PHENYLALANINE 4-MONOOXYGENASE AND THE *S*-OXIDATION OF *S*-CARBOXYMETHYL-L-CYSTEINE BY HUMAN CYTOSOLIC FRACTIONS

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SUMMARY

The purpose of this investigation was to reaction phenotype the identity of the cytosolic enzyme responsible for the *S*-oxidation of *S*-carboxymethyl-L-cysteine (SCMC) in female human hepatic cytosolic fractions. The identity of this enzyme in the female Wistar rat hepatic cytosolic fraction was found to be phenylalanine 4-monooxygenase (PAH). In pooled female human hepatic cytosolic fractions the calculated K_m and V_{max} for substrate (SCMC) activated PAH was 16.22 ± 11.31 mM and 0.87 ± 0.41 nmoles.min^{-1}mg^{-1}. The experimental data modelled to the Michaelis-Menten equation with non-competitive substrate inhibition. When the cytosolic fractions were activated with lysophophatidylcholine the V_{max} increased to 52.31 ± 11.72 nmoles.min^{-1}mg^{-1} but the K_m remained unchanged at 16.53 ± 2.32 mM. A linear correlation was seen in the production of Tyr and

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SCMC *R/S S*-oxide in 20 individual female hepatic cytosolic fractions for both substrate and lysophosphatidylcholine activated PAH (r_s >0.96). Inhibitor studies found that the specific chemical and antibody inhibitors of PAH reduced the production of Tyr and SCMC *R/S S*-oxide in these *in vitro* PAH assays. An investigation of the mechanism of interaction of SCMC with PAH indicated that the drug was a competitive inhibitor of the aromatic *C*-oxidation of Phe with a calculated K_i of 17.23 ± 4.15 mM. The requirement of BH_4 as cofactor and the lack of effect of the specific tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase inhibitors on the *S*-oxidation of SCMC all indicate that PAH was the enzyme responsible for this biotransformation reaction in human hepatic cytosolic fractions.

KEY WORDS

phenylalanine 4-monooxygenase, *S*-carboxymethyl-L-cysteine, *S*-oxidation, reaction phenotyping

INTRODUCTION

The cysteine analogue drug, *S*-carboxymethyl-L-cysteine (SCMC) is used extensively as a free radical scavenging therapeutic agent in chronic obstructive pulmonary disease (COPD) and otitis media with effusions (OME) /1-3/. The mechanism of free radical scavenging is the chemical oxidation of the thioether moiety of SCMC by the reactive oxygen species resulting in the formation of a stable *S*-oxide intermediate /1/. This is of considerable interest since the major metabolic fate of SCMC in the body is the formation of *S*-oxide metabolites /4/. This biotransformation reaction is under both pharmacogenetic and diurnal control in man /5,6/ which results in considerable variation in the bioavailability of SCMC /5,7/. The resulting variability in the bioavailability is probably responsible for the wide variation in clinical efficacy seen with patients with COPD and OME /2,3/. Previous work has identified that in the Wistar rat and HepG2 cells, phenylalanine 4-monooxygenase (PAH) was the enzyme responsible for this biotransformation reaction /8-10/.

PAH is a hepatic enzyme that is responsible for the oxidation of L-phenylalanine (Phe) to L-tyrosine (Tyr), and defects in the *PAH* gene,

with subsequent production of a faulty enzyme, are the cause of the vast majority of cases of phenylketonuria in man /11/. The molecular genetics of PAH in relation to phenylketonuria has been extensively investigated but the regulation of PAH at the proteomic level is only now being actively researched. The *PAH* gene can be found on chromosome 12, band region q22-q24.1, but the full genomic sequence of *PAH* spanning more than 90 kb has yet to be reported. However, the cDNA sequence has been found to contain 13 exons. The protein product of this cDNA sequence has a molecular mass of 52 kDa and is composed of 452 amino acids. The PAH protein is a tetramer comprised of four identical monomers /12/. Each tetramer is in fact a 'dimer of dimers' with each dimer composed of two monomers arranged in a 'head-to-tail' orientation relative to one another /13/. Each monomer contains an Fe^{2+} ion but possesses no catalytic activity itself; only the dimers and tetramers show any enzymatic activity. Every monomer contains a number of domains. These domains are the N-terminal regulatory domain (amino acids 1-142), catalytic domain (amino acids 143-410) and the C-terminal polymerisation domain (amino acids 411-452). It is the N-terminal regulatory domain that controls access to the enzyme's active site /14/. The conversion of PAH from a low activity to a high activity configuration is achieved by a number of different mechanisms: (1) co-operative binding of Phe to the catalytic domain results in an alteration of either K_m or V_{max} for Phe; (2) phosphorylation of Ser^{16} in the N-terminal regulatory domain leads to an alteration in the V_{max} for L-Phe; (3) limited proteolysis enables the conversion of the tetrameric protein into a dimeric form; (4) modification of Cys^{237} in the catalytic domain; (5) binding of lysophosphatidylcholine to PAH.

All of the above affect the kinetics of Phe metabolism by PAH, but mechanisms 3-5 result in the substrate specificity of PAH being altered, allowing the oxidation of the aliphatic amino acids, L-methionine (Met) and *S*-methyl-L-cysteine (SMC) to their *S*-oxide metabolites. Nor-leucine also undergoes aliphatic *C*-oxidation to its e-hydroxy derivative /8,10,11,15/. The physiological regulation of PAH with regard to Phe metabolism occurs via mechanisms 1 and 2. However, the role of PAH in xenobiotic biotransformation is probably regulated by mechanisms 3-5. To date the physiological mechanisms of activation of PAH to a xenobiotic metabolism role are unknown. The present paper describes the investigation into the identity of the

enzyme responsible for the *S*-oxidation of SCMC in human cytosolic fractions.

