

Uteroferrin Induces Lipid Peroxidation in Endometrial and Conceptus Microsomal Membranes and Is Inhibited by Apotransferrin, Retinol Binding Protein, and the Uteroferrin-Associated Proteins¹

Jeffrey L. Vallet²

USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska 68933

ABSTRACT

Iron-containing proteins catalyze lipid peroxidation when combined with either H₂O₂ or ascorbic acid (ASC). Microsomal membranes were prepared from Day 13 endometrial and conceptus tissues (5 pigs) and from Day 30 endometrial, placental, fetal liver, and fetus minus fetal liver tissues (5 pigs). Microsomal membranes were subjected to the following in vitro treatments: 1) no treatment, 2) 50 μM ASC, 3) 100 μM uteroferrin (UF), 4) 50 μM ASC + 100 μM UF, 5) 50 μM ASC + 100 μM UF + 10 μM apotransferrin (transferrin with no iron bound; ATF), and 6) 50 μM ASC + 100 μM UF + 10 μM holotransferrin (transferrin saturated with iron; HTF). For treatments 7 through 10, membranes were preincubated (0°C, 3 h) with either 7) no treatment, 8) 50 μM fetuin, 9) 50 μM holoretinol binding protein (holoRBP: retinol binding protein [HoloRBP] with retinol bound), or 10) 50 μM apoRBP (RBP with no retinol bound) followed by incubation with 50 μM ASC + 100 μM UF. Lipid peroxidation was measured in the samples as thiobarbituric acid reactive substances (TBARS). Endogenous TBARS were greater ($p < 0.05$) in Day 13 conceptus than in Day 13 endometrium and were highest ($p < 0.05$) on Day 30 in fetal liver. Combined ASC and UF caused a large increase ($p < 0.05$) in TBARS in all membranes except Day 30 placental membranes. Addition of ATF, but not HTF, decreased TBARS production in all membrane preparations. HoloRBP, but not fetuin or apoRBP, decreased ($p < 0.05$) TBARS production in all but Day 30 endometrial membranes. In other experiments, when combined with ASC, UF/UF-associated protein complex induced less ($p < 0.01$) lipid peroxidation in fetal liver microsomal membranes than did free UF. Catalase and superoxide dismutase had no effect on UF-induced lipid peroxidation in fetal liver membranes. These results indicate that 1) UF combined with ASC induces lipid peroxidation in Day 13 endometrial and conceptus and Day 30 endometrial, fetal liver, and fetus minus liver microsomal membranes, and 2) ATF, holoRBP, and the UF-associated proteins, but not catalase or superoxide dismutase, inhibit this reaction.

INTRODUCTION

Embryonic losses (losses occurring before Day 25 of pregnancy) in swine have been estimated at between 20% and 40% [1–3]. Further losses occur during later pregnancy when conceptuses are subjected to intrauterine crowding [4, 5]. The mechanisms responsible for these losses are not well understood.

It has been hypothesized that some aspect(s) of maternal recognition of pregnancy may be the cause of embryonic loss [6]. During maternal recognition of pregnancy in swine, the conceptus secretes estrogen [7], which then stimulates protein secretion from the endometrium [8, 9]. These endometrially secreted proteins include uteroferrin (UF), which transports iron to the developing conceptus, retinol binding protein (RBP), which transfers retinol [10], and the UF-associated proteins (UFAP), the functions of which are currently not understood. Changes in endometrial protein secretion or some other estrogen-induced change in the intrauterine milieu may be lethal to underdeveloped embryos [6]. Transfer of embryos that are 24 h less developed than the uterus results in successful pregnancy. However, co-transfer of blastocysts that are 24 h less developed with syn-

chronous blastocysts results in preferential loss of the asynchronous blastocysts [11]. Furthermore, total embryonic loss can be induced by administration of exogenous estrogen before the period when normal blastocyst estrogen production occurs [12, 13]. Estrogen treatment accelerates the normally occurring changes in endometrial protein secretion [14]. These data support the concept that estrogen production by more developed blastocysts in a litter may alter the intrauterine environment, making it inappropriate for less developed blastocysts.

Fetal losses between Days 25 and 40 of pregnancy increase when conceptuses are subjected to intrauterine crowding [15–18]. Although little information as to the cause of this mortality is available, decreased fetal and placental weights under crowded intrauterine conditions [18] suggest that development of crowded conceptuses is restricted compared to that of uncrowded conceptuses. Thus, as in early pregnancy, changes in the intrauterine environment that are beneficial to uncrowded conceptuses could be inappropriate for crowded, less developed conceptuses and may cause fetal loss.

These results are consistent with the possibility that some component of uterine secretions that is detrimental to smaller, possibly less developed conceptuses may be the cause of embryonic and fetal mortality. Such a component of uterine secretions may be UF. UF is a purple, iron-containing acid phosphatase, and, like free iron and many other iron-containing proteins, purple acid phosphatases have

Accepted August 14, 1995.

Received April 17, 1995.

¹Mention of trade names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

²Correspondence. FAX: (402) 762-4148.

been shown to induce lipid peroxidation when combined with hydrogen peroxide [19, 20]. Lipid peroxidation can be lethal to cells [21–24]. Increased UF secretion occurs at maternal recognition of pregnancy [8] and between Days 30 and 40 of pregnancy [25], periods coincident with the embryonic losses described above. Smaller, less developed conceptuses could have decreased defenses against lipid peroxidation, or could be less efficient in metabolizing UF, resulting in increased damage. Although the concentrations of hydrogen peroxide used by others [19, 20] would not be present in vivo, iron combined with ascorbic acid (ASC) can also catalyze lipid peroxidation [26, 27]. The effect of physiological concentrations of ASC on UF-induced lipid peroxidation has not been investigated.

If UF can induce lipid peroxidation, mechanisms must exist within the intrauterine environment to inhibit this reaction. In adult humans, iron-catalyzed lipid peroxidation is inhibited by transferrin [28], which binds iron with sufficient affinity to prevent lipid peroxidation reactions. Fetal transferrin concentrations in swine are low on Day 23 of pregnancy and increase to Day 37, after which concentrations stabilize but remain lower than adult concentrations [29, 30]. Transferrin production during the time of maternal recognition of pregnancy has not been investigated; however, a protein with characteristics similar to those of transferrin is secreted by the conceptus as early as Day 13 [31]. It has also been shown that transferrin takes up iron from UF in allantoic fluid if ASC is present [32, 33], suggesting that a protective inhibitory role for transferrin is likely. This possible role of transferrin has not been investigated.

RBP may be another defense against UF-induced lipid peroxidation. Retinol has been reported to have antioxidant activity in vivo [34] and when it is incorporated into membranes [35]; however, the antioxidant activity of retinol borne by RBP has never been investigated.

Another possible protective mechanism may be the UFAP. These proteins are secreted by the uterine endometrium and bind UF in the reduced state [36]. Their association with UF could inhibit the ability of UF to induce lipid peroxidation.

Superoxide dismutase, which catalyzes the degradation of the superoxide ion, and catalase, which catalyzes the degradation of hydrogen peroxide, are both part of the cellular defense against oxidants [28]. The effect of these enzymes on UF-induced lipid peroxidation is, therefore, of interest.

