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INDUCTION OF AVIAN SERUM APOLIPOPROTEIN II AND VITELLOGENIN BY TAMOXIFEN

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Summary- Apo II and vitellogenin were detected in sera of nonestrogenized roosters by radioimmunoassay. Tamoxifen (30mg/kg) raised basal serum apo II more than 50 fold and vitellogenin more than 15 fold. Serum accumulation of apo II was biphasic in response to increasing doses of tamoxifen. This biphasic response was reflected in the relative rates of hepatic apo II synthesis. The tamoxifen metabolites, hydroxytamoxifen and desmethyltamoxifen, and the triphenylethylene antiestrogen, CI 628, also increased serum apo II. These studies show that the expression of vitellogenin and apo II genes occurs at a basal rate prior to exogenous estrogen treatment. These experiments also demonstrate that the triphenylethylene antiestrogens have agonist activity in the chicken when very sensitive techniques are used to measure estrogen-regulated proteins.

In birds, estrogens stimulate the hepatic synthesis of apo II and vitellogenin and cause a dramatic accumulation of these proteins in plasma (1-3). While it is generally believed that vitellogenin synthesis in the rooster is completely dependent upon exogenous estrogens, there have been conflicting reports with respect to the hormone dependence of apo II synthesis. Chan and coworkers (1,2) reported that apo II is present in rooster plasma at relatively high levels in the absence of exogenous estrogens. In contrast, Kudzma et al. (4) failed to detect apo II in plasma lipoproteins from untreated birds by electrophoresis, and Wiskocil et al. (5) were unable to detect apo II mRNA prior to estrogen treatment. In the present study sensitive radioimmunoassays were used to determine whether apo II and vitellogenin are present in serum from untreated animals. The results show that both proteins are present at low levels prior to estrogen treatment. A low level of hepatic apo II synthesis was also detected. The antiestrogen, tamoxifen, was used to test whether basal apo II synthesis was due to endogenous estrogens, since previous studies (6-8) have shown tamoxifen to act as a pure antagonist in the chicken. We found, quite unexpectedly, that tamoxifen elevated plasma apo II and vitellogenin and increased hepatic apo II synthesis.

ABBREVIATIONS USED: Apo II: apolipoprotein II; apo B: apolipoprotein B; SDS: sodium dodecyl sulfate

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Materials and Methods

Chemicals- Tamoxifen citrate, 4-hydroxytamoxifen (free base), and desmethyltamoxifen citrate were provided by ICI Americas, Inc. (Wilmington, Del.). CI 628 was from Parke-Davis (Detroit, Mich.) and clomiphene from the University Hospital (Stony Brook, N.Y.). Tritiated L-[4,5-^{3}H] leucine (60Ci/mmole) was from New England Nuclear.

Animals- White leghorn roosters (SPAFAS, Norwich, CN) (0.9-2kg) were injected intramuscularly with various doses of antiestrogens in propylene glycol and killed at the indicated times. Control animals received an equal volume of vehicle or no treatment.

Antiserum- Apo II was purified from VLDL of estrogen treated roosters (9) and was free of detectable apo B as judged by gel electrophoresis, the absence of reactivity with anti-apo B serum, and the absence of histidine upon amino acid analysis. Nevertheless, extensive characterization of the rabbit anti-apo II serum showed that it contained both nonprecipitating antibody to apo II and precipitating antibody to apo B. Contaminating anti-apo B antibody was removed by two cycles of chromatography of the antiserum on Sepharose 6B to which apo B had been coupled by standard procedures (10). The final anti-apo II serum showed no detectable reactivity toward purified apo B or pulse-labeled apo B in liver extracts. Antiserum directed against the mixture of vitellogenin I and vitellogenin II was prepared as described (11).

Radioimmunoassays- Apo II and a mixture of vitellogenin I and II were iodinated using the chloramine T method (12) to specific activities of 24μCi/μg and 15μCi/μg for apo II and vitellogenin, respectively. Assays were carried out with antiserum sufficient to precipitate 40-50% of the ^{125}I-antigen. Incubation with primary antiserum was for 24 and 48 hours for apo II and vitellogenin, respectively. Subsequently, 2μl of nonimmune rabbit serum and 8μl of goat anti-rabbit γ-globulin were added and incubation was continued for 40 minutes. Immunoprecipitates were collected and washed (13), and counted in an Isodyne 1185 gamma counter (Searle).