MATERIALS AND METHODS

Chemicals

2,2′-Dipyridyl, Phe, SCMC, 4-chlorophenylalanine, 3-iodotyrosine, 6-fluorotryptophan and Tyr were purchased from Sigma-Aldrich Co. Ltd. (Poole, UK). Nitric oxide synthase (NOS) inhibitor set was purchased from Calbiochem (Nottingham, UK). Tetrahydrobiopterin (BH_4) was purchased from Schricks Laboratories (Basal, Switzerland). PH8, large aromatic amino acid hydroxylase monoclonal antibody, was obtained from Biodesign International (Saco, USA). HPLC grade methanol was from Rathburn Chemical Co. (Walkerburn, Scotland). SCMC (*R/S*) *S*-oxides were synthesised and isolated by previously reported methods /16,17/.

Enzyme source

Pooled female and individual female human cytosolic fractions were purchased from BD Biosciences (Woburn, USA).

In vitro PAH enzyme assays

Substrate activated and lysophosphatidylcholine activated PAH assays using Phe and SCMC as substrates were carried out as previously reported /8,9,18/.

Determination of SCMC (*R/S*) *S*-oxides

The *S*-oxide metabolites of SCMC were analysed and quantified by HPLC with fluorescence detection following pre-column derivatization with *o*-phthaldehyde/2-mercaptoethanol as previously reported /8,9/.

Determination of Tyr

Tyr was determined spectrophotometrically as previously reported /10,19/.

Data analysis

Statistical data analyses were performed with Sigma Stat 10.0. Enzyme kinetic data were analysed by WinNonLin 3.0 and Leonora 1.0.

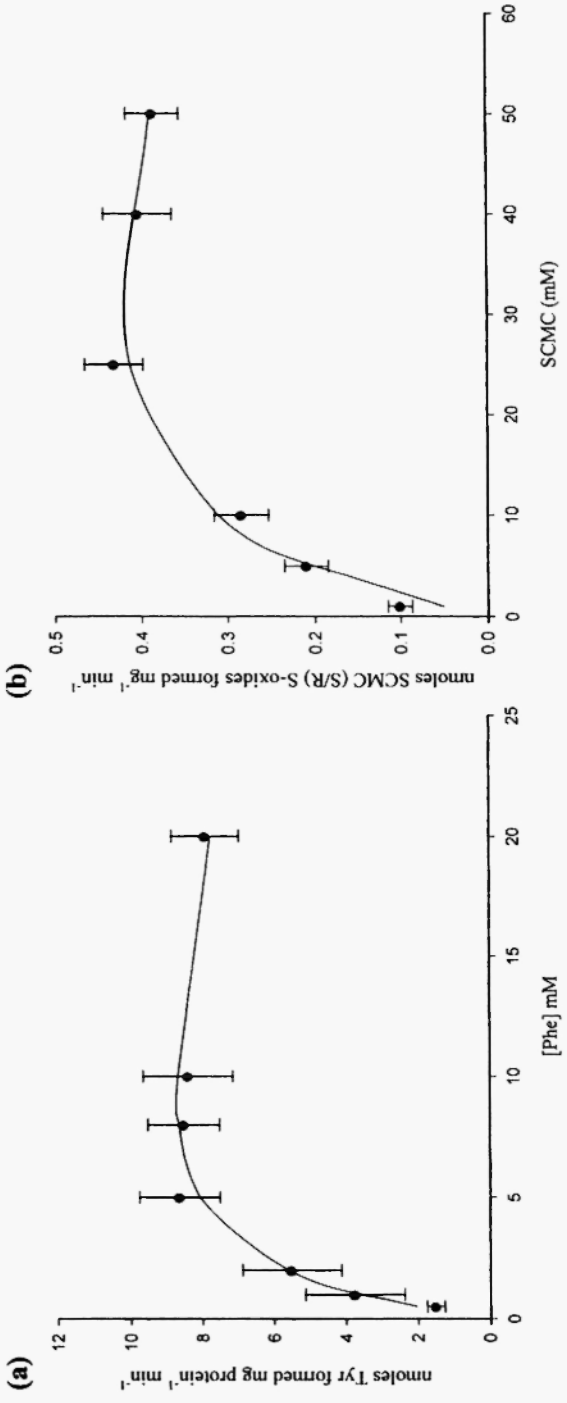
RESULTS

PAH and Phe enzyme kinetics

The effect of varying the pooled cytosolic protein concentration on both substrate and lysophosphatidylcholine activated PAH activity was found to be linear with respect to Tyr production in the range of 100-1,000 μg/ml (data not shown). Similarly, the effect of varying time on both substrate and lysophosphatidylcholine activated PAH activity with respect to Tyr production was found to be linear in the range 1-15 min (data not shown). The effect of varying Phe concentration on substrate activated pooled human female hepatic cytosolic fraction can be seen in Figure 1a. The data were modelled with both the Michaelis-Menten and Michaelis-Menten with non-competitive substrate inhibition equations. The best fit for the experimental data (solid circles) was the Michaelis-Menten equation with non-competitive substrate inhibition (solid line). The Leonora and WinNonLin calculated K_m, V_{max} and Cl_E (clearance via the enzyme, V_{max}/K_m) were 3.05 ± 0.94 mM, 14.23 ± 2.43 nmoles.min^{-1}mg^{-1} and 4.66 ± 0.47 μl. min^{-1}mg^{-1} (Table 1). The effect of lysophosphatidylcholine activation on Phe kinetics can be seen in Figure 1c and Table 1. Again the best fit to the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition. The Leonora and WinNonLin calculated K_m, V_{max} and Cl_E were 0.16 ± 0.06 mM, 29.10 ± 6.99 nmoles.min^{-1}mg^{-1} and 181.88 ± 15.28 μl.min^{-1}mg^{-1}.