The current experiments were designed with the following objectives: 1) to determine, by use of microsomal membranes from reproductive tissues as substrates, whether UF can induce lipid peroxidation in the presence of ASC; and 2) to examine possible tissue defense mechanisms available to inhibit this reaction such as apotransferrin, RBP, UFAP, catalase, and superoxide dismutase.

MATERIALS AND METHODS

Preparation of Microsomal Membranes

Tissues were placed into 20 ml of 50 mM Tris, 250 mM sucrose (pH 7.4), and homogenized. Five grams of tissue was used for endometrial, placental, and fetus minus liver tissues. Because the amount of tissue was limited, the total amount of tissue recovered was used for Day 13 conceptus and Day 30 fetal liver. Homogenates were then centrifuged ($1000 \times g$) for 10 min, and the supernatants were collected and centrifuged at $100\,000 \times g$ for 45 min. Pellets were rinsed once with 1 mM Tris (pH 8.2), 0.9% NaCl and then rehomogenized (2 ml for endometrial, fetal liver, and fetus minus liver tissues; 0.5 ml for conceptus and placental tissues) in the same buffer. Microsomal membranes were aliquoted, frozen in liquid nitrogen, and stored at -70°C until used. Concentration of membrane protein in each preparation was determined by the method of Lowry et al. [37] with BSA used as standard.

Preparation of UF, UF/UFAP, RBP, and Transferrin

UF, the UF/UFAP complex, and RBP were purified from Day 45 or Day 60 porcine allantoic fluid. The holoRBP (RBP with retinol bound) was purified by the procedure described by Vallet [38]. Proteins that do not bind to DEAE cellulose in the first step of RBP purification contain UF. UF and UF/UFAP complex were purified from this fraction according to the procedures of Buhi et al. [32] and Baumbach et al. [36]. Protein concentrations were determined [37] with BSA used as a standard. Purity of RBP, UF, and UF/UFAP complex were determined by SDS-PAGE (Fig. 3; RBP not shown). ApoRBP (RBP with no retinol bound) was prepared by extracting holoRBP twice with ether, followed by lyophilization. This treatment removed at least 95% of the bound retinol as measured by fluorescence at 330-nm excitation, 470-nm emission (not shown). No attempt was made to further saturate the holoRBP with retinol because of the potential difficulty in removing unbound retinol. For use in experiments, RBP and UF were dialyzed against distilled water, lyophilized, redissolved at appropriate concentrations in 1 mM Tris (pH 8.2) and 0.9% saline, and then aliquoted and frozen at -70°C until used. The UF/UFAP complex was dialyzed against 1 mM Tris, 0.9% saline and used without concentration to avoid disruption of the complex.

Bovine holo- and apotransferrin were purchased from Sigma Chemical Co. (St. Louis, MO). Transferrins were dissolved in 1 mM Tris (pH 8.2) and 0.9% saline, aliquoted, and frozen at -70°C until used.

Measurement of Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was monitored as TBARS, a measure of malondialdehyde production from breakdown of unsat-

urated fatty acid peroxides, by a modification of the method of Ohkawa et al. [39]. Briefly, 1 μ l of a solution of 10 mM alpha-tocopherol in ethanol was added to each sample to prevent further lipid oxidation. Then, 10 μ l 8% SDS, 75 μ l 20% acetic acid (pH 3.5), and 75 μ l 0.8% thiobarbituric acid (Sigma) were added sequentially to each sample. Samples were incubated in a boiling water bath for 20 min and allowed to cool to room temperature, and then were extracted with 1.5 ml butanol, mixed vigorously, and centrifuged in a microcentrifuge for 10 min. The butanol was recovered, and fluorescence of the butanol extract was determined at an excitation wavelength of 515 and an emission wavelength of 553. Data were expressed as relative fluorescence units per 10 μ g membrane protein.

Experiment 1: Preliminary Characterization of UF, Holotransferrin, and Free Iron-induced Lipid Peroxidation

A large pool of Day 45 endometrial microsomal membranes was used for preliminary characterization of lipid oxidation catalyzed by UF, free iron, and holotransferrin. Unless otherwise specified, reactions were performed in 1 mM Tris 1.9% saline (pH 8.2) with use of either 100 μ M UF, free iron, or holotransferrin, plus 50 μ M ASC along with 10 μ g membrane protein. Total reaction volume was 10 μ l, and reactions were incubated for 30 min at 37°C with air used as gas phase. The effect of amount of membranes (0–100 μ g membrane protein), reaction time (0–4 h), ASC concentrations (0–200 μ M), and iron/iron-containing protein concentrations (0–200 μ M) were determined. Also, a preliminary experiment to determine the effect of increasing concentrations of holo- vs. apoRBP on lipid peroxidation in the presence of UF and ASC was performed with holo and apoRBP concentrations ranging from 0 to 100 μ M. Preincubation of membranes with RBP treatments was performed for 3 h at 0°C (to allow the membranes to equilibrate with RBP), followed by the addition of UF and ASC (final concentration of RBP 0–50 μ M) and incubation at 37°C for 30 min. A preliminary trial to determine the effect of apo- vs. holotransferrin on UF-induced lipid peroxidation was performed, with concentrations of transferrin ranging from 0 to 50 μ M. All preliminary determinations were performed in triplicate in at least two separate experiments. Incubated samples were then processed to measure TBARS.

Experiment 2: Interaction between UF, RBP, and Transferrin

Pigs used were maintained according to established guidelines for animal care. To further investigate the ability of UF to induce lipid peroxidation and to determine the interaction of UF with transferrin and RBP, uteri were collected from gilts slaughtered on Days 13 (n = 5) and 30 (n = 5) of pregnancy. On Day 13 of pregnancy, each uterine

horn was flushed with 20 ml saline, and then 5 g endometrium was collected. Uterine flushings were centrifuged (1000 \times g) for 10 min to recover conceptus tissue, and the supernatants were retained for measurement of RBP by RIA [40] and measurement of retinol [41]. On Day 30, endometrium, placenta, and fetuses were collected, and fetal livers and the remainder of the fetus (fetus minus liver) were pooled separately. All tissues were then processed for recovery of microsomal membranes.

Day 13 endometrial and conceptus, and Day 30 endometrial, placental, fetal liver, and fetus minus liver microsomal membranes (because of limitations in tissue, 1.5 μ g conceptus microsomal membrane protein was used in this experiment, 10 μ g was used for all others) were incubated (37°C, 30 min) with the following treatments: 1) no treatment, 2) 50 μ M ASC, 3) 100 μ M UF, 4) 50 μ M ASC + 100 μ M UF, 5) 50 μ M ASC + 100 μ M UF + 10 μ M apotransferrin, and 6) 50 μ M ASC + 100 μ M UF + 10 μ M holotransferrin. For treatments 7 through 10, membranes were preincubated (0°C, 3 h) with either 7) no treatment, 8) 50 μ M fetuin (irrelevant protein control), 9) 50 μ M holoRBP, or 10) 50 μ M apoRBP followed by incubation (37°C, 30 min) with 50 μ M ASC + 100 μ M UF. Incubations were performed in triplicate for membranes from each pig. Samples were then processed to measure TBARS.

