DRAFT 05 08 03

Preliminary Data Summary 05 08 03

PHASE I
PRE-OPTIMIZATION EXPERIMENTS FOR SUBSTRATE CHARACTERIZATION FOR PORCINE MICROSOMES

EPA Contract Number 68-W-01-023
WA 2-24

May 8, 2003

PREPARED FOR

GARY E. TIMM
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

BATTELLE
505 KING AVENUE
COLUMBUS, OH 43201

Preliminary Data Summary 05 08 03

Work Assignment 2-24

Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

Phase I

Pre-Optimization Experiments for Substrate Characterization for Porcine Microsomes

1 Introduction

The pre-optimization experiments were designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments included characterizing the radiolabeled substrate and preparation of placental microsomes. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) were analyzed for protein concentration, cytochrome P450 (P450) content, and aromatase activity. The P450 content measurement confirms that the enzyme is present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay determination using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations were of sufficient activity to conduct the definitive optimization experiments.

This report includes the results of the pre-optimization experiments related to porcine placental microsomes.

2 Materials and Methods

2.1 Chemicals

Non-radiolabeled 4-androstene-3,17-dione (ASDN) was received through Battelle from Sigma (St. Louis, MO). [1β-^{3}H(N)]Androst-4-ene-3,17-dione ([^{3}H]ASDN) was obtained from Perkin Elmer Life Science, Boston, MA. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glycerol, niacinamide, dithiothreitol and bovine serum albumin were purchased

from Sigma. Sodium phosphate monobasic, sodium phosphate dibasic, sucrose and propylene glycol were from JT Baker. Ultima Gold scintillation cocktail was purchased from Packard Instruments. DC Protein assay kit was purchased from Biorad (Hercules, CA) .

2.2 Placental Microsome Preparation

All porcine placentas were received from NCSU's University Field Laboratories Swine Educational Unit. With the exception of the first placenta collected, which was at room temperature for an undetermined length of time before being placed on ice, each placenta was chilled (either in a cold room or on ice) within 5 minutes of delivery by the sow. Each placenta was transported on ice to RTI within about 1 h after collection. The tissue was placed on a ice-chilled board and the velvety tissue was scraped away from the membrane using a razor blade. The collected tissue was transfered into beakers containing ice-cold buffer (ca. 2:1, w/v; tissue :buffer; 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), 0.04 M niacinamide). The tissue was minced with scissors where necessary and then was homogenized in portions using a Polytron homogenizer. The homogenate was transferred to centrifuge tubes and centrifuged at a setting of 10,000*g* for 30 min at 4 °C in an IEC B-22M centrifuge. The supernatant was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm (which is equivalent to approximately 100,000*g*) in a refrigerated Beckman L5-50B Ultracentrifuge for 1 h to obtain the crude microsomal pellet. The supernatant was decanted and discarded and the microsomal pellet was resuspended in a chilled buffer containing 0.1 M sodium phosphate buffer, pH 7.4. The sample was centrifuged again at a setting of 35,000 rpm in the Beckman L5-50B for 1 h to wash the microsomes. This washing procedure was repeated one additional time. The twice-washed microsomal pellet was resuspended in chilled 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol and 0.05 mM dithiothreitol. The microsomal suspensions were divided into ca. 0.2-0.5 mL portions, flash frozen in liquid nitrogen, and stored at ca. -70 °C.

2.3 Protein determination

The protein concentration of the porcine placental microsome preparations were determined. A 6-point standard curve was prepared using BSA, ranging from 0.13 to 1.5 mg protein/mL on each day of analysis. Protein concentration was determined by using a DC Protein Assay kit. To a 25 µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples were allowed to sit at room temperature for at least 15 min to allow for color development. The absorbances are stable for about 1 h. Each sample (unknowns and standards) was transferred to disposable polystyrene cuvettes and the visible absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed from the absorbance of the protein standards.

2.4 P450 Content

P450 content was determined for the porcine placental microsome preparations. Using the carbon monoxide (CO) spectral assay of Omura and Sato (1964), a single experiment was conducted as described below.