PAH and SCMC enzyme kinetics

The effect of varying the pooled cytosolic protein concentration on both substrate and lysophosphatidylcholine activated PAH activity was found to be linear with respect to SCMC *R/S S*-oxide production in the range of 100-700 μg/ml (data not shown). Similarly, the effect of varying time on both substrate and lysophosphatidylcholine activated PAH activity with respect to SCMC *R/S S*-oxide production



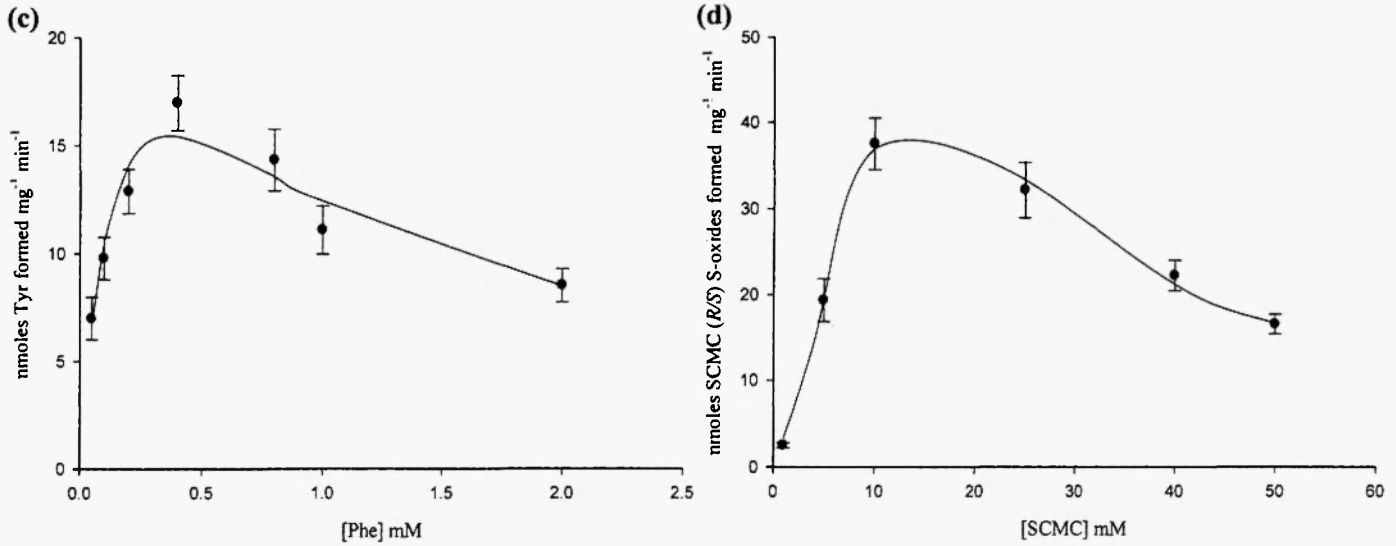


Fig 1: Effect of varying (**a**) Phe and (**b**) SCMC concentration on substrate activated pooled human female hepatic cytosolic PAH activity; effect of varying (**c**) Phe and (**d**) SCMC concentration on lysophosphatidylcholine activated pooled human female hepatic cytosolic PAH activity. V against S plots of varying Phe and SCMC substrate concentrations with BH_4 concentration fixed at 50.0 µM (Phe) and 80.0 µM (SCMC). Incubation times were 15 min for all assays and the concentration of pooled human hepatic cytosolic fractions was 500 µg/ml for each assay. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the means ± SD of 6 assays. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphatidylcholine for 3 min prior to starting the reaction. The experimental data (solid black circles) were modelled with the Leonora 1.0 software and the calculated V (solid black line) is displayed on each V against S plot. PAH = phenylalanine 4-monooxygenase; SCMC = *S*-carboxymethyl-L-cysteine.

TABLE I

Kinetic data for Phe and SCMC in human female pooled hepatic cytosolic fraction PAH assays

Substrate	Activation	K_m (mM)	V_{max} (nmoles.min^{-1}mg^{-1})	Cl_E (µl.min^{-1}mg^{-1})
Phe	substrate	3.05 ± 0.94	14.23 ± 2.43	4.66 ± 0.47
SCMC	substrate	16.22 ± 11.31	0.87 ± 0.41	0.05 ± 0.005
Phe	lysophosphatidylcholine	0.16 ± 0.06	29.10 ± 6.99	181.8 ± 15.28
SCMC	lysophosphatidylcholine	16.53 ± 2.32	52.31 ± 11.72	3.16 ± 0.11

Leonora 1.0 and WinNonLin 3.0 calculated K_m, V_{max} and Cl_E for the substrate and lysophosphatidylcholine activated PAH assays. The concentrations of BH_4 were fixed at 500 µM (Phe) and 800 µM (SCMC). Incubation times were 15 min for all assays and the concentration of pooled human hepatic cytosolic fractions was 500 µg/ml for each assay. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the means ± SD of 6 assays. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphatidy choline for 3 min prior to starting the reaction.
PAH = phenylalanine 4-monooxygenase; SCMC = S-carboxymethy-L-cysteine.