Experiment 3: Comparison of Catalytic Activity of UF and UF/UFAP Complex

Fetal liver microsomal membranes from the previous experiment were incubated for 30 min at 37°C with 1) no treatment, 2) 50 μ M ASC, 3) 40 μ M UF, 4) 40 μ M UF/UFAP complex (assuming a molecular weight of 80 000 for the complex), 5) 40 μ M UF + 50 μ M ASC, or 6) 40 μ M UF/UFAP complex + 50 μ M ASC, followed by measurement of TBARS. The UF and UF/UFAP complex preparations used were subjected to SDS-PAGE followed by staining with Coomassie blue dye and densitometry to compare the relative amounts of UF present. Finally, the effect of UF concentrations ranging from 1 to 100 μ M on TBARS in liver membranes was determined.

Experiment 4: Effect of Catalase and Superoxide Dismutase on UF-Catalyzed Lipid Peroxidation

Fetal liver membranes were incubated for 30 min at 37°C with 100 μ M UF + 50 μ M ASC and the following treatments: 1) no treatment, 2) 100 U/ml catalase, or 3) 100 U/ml superoxide dismutase. After incubation, samples were processed for TBARS.

Statistical Analyses

Preliminary data were summarized by analysis of variance with models that included effects of membrane protein amount, time, ASC concentration, and iron/iron-containing

protein concentration depending on the experiment, along with effects of iron/iron-containing protein and interactions with iron/iron-containing protein. Maximal TBARS production in the various trials was determined by use of contrasts. Similar analyses were used to summarize the effects of apo- vs. holoRBP and apo- vs. holotransferrin at various concentrations.

Data from experiment 2 were analyzed by analysis of variance after log transformation with use of a model that included effects of pig and treatment. The following contrasts were performed: 1) treatments 1 and 3 vs. 2 and 4 (main effect of ASC), 2) treatments 1 and 2 vs. 3 and 4 (main effect of UF), 3) treatments 1 and 4 vs. 2 and 3 (interaction of ASC and UF), 4) treatment 4 vs. treatment 6 (effect of holotransferrin), 5) treatments 4 and 6 vs. 5 (effect of apo-transferrin), 6) treatments 7 vs. 8 (effect of fetuin), 7) treatments 7 and 8 vs. 10 (effect of apoRBP), and 8) treatments 7, 8, and 10 vs. 9 (effect of holoRBP). In a further analysis, TBARS in untreated membranes (treatment 1) from the various tissue sources were analyzed separately by analysis of variance to assess endogenous lipid peroxidation. The model used included effects of day of pregnancy, tissue within day of pregnancy, and pig within day of pregnancy. The following contrasts were performed: 1) Day 13 endometrial tissue vs. conceptus tissue, 2) Day 30 fetus minus liver tissue vs. placental tissue, 3) Day 30 fetus minus liver and placental tissue vs. endometrial tissue, and 4) Day 30 fetus minus liver, placental, and endometrial tissue vs. fetal liver tissue.

Data from experiment 3 were log-transformed and then subjected to analysis of variance with a model that included effects of pig and treatment. The following contrasts were performed: 1) treatment 3 vs. 4; 2) treatment 5 vs. 6; 3) treatment 3 and 4 combined vs. treatment 1; and 4) treatment 5 and 6 combined vs. treatment 2. Densitometric measurements of UF in the UF and UF/UFAP complex were analyzed by analysis of variance. The effect of increasing concentrations of UF on lipid peroxidation in fetal liver microsomal membranes was analyzed by analysis of variance after log transformation of the data. The following contrasts were performed: 1) 0 vs. 1 μM UF, 2) 0 and 1 μM UF combined vs. 3 μM UF, 3) 30 vs. 100 μM UF, and 4) 30 and 100 μM UF combined vs. 10 μM UF. Data from experiment 4 were analyzed by analysis of variance and the following contrasts: 1) control vs. catalase and 2) control vs. superoxide dismutase.

RESULTS

Experiment 1: Preliminary Characterization of UF, Transferrin, and Free Iron-induced Lipid Peroxidation

Generally, UF was the most active in inducing lipid peroxidation followed by free iron and transferrin; either the

latter two were equal in activity (varying time or ASC), or free iron was more active (varying membrane protein) than transferrin. Statistical analysis indicated that maximal TBARS was obtained when 10 μg microsomal membrane protein was used (Fig. 1a); therefore, this amount was used for the rest of the experiments. Generation of TBARS was maximal at 30, 60, and 60 min for UF, transferrin, and free iron, respectively (Fig. 1b). Because the reaction for UF was maximal at 30 min, this time was chosen for further experiments. Increasing ASC concentrations (Fig. 1c) increased generation of TBARS to a maximum at 100 μM when 100 μM of either UF or free iron was used. Maximum TBARS for 100 μM transferrin was obtained at 50 μM ASC. With 50 μM ASC, maximum TBARS production was obtained at UF and transferrin concentrations of 50 μM (Fig. 1d). Little TBARS production was obtained with free iron concentrations of 50 μM or below. The maximum reaction was obtained at 100 μM iron. HoloRBP displayed antioxidant activity (i.e., inhibited TBARS production compared to apoRBP) only at the highest concentration used (50 μM final concentration; Fig. 1e). Apotransferrin was very effective at inhibiting TBARS production induced by UF; complete inhibition of the reaction induced by 100 μM UF was accomplished with 10 μM apotransferrin (Fig. 1f).

Experiment 2: Effect of RBP and Transferrin on UF-induced Lipid Peroxidation

Mean concentrations of RBP and retinol in uterine flushings collected on Day 13 were $159.8 \pm 10.4 \mu\text{g/ml}$ and $759.6 \pm 28.4 \text{ ng/ml}$, respectively. Assuming a molecular weight for RBP and retinol of 20 000 and 287, respectively, and a 1:1 ratio of retinol with RBP, average saturation of RBP was $33.4 \pm 1.4\%$.

Mean fluorescence values of TBARS formed in the presence of treatments 1 through 10 are summarized in Tables 1–3. Contrasts indicated that a statistical interaction between ASC and UF was present for microsomal membranes obtained from Day 13 endometrial ($p < 0.01$) and Day 30 endometrial ($p < 0.01$), fetal liver ($p < 0.01$), and placental ($p < 0.05$) tissues (Table 1). Examination of the results indicated that the interaction for membranes obtained from Day 13 and 30 endometrial and Day 30 fetal liver tissues was due to a synergistic effect of UF and ASC combined. In contrast, the interaction for membranes from placental tissues appeared to be due to a slight inhibitory effect of UF when combined with ASC compared to ASC alone, while treatment with UF alone had no effect. Main effects of both ASC and UF were obtained for Day 13 conceptus and Day 30 fetus minus liver membranes, indicating that the addition of both reagents stimulated TBARS production. The lack of an interaction for these tissues suggests that the effects of UF and ASC were additive.