Tissue synthesis- Tissue incubations and preparation of liver extracts were as described (13), except that the incubation medium contained 250μCi[^{3}H] leucine/0.1ml. Labeled apo II was immunoprecipitated from liver extracts using the double antibody technique (13). Immunoprecipitates were analyzed on SDS-20% polyacrylamide gels (9, system B) and radioactive bands were visualized by fluorography (14). Intensities of immunoprecipitated [^{3}H]-apo II bands were compared by densitometry (15).

Results

Basal serum apo II and vitellogenin levels- A sensitive radioimmunoassay was developed to examine serum apo II levels. The working range of this assay lay between 0.3-3ng of apo II (see antigen binding curve [solid line], Fig. I). Basal apo II levels ranged from 1-40ng/ml in serum samples from 20 animals. Replicate assays showed less than 10% variation; hence, the wide range of serum apo II may represent genuine animal to animal variation. Figure 1 shows that serum from an untreated rooster decreased antibody-bound ^{125}I-apo II in parallel to the standard curve generated with purified apo II, while equivalent volumes of fetal calf serum had no effect. The radioimmunoassay for vitellogenin had a working range between 1-10ng. Control serum, but not fetal calf serum, displaced ^{125}I-vitellogenin from its antibodies (data not shown). Values for basal serum vitellogenin ranged from 4-8ng/ml in 4 roosters, while 2 other roosters had levels of approximately 100 ng/ml; these animals also had elevated apo II levels.

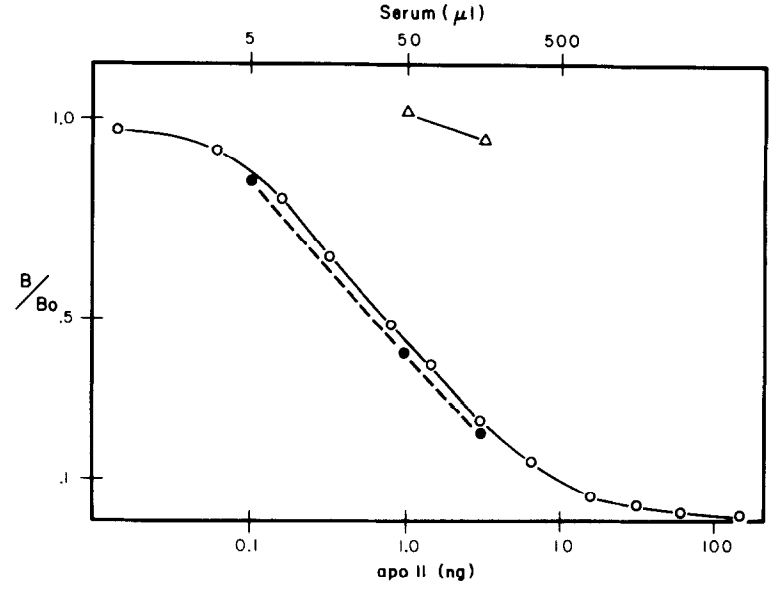

Fig. 1. Apo II radioimmunoassay. ^{125}I-apo II was displaced from anti-apo II antibodies by increasing amounts of unlabeled apo II (o-o) and increasing volumes of rooster serum (●---●, note top scale), but not fetal calf serum (▲—▲).

Basal hepatic apo II synthesis- When liver proteins were pulse-labeled in vitro to high specific activity (see Materials and Methods), a labeled protein that comigrated with plasma apo II on SDS gels was precipitated from liver extracts of untreated roosters by anti-apo II serum but not nonimmune rabbit serum. Visualization of this band required prolonged fluorographic exposure