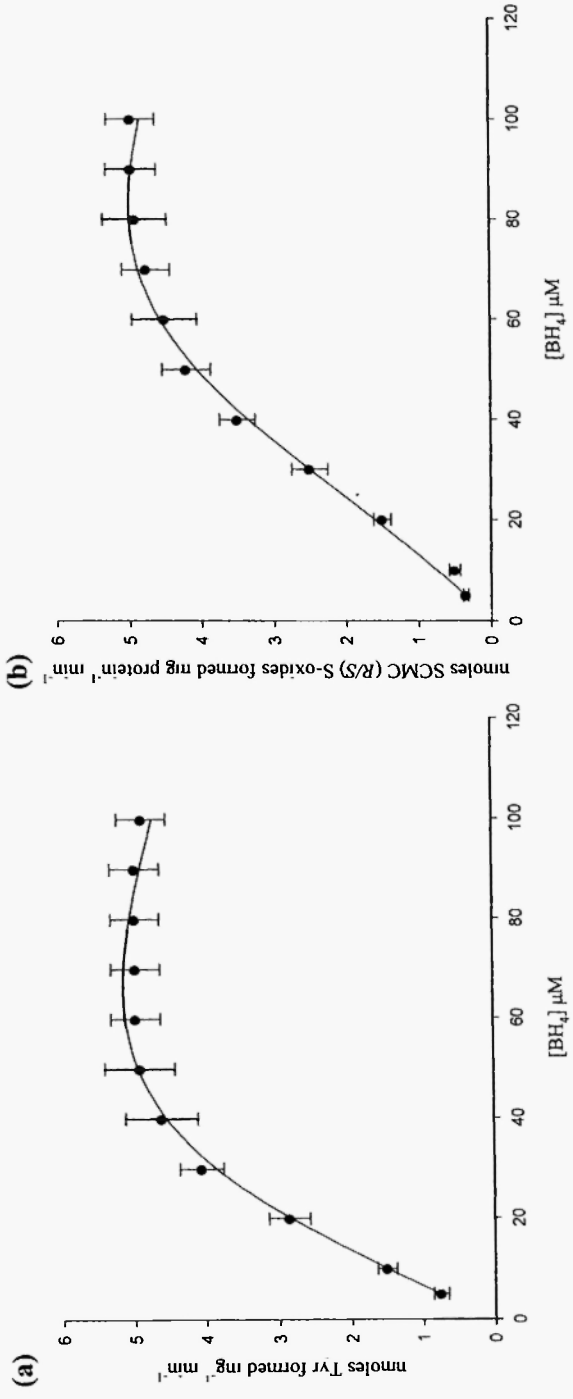
was found to be linear in the range 1-20 min (data not shown). The effect of varying SCMC concentration on substrate activated pooled human female hepatic cytosolic fraction can be seen in Figure 1b. The data were modelled with both the Michaelis-Menten and Michaelis-Menten with non-competitive substrate inhibition equations. The best fit for the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition. The Leonora and WinNonLin calculated K_m, V_{max} and Cl_E were 16.22 ± 11.31 mM, 0.87 ± 0.41 nmoles.min^{-1}mg^{-1} and 0.05 ± 0.005 µl.min^{-1}mg^{-1} (Table 1). The effect of lysophosphatidylcholine activation on SCMC kinetics can be seen in Figure 1d and Table 1. The best fit to the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition and the Leonora and WinNonLin calculated K_m, V_{max} and Cl_E were 16.53 ± 2.32 mM, 52.31 ± 11.72 nmoles.min^{-1}mg^{-1} and 3.16 ± 0.11 µl.min^{-1}mg^{-1}.

PAH and cofactor enzyme kinetics using Phe as substrate

The effect of varying both time and pooled hepatic cytosolic protein concentration on the linearity of Tyr production was investigated for both substrate and lysophosphatidylcholine activated PAH. Tyr production was linear in the range of 100-1,000 µg/ml and 1-15 min (data not shown). The effect of varying BH_4 concentration on Tyr production on substrate activated PAH can be seen in Figure 2a. The best fit to the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition and the Leonora and WinNonLin calculated K_m and V_{max} for BH_4 were 23.10 ± 6.18 mM and 6.53 ± 0.54 nmoles.min^{-1}mg^{-1} (Table 2). The effect of varying BH_4 concentration on Tyr production on lysophosphatidylcholine activated PAH can be seen in Figure 2c. The best fit to the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition and the Leonora and WinNonLin calculated K_m and V_{max} for BH_4 were 23.33 ± 6.03 mM and 26.33 ± 2.11 nmoles. min^{-1}mg^{-1} (Table 2).

PAH and cofactor enzyme kinetics using SCMC as substrate

The effect of varying both time and pooled hepatic cytosolic protein concentration on the linearity of SCMC *R/S S*-oxide production was investigated for both substrate and lysophosphatidylcholine