Addition of holotransferrin to UF- and ASC-treated membranes had no effect on TBARS production for any mem-

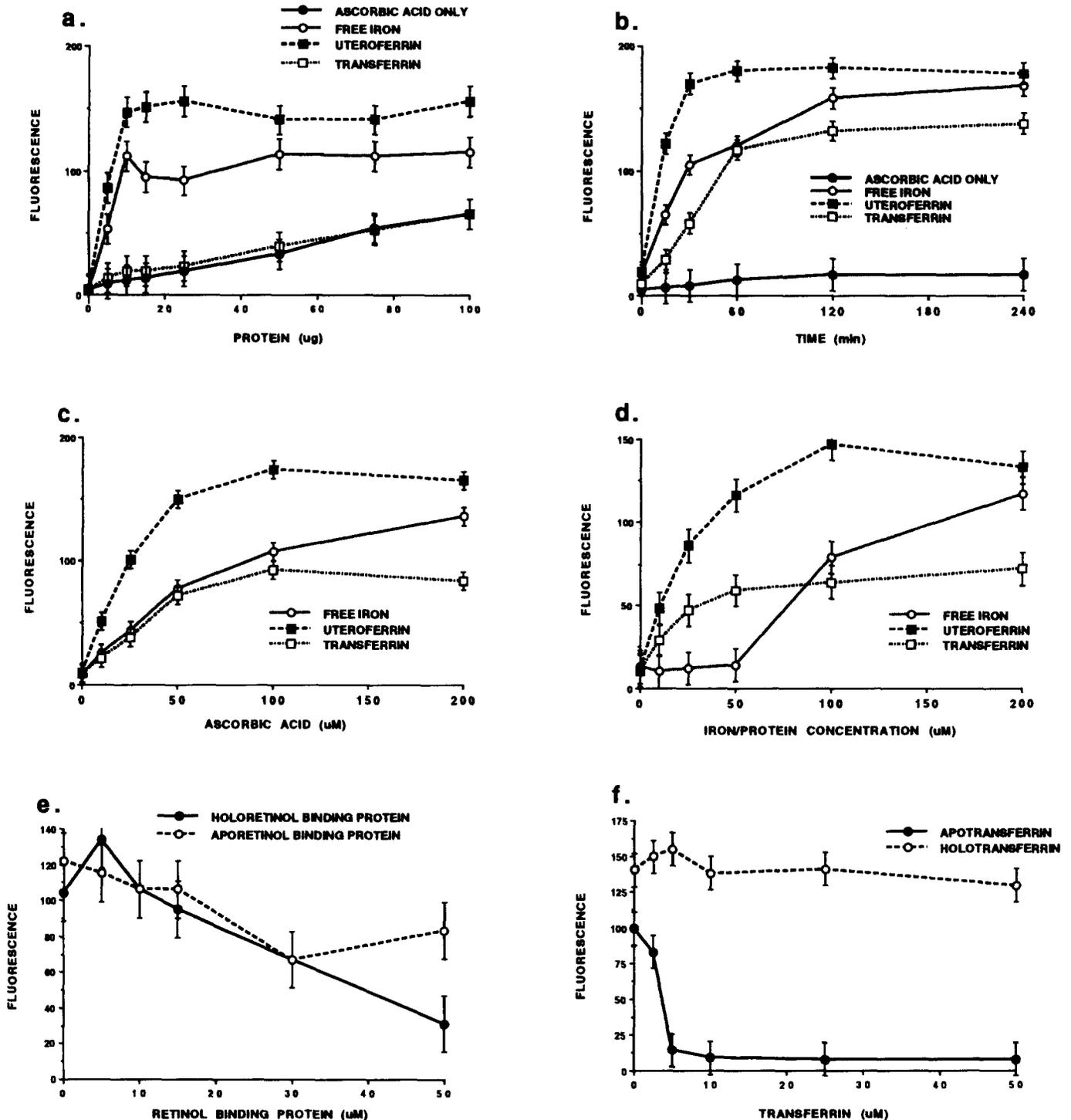


FIG. 1. Results of preliminary experiments to determine effect of (a) endometrial microsomal membrane protein amount, (b) reaction time, (c) ASC concentration, (d) amount of iron/iron-containing protein, (e) concentration of holo- vs. apoBPP, and (f) concentration of apo- vs. holo-transferrin, on TBARS (measured as relative fluorescence) generated by UF (all graphs), holo-transferrin (graphs a-d), or free iron (graphs a-d) by use of a pool of microsomal membranes prepared from endometrial tissue collected on Day 45 of pregnancy as substrate. Means are least squares means; error bars are SEM from analysis of variance of at least two experiments performed in triplicate. Unless otherwise indicated, 100 μ M UF, free iron, or transferrin; 50 μ M ASC; and 10 μ g membrane protein were used.

TABLE 1. Mean (\pm pooled SEM from analysis of variance for each tissue source; n = 5) fluorescence (relative units) per 10 μ g microsomal membrane protein (a measure of thiobarbituric acid reactive substances) from experiment 2 are summarized for the following treatments: no treatment (NT), 50 μ M ascorbic acid (ASC), 100 μ M UF, 50 μ M ascorbic acid plus 100 μ M UF (UF + ASC).

| Microsomal membrane source | Treatment | | | |
|---|-----------------|-------|------|----------|
| | NT | ASC | UF | UF + ASC |
| Day 13 endometrium ^a | 5.4 \pm 17.6 | 10.6 | 11.2 | 129.4 |
| Day 13 conceptus ^{b,c} | 18.4 \pm 18.5 | 77.4 | 29.4 | 130.6 |
| Day 30 endometrium ^a | 9.0 \pm 16.8 | 14.0 | 13.2 | 156.0 |
| Day 30 placenta ^d | 11.8 \pm 6.1 | 49.2 | 14.8 | 40.4 |
| Day 30 fetal liver ^a | 40.6 \pm 13.9 | 105.6 | 60.0 | 245.0 |
| Day 30 fetus minus liver ^{b,c} | 11.2 \pm 13.0 | 30.2 | 43.2 | 133.6 |

^aInteraction between UF and ascorbic acid ($p < 0.01$).

^bMain effect of ascorbic acid ($p < 0.01$).

^cMain effect of UF ($p < 0.01$).

^dInteraction between UF and ascorbic acid ($p < 0.05$).

brane; addition of apotransferrin significantly inhibited ($p < 0.01$) TBARS production for all membranes examined (Table 2).

No effect of fetuin or apoRBP was obtained on TBARS production for any of the tissues examined. The production of TBARS was significantly decreased when holoRBP was added to membranes prepared from Day 13 endometrial ($p < 0.01$) and conceptus ($p = 0.01$) and Day 30 fetal liver ($p < 0.01$), placental ($p < 0.01$), and fetus minus liver ($p < 0.01$) tissues, but holoRBP had no effect on TBARS production in Day 30 endometrial tissue microsomal membranes (Table 3).

Comparisons were made between endogenous TBARS in microsomal membrane preparations on each of the days examined by analyzing TBARS in untreated membranes (treatment 1). On Day 13, analysis indicated that conceptus microsomal membranes contained more ($p < 0.01$) TBARS per mg protein than did endometrial membranes. On Day 30 of pregnancy, TBARS in fetus minus liver, placenta, and endometrium did not differ from each other, and were significantly less than TBARS in fetal liver membranes.

