygenase, on the lactoperoxidase-catalyzed reaction and found it to be inhibitory for the oxidation of estradiol by lactoperoxidase even at very low concentration (0.03 mM). Presumably, this is due to NADPH reducing any generated H_2O_2 and could be non-enzymatic. Ascorbate, which had been shown previously [16, 23] to have little effect on estradiol hydroxylation, also inhibited both reactions. It is therefore unlikely that a true peroxidase-catalyzed

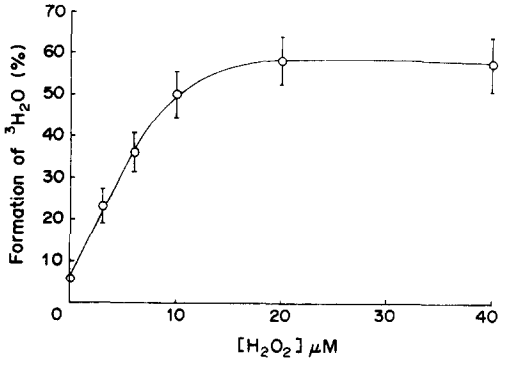

Fig. 3. Effect of hydrogen peroxide on the formation of ^3H_2O from [2-^{3}H]estradiol by lactoperoxidase. [2-^{3}H]E_2 was incubated with lactoperoxidase and various concentrations of H_2O_2 under the conditions described in Fig. 2. Values are the means ± SE from 3–4 experiments.

Table 3. Modulation of the formation of ^3H_2O or water-soluble products from [2-^{3}H]estradiol by lactoperoxidase in the presence or absence of glutathione

Additions to lactoperoxidase	GSH (2 mM)	^{3}H transfer into ^3H_2O (%)	Inhibition (%)
—	—	11.6 ± 1.0	—
	+	15.7 ± 1.0	—
Ascorbate (2mM)	—	0.5	96
	+	0.4	97
NADH or NADPH	—	2.2 ± 0.1	82
(0.3 mM)	+	14.9 ± 1.7	5
Catalase (200 μg)	—	3.3 ± 1.8	72
	+	8.1 ± 0.2	48
Superoxide dismutase	—	5.4 ± 0.8	53
(100 μg)	+	6.7 ± 0.7	57
Boiled lactoperoxidase	—	2.4	78
	+	2.1	87

[2-^{3}H]E_2 (9.2 μM) was incubated with lactoperoxidase (12.5 μg/ml) in the presence of the compounds listed, under the same conditions as in Table 1. The amount of ^3H_2O formed was determined as described in the text. NAD or NADP at 0.3 mM showed no effect but NADPH (0.3 mM) still inhibited ^3H_2O formation by 80%. Data are presented as means of 2 experiments or means ± SE of 4–6 experiments.

reaction would interfere with the radiometric assay for the formation of catechol estrogens by monooxygenase since this reaction is always carried out in the present of NADPH and, in most cases, with added ascorbate. If the latter is omitted, the catechol estrogen formed can result in adduct formation with loss of ^3H. In liver, such spurious loss of the isotope has been shown and accounts for some discrepancy with [4-^{3}H]E_2 when the radiometric assay is compared to one using gas chromatography/mass spectrometry [29]. In brain however, where the level of cytochrome *P*-450 is very low [30] displacement of ^3H from ^3H-labeled steroids may occur as a result of lipid peroxidation of unsaturated fatty acids in membranes [31].

The results with catalase and superoxide dismutase which inhibited the formation of ^3H_2O from [2-^{3}H]E_2 partially, suggest that some externally generated O_2- or H_2O_2 may be involved in the displacement of ^3H catalyzed by lactoperoxidase. Further work is in progress to determine the mechanisms involved and to assess whether other mammalian peroxidases behave in a similar way. In the presence of H_2O_2, displacement of ^3H as ^3H_2O from [2-^{3}H]E_2 provides a sensitive assay to measure peroxidase activity in tissues rich in this enzyme such as the estrogen-stimulated rodent uterus [6–8].

Acknowledgements—We thank Anne-Marie Newcombe for excellent technical assistance. This work was supported by grants MT 7688 from the Medical Research Council of Canada (P.H.J.) CA 39734, 44458 and TC 2016 (H.L.B.).

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