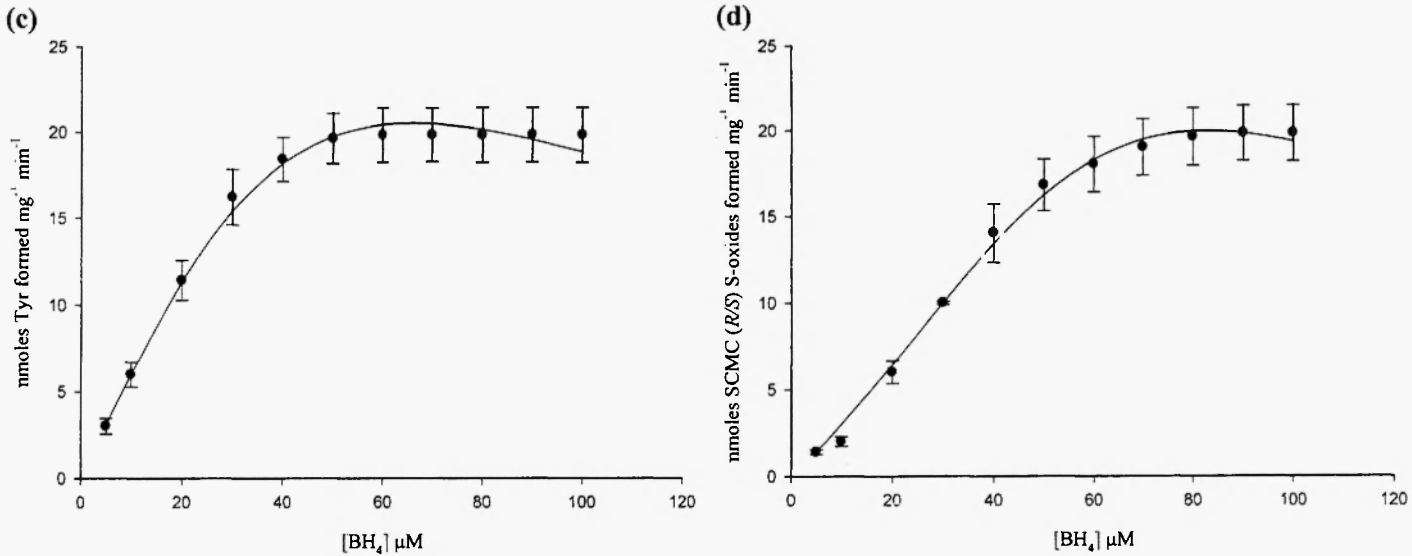


Fig. 2: The V against S plots of varying BH_4 cofactor concentrations with the substrate concentration fixed at 5.0 mM (Phe) and 25.0 mM (SCMC). Incubation times were 15 min. for all assays and the concentration of pooled human hepatic cytosolic fractions was 500 μg/ml for each assay. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the means ± SD of 6 assays. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphatidylcholine for 3 min prior to starting the reaction. The experimental data (solid black circles) were modelled with Leonora 1.0 software and the calculated V (solid black line) is displayed on each V against S plot. (**a**) Phe and (**b**) SCMC on substrate activated pooled human female hepatic cytosolic PAH activity; (**c**) Phe and (**d**) SCMC on lysophophatidylcholine activated pooled human female hepatic cytosolic PAH activity. PAH = phenylalanine 4-monooxygenase; BH_4 = tetrahydrobiopterin, SCMC = *S*-carboxymethyl-L-cysteine.

TABLE 2

Kinetic data for BH_4 in human female pooled hepatic cytosolic fraction PAH assays

Cofactor	Substrate	Activation	K_m (μM)	V_{max} (nmoles. $min^{-1}mg^{-1}$)
BH_4	Phe	substrate	23.10 ± 6.18	6.53 ± 0.54
BH_4	SCMC	substrate	78.12 ± 11.58	9.59 ± 2.09
BH_4	Phe	lysophosphatidyl-choline	23.33 ± 6.03	26.33 ± 2.11
BH_4	SCMC	lysophosphatidyl-choline	73.12 ± 10.27	38.37 ± 8.39

Leonora 1.0 and WinNonLin 3.0 calculated K_m and V_{max} for the substrate and lysophosphatidylcholine activated PAH assays. The concentrations of substrate were fixed at 5.0 mM (Phe) and 25.0 mM (SCMC). Incubation times were 15 min for all assays and the concentration of pooled human hepatic cytosolic fractions was 500 μg/ml for each assay. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the mean ± SD of 6 assays. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphatidylcholine for 3 min prior to starting the reaction.
PAH = phenylalanine 4-monooxygenase; BH_4 = tetrahydrobiopterin; SCMC = *S*-carboxymethyl-L-cysteine.

activated PAH. SCMC *R/S S*-oxide production was linear in the range of 100-700 mg/ml and 1-20 min (data not shown). The effect of varying BH_4 concentration on SCMC *R/S S*-oxide production on substrate activated PAH can be seen in Figure 2b. The best fit to the experimental data was the Michaelis-Menten equation with non-competitive substrate inhibition. The Leonora and WinNonLin calculated K_m and V_{max} for BH_4 were 78.12 ± 11.58 mM and 9.59 ± 2.09 nmoles.$min^{-1}mg^{-1}$ (Table 2). The effect of varying BH_4 concentration on SCMC *R/S S*-oxide production in lysophosphatidylcholine activated PAH can be seen in Figure 2d. The best fit to the experimental data was the Michaelis-Menten equation with non-competitive sub-

strate inhibition. The Leonora and WinNonLin calculated K_m and V_{max} for BH_4 were 73.12 ± 10.27 mM and 38.37 ± 8.39 nmoles.min^{-1}mg^{-1} (Table 2).

PAH correlation studies on metabolite production

The correlation of PAH generated Tyr and SCMC *R/S S*-oxide in 20 individual female hepatic cytosolic fractions can be seen in Figure 3 for substrate activated and lysophosphatidylcholine activated PAH. The correlation of metabolite formation in the substrate activated (Phe and SCMC) female hepatic cytosolic fractions has an r_S value of 0.96 (Spearman's Rank correlation coefficient, $p < 0.001$). A similar set of results was seen in the lysophosphatidylcholine activated individual female hepatic cytosolic fractions with an r_s value of 0.98 (Spearman's Rank correlation coefficient, $p < 0.001$).

PAH inhibitor studies

The effect of various inhibitors on lysophosphatidycholine activated pooled female hepatic cytosolic fractions PAH activity using both Phe and SCMC as substrates can be seen in Table 3. The specific PAH inhibitor, 4-chlorophenylalanine, caused inhibition of both Tyr and SCMC *R/S S*-oxide production at all concentrations used (10.0, 100.0 1,000.0 μM) but the tyrosine hydroxylase and tryptophan hydroxylase specific inhibitors (3-iodotyrosine and 6-fluorotryptophan) showed only a marginal affect at 1,000 μM. PH8, the large aromatic amino acid hydroxylase monoclonal antibody, inhibited the production of both Tyr and SCMC *R/S S*-oxide (>98%) at both 100.0 and 200.0 μg/ml concentration.