TABLE 2. Mean (\pm pooled SEM from analysis of variance for each tissue source; n = 5) fluorescence (relative units) per 10 μ g microsomal membrane protein (a measure of thiobarbituric acid reactive substances) from experiment 2 are summarized for the following treatments: 100 μ M UF plus 50 μ M ASC (UF + ASC), 100 μ M UF plus 50 μ M ascorbic acid plus 10 μ M apotransferrin (UF + ASC + ATF) and 100 μ M UF plus 50 μ M ascorbic acid plus 10 μ M holotransferrin (UF + ASC + HTF).

| Microsomal membrane source | Treatment | | |
|---------------------------------|------------------|----------------|----------------|
| | UF + ASC | UF + ASC + ATF | UF + ASC + HTF |
| Day 13 endometrium ^a | 129.4 \pm 17.6 | 11.2 | 147.2 |
| Day 13 conceptus | 130.6 \pm 18.5 | 34.6 | 125.4 |
| Day 30 endometrium | 156.0 \pm 16.8 | 15.8 | 109.0 |
| Day 30 placenta | 40.4 \pm 6.1 | 16.4 | 43.2 |
| Day 30 fetal liver | 245.0 \pm 13.9 | 41.8 | 256.6 |
| Day 30 fetus minus liver | 133.6 \pm 13.0 | 16.4 | 142.8 |

^aUF + ASC and UF + ASC + HTF were not different; UF + ASC + ATF was different ($p < 0.01$) from UF + ASC and UF + ASC + HTF combined for all microsomal membrane sources.

TABLE 3. Mean (\pm pooled SEM from analysis of variance of each tissue source; n = 5) fluorescence (relative units) per 10 μ g microsomal membrane protein (a measure of thiobarbituric acid reactive substances) from experiment 2 are summarized for the following treatments: preincubation with no treatment (NT), 50 μ M fetuin (FET), 50 μ M holoRBP (HRBP) or 50 μ M apoRBP (ARBP) followed by incubation with 100 μ M UF and 50 μ M ascorbic acid.

| Microsomal membrane source | Treatments | | | |
|---------------------------------|------------------|-------|-------|-------|
| | NT | FET | HRBP | ARBP |
| Day 13 endometrium ^a | 109.4 \pm 17.6 | 93.8 | 17.0 | 157.0 |
| Day 13 conceptus | 73.4 \pm 18.5 | 116.0 | 46.0 | 85.2 |
| Day 30 endometrium | 184 \pm 16.8 | 233.8 | 199.4 | 196.6 |
| Day 30 placenta | 45.2 \pm 6.1 | 43.8 | 26.4 | 46.2 |
| Day 30 fetal liver | 300.4 \pm 13.9 | 343.0 | 265.6 | 355.6 |
| Day 30 fetus minus liver | 107.8 \pm 13.0 | 141.6 | 79.8 | 129.2 |

^aHRBP was different ($p < 0.01$) from NT, FET and ARBP combined for all tissues but Day 30 endometrium. NT, FET and ARBP were not different for all tissues.

Experiment 3: Comparison of Catalytic Activity of UF and UF/UFAP Complex

Results of this experiment are summarized in Figure 2. The TBARS production with UF and with the UF/UFAP complex without ASC were similar and resulted in a small but significant ($p < 0.01$) increase compared to the no treatment controls. UF/UFAP complex stimulated less ($p < 0.01$) TBARS than did UF when each was combined with ASC. The SDS-PAGE analysis indicated that the UF/UFAP complex preparation contained more UF than did the UF prep-

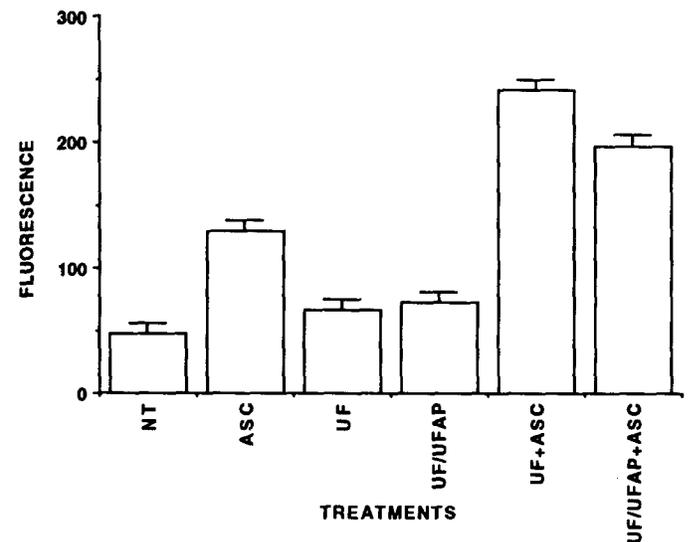


FIG. 2. Mean fluorescence (measure of TBARS) per 10 μ g fetal liver microsomal membrane protein (n = 5 per treatment) treated with either no treatment (NT), 50 μ M ASC, 40 μ M UF, 40 μ M UF/UFAP complex, 40 μ M UF plus 50 μ M ASC (UF + ASC), and 40 μ M UF/UFAP complex plus 50 μ M ASC (UF/UFAP + ASC). UF and UF/UFAP treatments were similar and stimulated ($p < 0.01$) TBARS production compared to control. UF/UFAP + ASC treatment stimulated less ($p < 0.01$) TBARS production than UF + ASC treatment. UF/UFAP + ASC and UF + ASC treatments combined stimulated greater ($p < 0.01$) TBARS production than ASC treatment. Error bars are pooled SEM from analysis of variance.

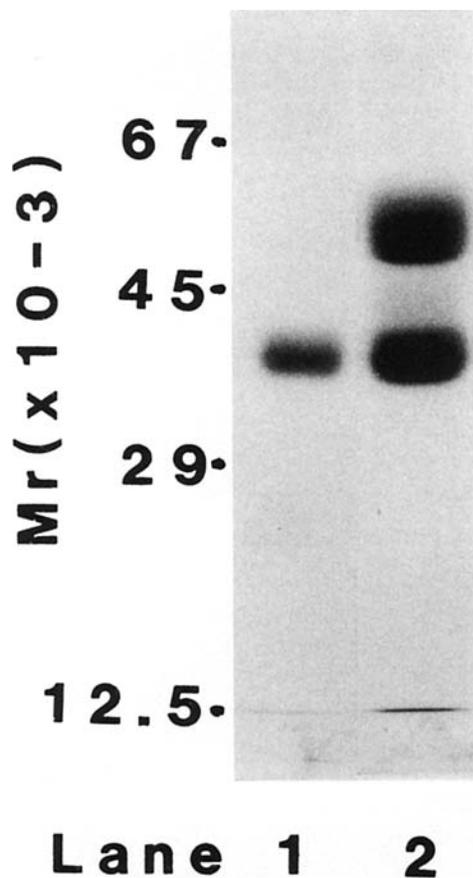


FIG. 3. SDS-PAGE gel comparing UF (lane 1) and UF/UFAP complex (lane 2) preparations used in experiment 3. Five microliters of 80- μ M solution of each was loaded. Densitometry indicated that UF/UFAP complex preparation contained 2.2 times more UF than did UF preparation ($n = 4$).

aration (Fig. 3). Densitometric analysis indicated that the UF/UFAP complex contained approximately 2.2 times the amount of UF in the UF preparation (38.3 ± 1.74 vs. 16.8 ± 1.74 relative units, respectively; $p < 0.01$).