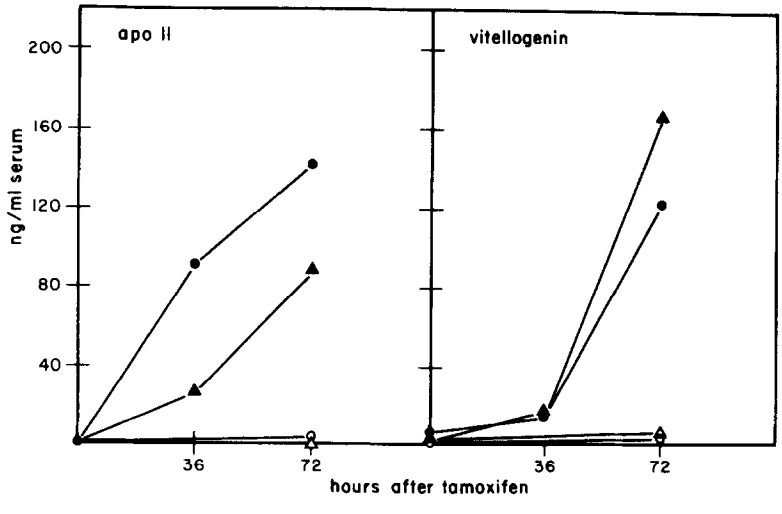

Fig. 2. Kinetics of serum apo II and vitellogenin accumulation. Two roosters received tamoxifen citrate (30mg/kg) (●,▲) or propylene glycol (o,▲) and apo II (left panel) and vitellogenin (right panel) were measured in serum samples taken at the indicated times.

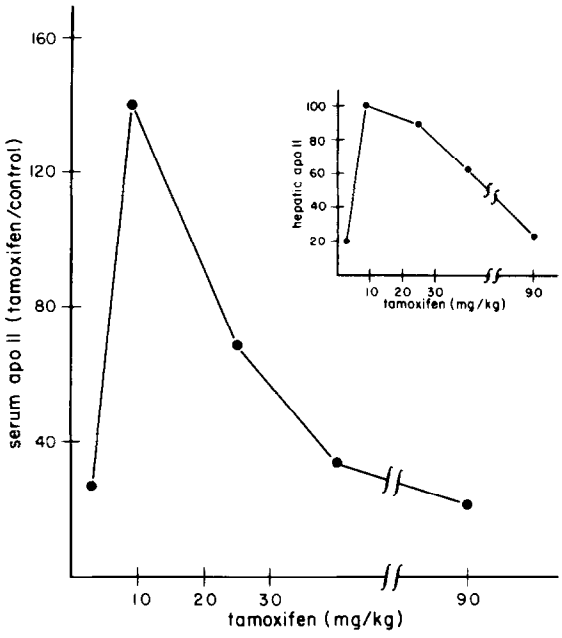

Fig. 3. Dose response characteristics of apo II synthesis and serum accumulation. Roosters received the indicated doses of tamoxifen citrate. Plasma apo II was measured at 0 and 48 hours. Results are expressed as a ratio of apo II levels before and after treatment. Each point represents the average of apo II values from two roosters. Insert: At 48 hours hepatic apo II synthesis was determined as described in Materials and Methods. Immunoprecipitates were analyzed on SDS gels by fluorography and the intensities of radioactive apo II bands were compared by densitometry. Units are arbitrary. Immunoprecipitates were from liver extracts with equivalent trichloroacetic acid precipitable protein radioactivity.

(2-4 weeks). This band was not immunoprecipitated from kidney, intestinal, adipose, adrenal, or skeletal muscle extracts. Rates of apo II synthesis in uninduced liver were estimated to be 0.01-0.1% of hepatic protein synthesis by comparing the intensities of labeled apo II bands from control liver with bands representing known rates of apo II synthesis from estrogen-induced livers.

Tamoxifen treatment- Figure 2 shows that tamoxifen (30mg/kg) increased apo II more than 50 fold and vitellogenin more than 15 fold in the sera of 2 representative roosters. A dose-response curve for tamoxifen exhibited an unusual biphasic character. A maximum response was seen with 9mg/kg, while the response declined markedly with higher doses (Fig. 3). When pulse labeled apo II was immunoprecipitated from liver extracts of these roosters and analyzed by SDS gel electrophoresis, densitometry showed that the relative rates of apo II synthesis followed a similar biphasic curve (Fig. 3, insert). Analysis of the serum samples in Fig. 3 for vitellogenin yielded a similar biphasic response curve (not shown).