SCMC was found to inhibit the production of Tyr by PAH but only at 1,000 and 5,000 μM concentrations, but Phe was found to inhibit the production of SCMC *R/S S*-oxide at all concentrations investigated (10.0, 100.0, 1,000 μM). The Fe^{2+} chelator, 2,2′-dipyridyl was also found to inhibit both Tyr and SCMC *R/S S*-oxide formation by PAH at all concentrations investigated (10.0, 100.0, 1,000 μM). Finally, the NOS inhibitor set was found to have no effect on the production of both Tyr and SCMC *R/S S*-oxide by lysophosphatidylcholine activated pooled female human hepatic cytosolic fractions at all concentrations investigated (5.0-100.0 μM).

TABLE 3

Inhibition data for lysophosphatidylcholine activated human female pooled hepatic cytosolic fraction PAH assays

Inhibitor	Concentration (μM)	Substrate	% inhibition	Substrate	% inhibition
4-Chlorophenylalanine	10.0	Phe	23.5 ± 4.2	SCMC	54.3 ± 7.5
	100.0	Phe	60.0 ± 7.1	SCMC	87.0 ± 4.7
	1,000.0	Phe	97.2 ± 2.3	SCMC	99.2 ± 1.0
3-Iodotyrosine	10.0	Phe	0.0	SCMC	0.0
	100.0	Phe	0.0	SCMC	0.0
	1,000.0	Phe	3.0 ± 1.5	SCMC	5.2 ± 2.2
6-Fluorotryptophan	10.0	Phe	0.0	SCMC	0.0
	100.0	Phe	0.0	SCMC	0.0
	1,000.0	Phe	2.5 ± 1.0	SCMC	4.5 ± 3.1
PH8	100.0 μg/ml	Phe	9.0 ± 1.2	SCMC	98.4 ± 1.5
	200.0 μg/ml	Phe	99.0 ± 1.5	SCMC	99.3± 0.75
SCMC	10.0	Phe	0.0	NA	NA
	100.0	Phe	0.0	NA	NA
	1,000.0	Phe	12.5 ± 2.7	NA	NA
	5,000.0	Phe	25.2 ± 4.1	NA	NA

Inhibitor	Concentration (μM)	Substrate	% inhibition	Substrate	% inhibition
Phe	10.0	NA	NA	SCMC	20.5 ± 4.3
	100.0	NA	NA	SCMC	63.1 ± 5.6
	1,000.0	NA	NA	SCMC	92.8 ± 7.4
2,2′-Dipyridyl	10.0	Phe	40.8 ± 3.2	SCMC	48.3 ± 7.2
	100.0	Phe	75.3 ± 6.7	SCMC	81.0 ± 4.7
	1,000.0	Phe	99.0 ± 1.3	SCMC	99.0 ± 1.1
NOS inhibitor set	Concentration 1	Phe	0.0	SCMC	0.0
	Concentration 2	Phe	0.0	SCMC	0.0

The effect of various inhibitors on lysophosphatidylcholine activated PAH activity in pooled female human hepatic cytosolic fractions. Phe concentration was 5.0 mM and BH_4 concentration 50.0 μM; SCMC concentration 25.0 mM and BH_4 concentration 80.0 μM. Time of incubation was 15 min and the pooled female human hepatic cytosolic protein concentration was 500.0 μg/ml. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphaticylcholine for 3 min prior to starting the reaction. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the means ± SD of 6 assays

NOS inhibitor set Concentration 1: L-N^5-(1-iminoethyl)ornithine (50 μM), *S*-methyl-L-thiocitrulline (5 μM) N^G-monomethyl-L-arginine (50 μM), N^G-monomethyl-D-arginine (50 μM), 7-nitroindazole (50 μM), L-thiocitrulline (5 μM).

NOS inhibitor set Concentration 2: L-N^5-(1-iminoethyl) ornithine (100 μM), *S*-methyl-L-thiocitrulline (10 μM), N^G-monomethyl-L-arginine (100 μM), N^G-monomethyl-D-arginine (100 μM), 7-nitroindazole (100 μM), L-thiocitrulline (10 μM).

PAH = phenylalanine 4-monooxygenase; BH_4 = tetrahydrobiopterin, SCMC = *S*-carboxymethyl-L-cysteine; NA = not applicable; PH8 = large aromatic amino acid hydroxylase monoclonal antibody; NOS = nitric oxide synthase.