Subsequent analysis of the effect of increasing UF concentrations on TBARS in liver membranes indicated that as little as 3 μ M caused a significant ($p < 0.05$) increase in TBARS production when in the presence of 50 μ M ASC (Fig. 4). Maximum TBARS was obtained at 30- μ M concentration. Furthermore, TBARS production was not linearly related to UF concentration but instead was linear with the log of UF concentration (note the log scale in Fig. 4).

Experiment 4: Effect of Catalase and Superoxide Dismutase on UF-induced Lipid Peroxidation

Catalase (316.0 ± 6.1) and superoxide dismutase (318.4 ± 6.1) had no effect on UF-induced lipid peroxidation measured as TBARS (control, 315.2 ± 6.1).

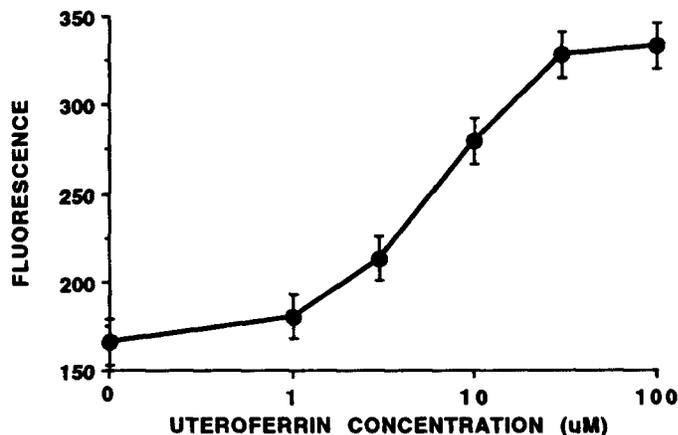


FIG. 4. Effect of UF concentration on TBARS (measured as relative fluorescence) in fetal liver microsomal membranes. TBARS production is linear, with log of UF concentration between 1 and 30 μ M ($n = 3$ per concentration). Error bars are pooled SEM from analysis of variance.

DISCUSSION

The results indicate that UF can induce lipid peroxidation in microsomal membranes of reproductive tissues in the presence of physiological concentrations of ASC. These experiments also demonstrate that transferrin, RBP, and UFAP, but not catalase or superoxide dismutase, may play a role in inhibiting this reaction in reproductive tissues.

A possible criticism of this experiment may be that an indirect assay (i.e., TBARS) was used to measure lipid peroxidation. This criticism is unlikely to be valid for three reasons: 1) a substantial body of research exists in which this assay has been used to monitor lipid peroxidation; 2) we are confirming that UF is capable of inducing lipid peroxidation, an observation that has been made by others using other methods [19, 20]; and 3) fluorescence of TBARS was used to decrease the likelihood of measurement of substances unrelated to lipid peroxidation.

Microsomal membranes from Day 13 endometrial and conceptus and Day 30 endometrial, fetal liver, and fetus minus liver tissues were susceptible to UF-induced lipid peroxidation, as measured by production of TBARS. Uncontrolled lipid peroxidation in tissues can be lethal, because of effects on permeability of cell membranes and on the function of proteins [21–24]. These results, coupled with the fact that periods of embryonic [1–3] and fetal [15–18] loss coincide with periods of increased UF production by the porcine endometrium [8, 25], are consistent with the hypothesis that UF may cause some embryonic and fetal loss. It may be further hypothesized that tissues known to bind UF, i.e., erythropoietic cells, should be most susceptible. Receptors that bind UF are present on fetal liver cells [42], and both the endogenous amount of TBARS production and the UF-induced TBARS production were greatest for this tissue. Erythropoietic tissues are also present in the early

blastocyst after approximately Day 13 of pregnancy, coincident with mesodermal outgrowth [43]. It therefore seems possible that erythropoietic tissues, because they bind UF, may be the most sensitive to this detrimental reaction.

Results indicate that for Day 13 conceptus and Day 30 fetus minus liver tissues, the effects of ASC and UF were both significant and additive (i.e., no statistical interaction was present), indicating that ASC treatment and UF treatment of membranes of these tissues significantly stimulated TBARS production. Likewise, results indicated that ASC treatment stimulated TBARS production in Day 30 fetal liver and placental tissues. It may be speculated that increased TBARS due to ASC is caused by interaction of ASC with iron-containing proteins that are endogenous to the microsomal membranes. This possibility is supported by the fact that apotransferrin decreased TBARS production to below that obtained with ASC alone, indicating that the endogenous ASC-dependent production of TBARS is iron-dependent. Candidates for the endogenous iron-containing proteins may include cytochromes, including both enzymes in the steroidogenic pathway and proteins in the respiratory chain; cyclooxygenases and lipoxygenases. The contribution of these proteins to lipid peroxidation *in vivo* is difficult to assess because these proteins may not have access to ASC inside the cell and thus would not be catalytic. Thus, the endogenous lipid peroxidation activated by ASC could be an artifact generated by disruption of the cells. This reasoning does not apply to UF, which because of its extracellular location would have access to ASC.

Results indicate that apotransferrin, but not holotransferrin, effectively prevented both UF-induced and endogenously induced TBARS production. This result is similar to previous reports [28] and suggests that control of this reaction *in vivo* may depend on both the concentration and the saturation level of the transferrin present. Limited information on the concentrations of transferrin in swine fetal plasma is available in the literature [29, 30]. These reports indicate that transferrin concentrations are low in fetal plasma on Day 23 of pregnancy and then increase to a stable level by Day 37 of pregnancy. No information regarding transferrin production by the conceptus before Day 23 is available. However, an unidentified protein with characteristics similar to those of transferrin is secreted by the porcine blastocyst on Day 13 of pregnancy but not before [31]. No information on saturation of fetal transferrin during pregnancy in swine is available. These aspects of conceptus iron metabolism are currently under investigation.