A sample of each microsomal preparation was diluted 1:20 (on one occasion a 1:10 dilution was assayed because of low protein content in the microsomal preparation) in 0.1 M phosphate buffer (pH 7.4). The diluted samples were gently bubbled with carbon monoxide for approximately 10 s and then each sample was divided between a pair of matched cuvettes (1 mL/cuvette). Next, a few grains of solid sodium dithionite was added to the sample cuvette with gentle mixing by inversion. The difference spectrum was then recorded from 400 to 500 nm using an Aminco split-beam spectrophotometer.

The concentration (nmol/mL) of P450 was calculated according to Beer's Law using an extinction coefficient value for P450 of 100 mM^{-1} cm^{-1}. The specific content (nmol/mg protein) was calculated by multiplying the P450 concentration (nmol/mL) times the dilution factor and dividing this product by the protein content (mg/mL) of the original sample.

2.5 Aromatase Activity

Aromatase activity was determined for each porcine placental microsome preparation. A single experiment (for each preparation) was conducted using only the substrate ([^{3}H]ASDN/ASDN). The assay was conducted as described in the following paragraph.

The [^{3}H]ASDN/ASDN substrate solution was prepared by combining solutions of [^{3}H]ASDN and ASDN. A 1 mg/mL solution of ASDN was prepared in ethanol. Serial dilutions of this solution were prepared in assay buffer to yield a solution containing ca. 1 μg ASDN/mL. The [^{3}H]ASDN stock was diluted 1:100 in assay buffer to yield a solution containing ca. 10 μCi/mL. The substrate solution was prepared by combining 275 μL of the 1 μg ASDN/mL solution, 100 μL of the 10 μCi [^{3}H]ASDN/mL solution and 625 μL buffer.

The assay was performed in duplicate in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 μL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 μCi, 50 nM), was added to the tubes. An NADPH-generating system comprised of NADP (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) was added to each tube. The tubes were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of the diluted microsomal suspension (~0.1 mg microsomal protein/mL). The total volume was 2.0 mL, and the tubes were incubated for 30 min. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for about 30 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 min at a setting of 1000 rpm (which is approximately equivalent to 230*g*). The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride

(2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers were transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution.

The radiochemical content of the substrate solution was determined by analyzing 5 weighed aliquots by LSS. The substrate solution specific activity was determined by dividing the radiochemical content of the substrate solution (dpm/g) by the total concentration of ASDN in the solution (ASDN + [^{3}H]ASDN; nmol/g solution).

Analysis of the samples was performed using LSS as described in SOP METAB-610. Radiolabel found in the aqueous fractions represents ^3H_2O formed, and that in the methylene chloride fractions represents unreacted substrate.

The amount of estrogen product formed was determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate solution (expressed in dpm/nmol). The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 30 min.

3 Results and Discussion

Six porcine placentas were obtained (each on a separate day) from a local research farm and microsomes were prepared. A sample of each microsome preparation was thawed rapidly in a water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. Only one of the six preparations had appreciable aromatase activity. The protein content for that preparation was determined to be ca. 28 mg/mL. The total protein yield for the preparation was calculated to be ca. 126 mg (ca. 4.5 mL microsomal suspension). P450 content of that preparation was determined to be ca. 0.053 nmol/mg protein. The aromatase activity of the porcine placental microsomes was ca. 3 pmol estrogen formed/mg protein/min; this value matches that found earlier for the bovine placental microsome preparation. No literature value for porcine placenta microsomal aromatase activity was found.

4 Conclusion

Isolation of microsomes with aromatase activity from porcine placentas was problematic at best. Collection of the thin layer of tissue was difficult and time-consuming. Of the five placentas that were collected and chilled promptly after delivery by the sow, only one yielded microsomes with appreciable aromatase activity. No experimental variables were identified that led to this low activity. In fact, with the exception of the first placenta, which remained at room temperature for an undetermined length of time prior to work-up, all placentas were processed by

the same method. Additional placental microsomes with sufficient aromatase activity would be needed for the conduct of the other phases of this project. The unavailability of literature values for aromatase activity in porcine placental microsomes makes it difficult to determine whether the aromatase activity found here is in the 'normal' range.

5 References

Omura, T.; Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 1964**,** ***239,*** 2370-2378.