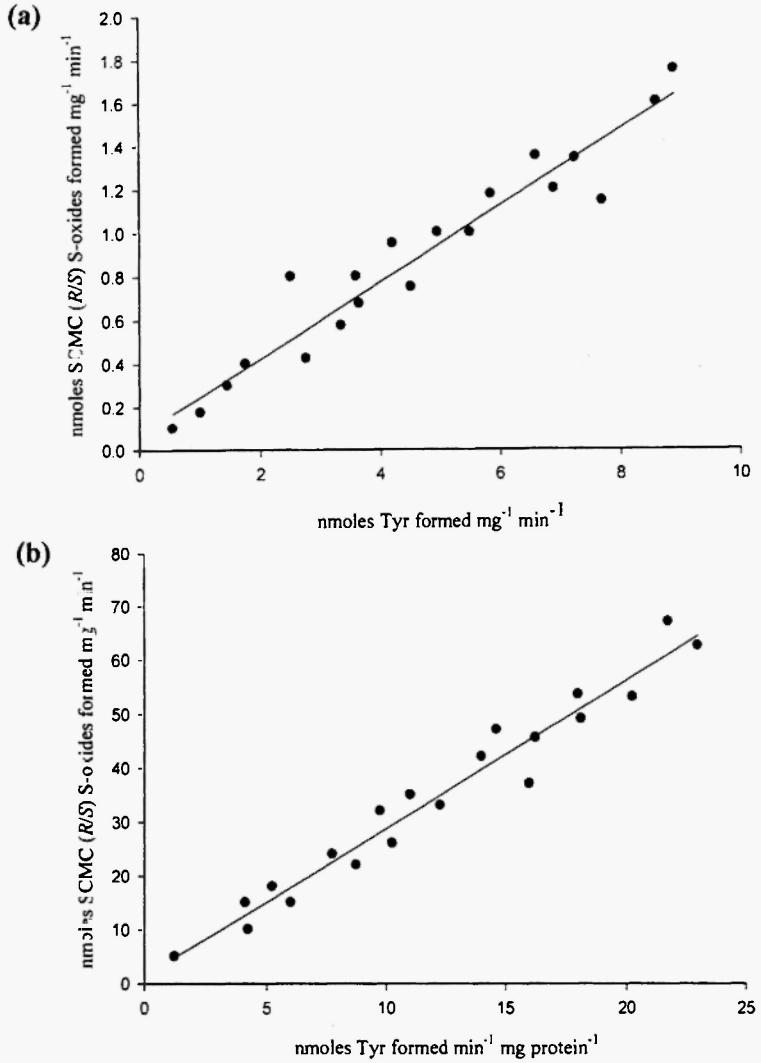


Fig. 3: Metabolite correlation of substrate (**a**) and lysophosphatidylcholine (**b**) activated PAH activity in 20 individual female human hepatic cytosolic fractions. Phe concentration was 5.0 mM and BH_4 concentration 50.0 μM; SCMC concentration 25.0 mM and BH_4 concentration 80.0 μM. Time of incubation was 15 min and the female human hepatic cytosolic protein concentration was 500.0 μg/ml. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. The lysophosphatidylcholine activated cytosolic fractions were incubated with 1 mM lysophosphatidylcholine for 3 min prior to starting the reaction. PAH = phenylalanine 4-monooxygenase; BH_4 = tetrahydrobiopterin; SCMC = *S*-carboxymethyl-L-cysteine.

The mechanism of inhibition of PAH by both Phe and SCMC in terms of Tyr and SCMC *R/S S*-oxide production was investigated in substrate activated pooled female human hepatic cytosolic fractions and the results can be seen in Figure 4. The Dixon plots indicate that both SCMC and Phe were competitive inhibitors of PAH when Phe (with SCMC as inhibitor) or SCMC (with Phe as inhibitor) was used as substrates with a Leonora calculated K_i for SCMC of 17.23 ± 4.15 mM (Fig. 4a) and a Leonora calculated K_i for Phe of 2.55 ± 1.05 mM (Fig. 4b).

DISCUSSION

The results from this kinetic investigation provide very strong experimental support for the fact that a single enzyme present in human female cytosolic fraction was responsible for the *S*-oxidation of the mucoactive drug, SCMC. The concentration range of SCMC used was from 1.0-50.0 mM but only one V_{max} and K_m could be detected in the substrate (SCMC) activated and lysophosphatidylcholine activated PAH assays (Fig. 1b and d). Similarly only one enzyme was involved in the aromatic *C*-oxidation of Phe in both the substrate (Phe) activated and lysophosphatidylcholine activated PAH assays (Fig. 1a and c). Both substrates (Phe and SCMC) showed non-typical enzyme kinetics, with the best model for the experimental data being provided by the Michaels-Menten equation with non-competitive substrate inhibition. Similar kinetic profiles were seen in pooled female Wistar rat hepatic cytosolic fraction and HepG2 cytosolic fraction activated with lysophosphatidycholine using Phe and SCMC as substrates /8-10/. The kinetic data clearly showed that the physiological substrate, Phe, had a greater affinity for PAH compared to SCMC in the lysophosphatidylcholine activated PAH assay (K_m Phe 3.05 ± 0.94 mM versus SCMC 16.22 ± 11.31) (substrate activated, $p > 0.05$, Student's t-test) and Phe 0.16 ± 0.06 mM versus SCMC 16.53 ± 2.32 (lysophosphatidylcholine activated, $p < 0.05$, Student's t-test). This was also seen in the turnover of the two substrates with PAH clearing 4.66 ± 0.47 $\mu l.min^{-1}mg^{-1}$ of Phe compared to 0.05 ± 0.005 $\mu l.min^{-1}mg^{-1}$ of SCMC when substrate activated ($p < 0.001$, Student's t-test) (Table 1). In the lysophosphatidylcholine activated PAH assays, Cl_E was 181.88 ± 15.28 $\mu l.min^{-1}mg^{-1}$ for Phe compared to 3.16 ± 0.11 $\mu l.min^{-1}mg^{-1}$ for SCMC ($p < 0.001$, Student's t-test) (Table 1). Thus SCMC may be a poor