TBARS production was significantly inhibited by holoRBP in all tissues examined except Day 30 endometrial tissue. Thus, one of the functions of the RBP secreted by the endometrium and conceptus during pregnancy may be to provide antioxidant activity. Retinol has been reported to be an effective antioxidant [34, 35]. Although the concentration required for manifestation of this activity is high [50

μM), results indicate that intrauterine RBP concentrations are within this range. Assuming an intrauterine volume of 1 ml per uterine horn and complete equilibration of the 20-ml flush volume, mean concentration of RBP in the uterine lumen was 3.2 mg/ml. Assuming a molecular weight of 20 000 for RBP, this concentration equals 160 μM . The saturation of RBP within the lumen is similar to that of purified holoRBP (40%; unpublished results) and is similar to that obtained for serum (47%, [44]) and allantoic fluid (27%, [45]) in other experiments. This saturation level suggests that intrauterine concentrations of retinol may be as high as 50 μM . It is also possible that these high concentrations may be generated at other sites of synthesis of RBP, providing cells that secrete RBP with extracellular antioxidant activity. The possibility that RBP may be part of the defense against lipid peroxidation induced by UF is supported by the observation that tissues exposed to UF or involved in UF metabolism also secrete RBP. Day 13 trophoblast secretes RBP and would be exposed to high intrauterine concentrations of UF. Both RBP and RBP mRNA are present in Day 13 yolk sac and Day 30 and 60 fetal liver; all are erythropoietic tissues and therefore may concentrate UF. Day 60 fetal kidney, which binds UF and transports it into the allantoic sac, also contains both RBP and mRNA for RBP [46, 47]. The inability of holoRBP to prevent lipid peroxidation in Day 30 endometrial microsomal membranes is surprising. It is possible that greater concentrations are necessary to protect Day 30 endometrium. Concentrations of mRNA for RBP are greater on Day 30 than during early pregnancy [47]. Secretion of RBP by endometrial explants is also greater on Day 30 than in early pregnancy (R.K. Christenson et al., unpublished observations).

Two possible biochemical mechanisms whereby UF induces lipid peroxidation could be suggested. One possibility is that combining ASC with UF results in removal of iron from UF and that it is ASC chelated iron that actually catalyzes lipid peroxidation. It has previously been reported that incubation of UF with ASC results in association of iron with ASC [32]. If this were the mechanism, one might expect that UF would be less effective than free iron in inducing lipid peroxidation. Our results suggest that UF is more effective, especially at low concentrations. However, this comparison is not conclusive because free iron is actually insoluble due to interaction of the iron with water, and the actual "free" iron in aqueous solutions is probably low [48]. A previous report [20] could be interpreted to be in favor of release of the ferrous iron from UF followed by catalysis by free ferrous iron, because the ferrous iron chelator α, α' -bipyridyl inhibited lipid peroxidation induced by a related protein, human purple acid phosphatase, but did not inhibit acid phosphatase activity. However, it was also reported that either reduction of the protein with dithiothreitol or incubation of the protein with the ferric chelator 1,2-dimethyl-3-hydroxypyrid-4-one inhibits the acid phosphatase

activity of the human acid phosphatase, which suggests that this protein differs from UF [49, 50] in that the acid phosphatase activity of the human acid phosphatase used requires a diferric iron center for acid phosphatase activity. Thus, the observation that the ferrous chelator inhibits the lipid peroxidation activity of the human acid phosphatase without inhibiting acid phosphatase activity may be explained by the different oxidation states of the iron center required for the two activities and does not imply that the lipid peroxidation activity is a direct or indirect activity of either the human acid phosphatase or, by inference, UF. Thus, an alternative possibility to explain UF-induced lipid peroxidation is that this activity may be a direct function of reduced UF. In either hypothesis, oxidation of lipids does not appear to proceed via generation of superoxide or hydrogen peroxide, since neither catalase nor superoxide dismutase is effective in inhibiting the reaction. These results are therefore similar to those found previously with use of free iron [26, 27] and suggest that the reduced iron/UF may interact with lipids directly. Further experimentation is necessary to answer these questions.

Microsomal membranes prepared from Day 30 placental tissue appear to be resistant to UF-induced lipid peroxidation. However, the microsomal membranes were susceptible to lipid peroxidation induced presumably by endogenous iron-containing proteins as indicated by the effect of ASC alone and the inhibition obtained with apotransferrin. This suggests that the placental membranes either inhibited UF specifically or prevented external proteins from gaining access to membrane lipids. If present throughout pregnancy, this effect could serve to protect the placenta from damage caused by UF. Further characterization of this UF inhibitory activity is required to establish its identity and mode of action.

Results indicate that the UF/UFAP complex is less active in inducing lipid peroxidation than is free UF. Thus, one function of these proteins may be to protect lipids from oxidative damage. The amount of inhibition may appear to be minor. However, results of the SDS-PAGE analysis indicated that the UF/UFAP complex actually contained over twice as much UF as the UF preparation, possibly because of low reactivity of the UFAP in the protein assay used. Also, results indicate that TBARS production in fetal liver microsomal membranes is not linear with increasing UF concentrations but is instead linear with the log of UF concentrations. Thus, large reductions in active UF would be required to measure a moderate reduction in TBARS production. Taken together, these data suggest that the UFAP may inhibit a substantial portion of the UF present.

In conclusion, it has been demonstrated that UF is capable of inducing lipid peroxidation in microsomal membranes of reproductive tissues in the presence of physiological concentrations of ASC. Transferrin and to a lesser extent RBP and the UFAP represent three factors that can

inhibit this reaction and may therefore be part of *in vivo* mechanisms that protect reproductive tissues from the high concentrations of UF present within the intrauterine environment during pregnancy in pigs. Although no data directly indicating that UF causes embryonic loss are currently available, this information, coupled with the temporal association of UF secretion with two periods of conceptus loss in swine, suggests the hypothesis that failure of adequate control of UF-induced lipid peroxidation could be responsible for some embryonic/fetal loss. This hypothesis is currently under investigation.

REFERENCES

- Hanly S. Prenatal mortality in farm animals. *J Reprod Fertil* 1961; 2:182-194.
- Perry JS, Rowlands IW. Early pregnancy in the pig. *J Reprod Fertil* 1962; 4:175-188.
- Polge C. Embryo transplantation and preservation. In: Cole DJA, Foxcroft GR (eds.). *Control of Pig Reproduction*. London: Butterworth Scientific; 1982: 277-291.
- Johnson RK, Zimmerman DR, Kittok RJ. Selection for components of reproduction in swine. *Livest Prod Sci* 1984; 11:541-558.
- Longenecker DE, Day BN. Fertility level of sows superovulated at post-weaning estrus. *J Anim Sci* 1968; 27:709-711.
- Pope WF, Xie S, Broermann DM, Nephew KP. Causes and consequences of early embryonic diversity in pigs. *J Reprod Fertil Suppl* 1990; 40:251-260.
- Perry JS, Heap RB, Burton RD, Gadsby JE. Endocrinology of the blastocyst and its role in the establishment of pregnancy. *J Reprod Fertil Suppl* 1976; 25:85-104.
- Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig. I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod* 1982; 27:925-939.
- Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig. III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. *Biol Reprod* 1982; 27:957-965.
- Roberts RM, Bazer FW. The functions of uterine secretions. *J Reprod Fertil* 1988; 82:875-892.
- Wilde MH, Xie S, Day ML, Pope WF. Survival of small and large littermate blastocysts in swine after synchronous and asynchronous transfer procedures. *Theriogenology* 1988; 30:1069-1074.
- Pope WF, Lawyer MS, Butler WR, Foote RH, First NL. Dose-response shift in the ability of gilts to remain pregnant following exogenous estradiol-17 β exposure. *J Anim Sci* 1986; 63:1208-1210.
- Morgan GL, Geisert RD, Zavy MT, Shawley RV, Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. *J Reprod Fertil* 1987; 80:125-131.
- Blair RM, Geisert RD, Zavy MT, Yellin T, Fulton RW, Short EC. Endometrial surface and secretory alterations associated with embryonic mortality in gilts administered estradiol valerate on days 9 and 10 of gestation. *Biol Reprod* 1991; 44:1063-1079.
- Knight JW, Bazer FW, Thatcher WW, Franke DE, Wallace HD. Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. *J Anim Sci* 1977; 44:620-637.
- Chen Z-Y, Dziuk PJ. Influence of initial length of uterus per embryo and gestation stage on prenatal survival, development, and sex ratio in the pig. *J Anim Sci* 1993; 71:1895-1901.
- Webel SK, Dziuk PJ. Effect of stage of gestation and uterine space on prenatal survival in the pig. *J Anim Sci* 1974; 38:960-965.
- Vallet JL, Christenson RK. Uterine space affects placental protein secretion in swine. *Biol Reprod* 1993; 48:575-584.
- Sibille J-C, Doi K, Aisen P. Hydroxyl radical formation and iron-binding proteins, stimulation by the purple acid phosphatases. *J Biol Chem* 1987; 262:59-62.
- Hayman AR, Cox TM. Purple acid phosphatase of the human macrophage and osteoclast, characterization, molecular properties, and crystallization of the recombinant Di-iron-oxo protein secreted by baculovirus-infected insect cells. *J Biol Chem* 1994; 269:1294-1300.
- Halliwell B, Gutteridge JML. Lipid peroxidation: a radical chain reaction. In: *Free Radicals in Biology and Medicine*. Oxford: Clarendon; 1989: 188-276.
- Benedetti A, Comparsi M. Formation, reactions and toxicity of aldehydes produced in

- the course of lipid peroxidation in cellular membranes. *Bioelectrochem Bioenerg* 1987; 18:187–202.
23. Frankel EN. Biological significance of secondary lipid oxidation products. *Free Radical Res Comm* 1987; 3:213–225.
 24. Sies H. Biochemistry of oxidative stress. *Angew Chem Int Ed Engl* 1986; 25:1058–1071.
 25. Basha SMM, Bazer FW, Roberts RM. The secretion of a uterine specific, purple phosphatase by cultured explants of porcine endometrium. Dependency upon the state of pregnancy of the donor animal. *Biol Reprod* 1979; 20:431–441.
 26. Brauhgler JM, Duncan LA, Chase RL. The involvement of iron in lipid peroxidation, importance of ferric to ferrous ratios in the initiation. *J Biol Chem* 1986; 261:10282–10289.
 27. Miller DM, Aust SD. Studies of ascorbate-dependent, iron catalyzed lipid peroxidation. *Arch Biochem Biophys* 1989; 271:113–119.
 28. Halliwell B, Gutteridge JMC. The chemistry of oxygen radicals and other oxygen-derived species. In: *Free Radicals in Biology and Medicine*. Oxford: Clarendon; 1989: 22–85.
 29. Cavanaugh ME, Cornelis ME, Dziegielewska KM, Luff AJ, Lai PCW, Lorscheider FL, Saunders NR. Proteins in cerebrospinal fluid and plasma of fetal pigs during development. *Dev Neurosci* 1982; 5:492–502.
 30. Lampreave F, Pineiro A. Concentrations of major plasma proteins in serum and whole-tissue extracts of porcine fetuses during development. *J Reprod Fertil* 1992; 95:441–449.
 31. Godkin JD, Bazer FW, Lewis GS, Geisert RD, Roberts RM. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. *Biol Reprod* 1982; 27:977–987.
 32. Buhi WC, Ducsay CA, Bazer FW, Roberts RM. Iron transfer between the purple phosphatase uteroferrin and transferrin and its possible role in iron metabolism of the fetal pig. *J Biol Chem* 1982; 257:1712–1723.
 33. Nuttleman PR, Roberts RM. Transfer of iron from uteroferrin (purple acid phosphatase) to transferrin related to acid phosphatase activity. *J Biol Chem* 1990; 265:12192–12199.
 34. Ciaccio M, Valenza M, Tesoriere L, Bongiorno A, Albiero R, Livrea MA. Vitamin A inhibits doxorubicin-induced membrane lipid peroxidation in rat tissues in vivo. *Arch Biochem Biophys* 1993; 302:103–108.
 35. Tesoriere L, Ciaccio M, Bongiorno A, Riccio A, Pintaudi AM, Livrea MA. Antioxidant activity of all-trans-retinol in homogeneous solution and in phosphatidylcholine liposomes. *Arch Biochem Biophys* 1993; 307:217–223.
 36. Baumbach GA, Ketcham CM, Richardson DE, Bazer FW, Roberts RM. Isolation and characterization of a high molecular weight stable pink form of uteroferrin from uterine secretions and allantoic fluid of pigs. *J Biol Chem* 1986; 261:12869–12878.
 37. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265–275.
 38. Vallet JL. Purification and properties of porcine allantoic fluid retinol binding protein. *Biol Reprod* 1993; 48(suppl 1):140.
 39. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95:351–358.
 40. Vallet JL. Technical note: a radioimmunoassay for porcine retinol binding protein. *J Anim Sci* 1994; 72:2449–2454.
 41. Selvaraj JR, Sushella TP. Estimation of serum vitamin A by a microfluorometric procedure. *Clin Chim Acta* 1970; 27:165–170.
 42. Saunders PTK, Renegar RH, Raub TJ, Baumbach GA, Atkinson PH, Bazer FW, Roberts RM. The carbohydrate structure of porcine uteroferrin and the role of the high mannose chains in promoting uptake by the reticuloendothelial cells of the fetal liver. *J Biol Chem* 1985; 260:3658–3665.
 43. Perry JS. The mammalian fetal membranes. *J Reprod Fertil* 1981; 62:321–335.
 44. Vallet JL, Christenson RK, Bartol FF, Wiley AA. Effect of retinyl palmitate, progesterone, oestradiol and tamoxifen treatment on secretion of a retinol binding protein-like protein during uterine gland development in neonatal swine. *J Reprod Fertil* 1995; (in press).
 45. Vallet JL, Christenson RK. Effect of estrone treatment from day 30 to 45 of pregnancy on endometrial protein secretion and uterine capacity. *J Anim Sci* 1994; 72:3188–3195.
 46. Harney JP, Miranda MA, Smith LC, Bazer FW. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol Reprod* 1990; 42:523–532.
 47. Harney JP, Smith LC, Simmen RCM, Fliss AE, Bazer FW. Retinol-binding protein: immunolocalization of protein and abundance of messenger ribonucleic acid in conceptus and maternal tissues during pregnancy in pigs. *Biol Reprod* 1994; 50:1126–1135.
 48. Aisen P, Listowsky I. Iron transport and storage proteins. *Annu Rev Biochem* 1980; 49:357–393.
 49. Schlosnagle DC, Sander EG, Bazer FW, Roberts RM. Requirement of an essential thiol group and ferric iron for the activity of the progesterone-induced porcine uterine purple phosphatase. *J Biol Chem* 1976; 251:4680–4685.
 50. Debrunner PG, Hendrich MP, Dejersey J, Keogh DT, Sage JT, Zerner B. Mossbauer and EPR study of the binuclear iron center in purple acid phosphatase. *Biochim Biophys Acta* 1983; 745:103–106.