Fig. 4: Dixon plots for SCMC as an inhibitor of substrate (Phe) activated PAH activity (**a**) and Phe as an inhibitor of substrate (SCMC) activated PAH activity (**b**) in pooled human female hepatic cytosolic fractions. The concentrations of BH_4 were fixed at 50.0 µM (Phe) and 80.0 µM (SCMC). Incubation times were 15 min for all assays and the concentration of pooled human hepatic cytosolic fractions was 500 µg/ml for each assay. Each assay was carried out in duplicate with heat inactivated pooled human female hepatic cytosolic fractions as blanks. The results are the means ± SD of 6 assays. The substrate activated cytosolic fractions were incubated with substrate at 37°C for 3 min prior to starting the reaction. Leonora modelling of the kinetic data indicated that SCMC was a competitive inhibitor of Phe metabolism with the Leonora calculated K_i = 15.72 ± 3.92 mM and Leonora calculated K_m = 16.22 ± 11.31 mM ($p > 0.05$, Studen's t-test), and Phe was a competitive inhibitor of SCMC metabolism with the Leonora calculated K_i = 1.59 ± 0.21 mM and Leonora calculated K_m = 3.05 ±0.94 mM ($p > 0.05$, Studen's t-test). PAH = phenylalanine 4-monooxygenase; BH_4 = tetrahydrobiopterin; SCMC = *S*-carboxymethyl-L-cysteine.

substrate for PAH but it appears to be the only cytosolic enzyme involved in the *S*-oxidation of this drug in humans.

Further experimental evidence for the involvement of PAH in the *S*-oxidation of SCMC was seen by the requirement for BH_4. Unlike the differences seen in both K_m (substrate activated, 5.31-fold difference and lysophosphatidylcholine activated, 103.31-fold difference) and V_{max} (substrate activated, 16.35-fold difference and lysophosphatidylcholine activated, 1.79-fold difference) (Table 1) values for the two substrates, the effect that the two substrates had on the cofactor were smaller for both the K_m (substrate activated, 3.38-fold difference and lysophosphatidylcholine activated, 3.13-fold difference) and the V_{max} (substrate activated, 1.46-fold difference and lysophosphatidylcholine activated, 1.45-fold difference). BH_4, however, is the cofactor for a number of other enzymes: tyrosine hydroxylase, tryptophan hydroxylase, NOS (inducible [iNOS], endothelial [eNOS] and neuronal [nNOS]) and 1-*O*-alkyl-glycerol oxidase all use BH_4 as cofactor. The last enzyme is located in the hepatic endoplasmic reticulum and so is not present in the cytosolic fraction. Both tyrosine hydroxylase (adrenal/central nervous system) and tryptophan hydroxylase (central nervous system) are also not reported to be present in the liver /13/. Thus the only other enzyme that utilises BH_4 as a cofactor in the hepatic cytosolic fraction is iNOS /20/.These results, however, still do not exclude the possibility that iNOS was involved in the *S*-oxidation of SCMC. However, using broad spectrum NOS inhbitors (inhbitors of iNOS, eNOS and nNOS) no effect was seen at the concentrations investigated (5-100 μM) on either Tyr or SCMC *R/S S*-oxide production. At these concentrations no NOS activity would be expected (Table 3).

The correlation of the production of the *S*-oxide metabolite, SCMC *R/S S*-oxide, with the production of a metabolite from a specific substrate in a number of individual subcellular fractions is a good indicator that the same enzyme was involved in the production of both metabolites. This can be seen in Figure 3. A linear correlation was seen in 20 human female hepatic cytosolic fraction PAH assays using both substrate and lysophosphatidylcholine activated enzyme with Phe and SCMC as substrates. The final part of the reaction phenotyping investigation was to study the effects of specific inhibitors (antibody and chemical) on the production of the metabolites from both substrates /21/. This can be seen in Table 3 and Figure 4. The specific

PAH inhibitor, 4-chlorophenylalanine, was effective at inhibiting both Tyr and SCMC *R/S S*-oxide formation at all concentrations used (10.0, 100.0 and 1,000 μM). However, the tyrosine hydroxylase specific inhibitor (3-iodotyrosine) and the tryptophan hydroxylase specific inhibitor (6-fluorotryptophan) produced <6% inhibition at 1,000 μM and no inhibition at 10.0 and 100.0 μM. PH8, the large aromatic amino acid hydroxylase monoclonal antibody, inhibits all three large aromatic amino acid hydroxylases, and completely (>98%) inhibited both Tyr and SCMC *R/S S*-oxide formation at both concentrations used.

The production of Tyr and SCMC *R/S S*-oxide from their substrates is Fe^{2+} dependent since the Fe^{2+} chelator, 2,2′-dipyridyl was an effective inhibitor at all concentrations used (10.0, 100.0 and 1,000 μM). PAH is an Fe^{2+} dependent enzyme /13/. The final supporting evidence of PAH being responsible for the *S*-oxidation of SCMC was the mechanism of inhibition of Phe with SCMC *S*-oxidation and the mechanism of inhibition of SCMC with aromatic *C*-oxidation of Phe. SCMC was found to be a weak inhibitor of Tyr production but Phe was found to be a very effective inhibitor of SCMC *R/S S*-oxide production (Table 3). These observations were further investigated and the mechanism of inhibition was elucidated by the construction of Dixon plots (Fig. 4). SCMC was found to be a competitive inhibitor of Phe aromatic *C*-oxidation and Phe was found to be competitive inhibitor of SCMC *S*-oxidation. The Leonora calculated K_is were found to be 17.23 ± 4.15 mM for SCMC and 2.55 ± 1.05 mM for Phe.

CONCLUSIONS

The results of these investigations have identified PAH as the enzyme responsible for the *S*-oxidation of SCMC in human female hepatic cytosolic fractions and complement the initial investigation in female Wistar rats /8,9/ and HepG2 cells /10/ that have also identified PAH as the enzyme responsible for the *S*-oxidation of SCMC. Work is now underway to investigate the substrate specificity of human cDNA expressed PAH with regard to SCMC *S*-oxidation and other thioether substrates.

ACKNOWLEDGEMENTS

BB, GBS and BF would like to thank the Royal Thai Government for funding BB's Ph.D. studentship.

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