Tamoxifen metabolites and other antiestrogens- Both hydroxytamoxifen and

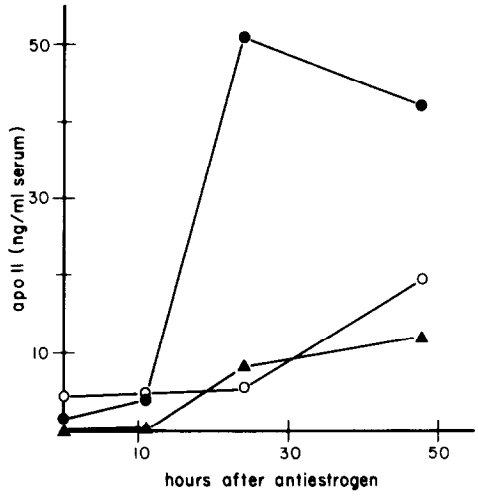

Fig. 4. Accumulation of serum apo II after treatment with hydroxytamoxifen, desmethyltamoxifen, or CI 628. Individual roosters received either 3mg hydroxytamoxifen (●), 3mg desmethyltamoxifen (o), or 30mg CI 628 (▲).

desmethyltamoxifen raised serum apo II levels (Fig. 4). Hydroxytamoxifen was more potent than desmethyltamoxifen at doses of 2.5,3, and 30mg/kg. Figure 4 only shows the 3mg/kg dose. The relative rate of hepatic apo II synthesis was also higher in hydroxytamoxifen than desmethyltamoxifen-treated animals (data not shown). The triphenylethylene antiestrogen, CI 628, significantly raised serum apo II levels (Fig. 4), while clomiphene (30mg/kg) produced only a marginal response (data not shown).

Discussion

Our results show that both apo II and vitellogenin are synthesized in the absence of estrogen treatment. However, the serum apo II levels in untreated roosters are much lower than reported by Chan et al. (2). The reason for this discrepency is unclear. Failure of other methods (4,5) to produce evidence for basal apo II or vitellogenin synthesis may be due to lower sensitivity of these techniques. Even the relatively sensitive hybridization measurements made by Wiskocil et al. (5) cover only a range of 2 x 10^4 from the maximum of the estrogenic response to the limit of detection, while we found that plasma apo II concentrations in untreated and fully estrogenized roosters vary over a range of 10^7. While it is generally believed that estrogenic stimulation of vitellogenin synthesis in the rooster involves de novo gene activation, the data presented here call for a reexamination of this point. Based on the composition of iodinated vitellogenin (11) and the extent of competition in the radioimmunoassay by control rooster serum, it is likely that both vitellogenin I and vitellogenin II are present in the basal state. This point, however, requires further investigation.

Our experiments did not clarify whether basal synthesis is estrogen dependent. We emphasize, however, that it is unlikely that these roosters were exposed to exogenous estrogens, since both the breeder and our animal facility use feeds lacking animal byproducts. Therefore, if the basal synthesis of apo II and vitellogenin is estrogen dependent, it is most likely due to low levels of endogenous estrogens.

A second finding in these experiments is that triphenylethylene antiestrogens do not act as pure antagonists in rooster liver. Our results suggest that serum apo II accumulation in response to these antiestrogens is due to increased hepatic apo II synthesis. Agonist activity of triphenylethylene antiestrogens in the rooster may be a general phenomenon since vitellogenin also accumulates in response to tamoxifen. It should be noted, however, that the maximum agonist activity seen here is still 1000 times less than the maximum response to estradiol, indicating that these compounds act as very weak partial agonists.

The biphasic character of the tamoxifen dose-response relationship (Fig. 3) is of considerable interest with respect to the molecular action of these drugs. One potential explanation for the biphasic character is the limited generation of a tamoxifen metabolite that has only agonist activity while tamoxifen is a partial agonist or a pure antagonist. In this case, the rising phase of the response curve could be due to the agonist metabolite, while the descending phase could be due to the unmetabolized tamoxifen. The unmetabolized tamoxifen would increase in relation to the metabolite when the administered dose saturated the metabolic pathway which generates the metabolite. This explanation ascribes the agonist and antagonist activities to different effector molecules. Alternatively, tamoxifen or its metabolites may each exhibit dual activities via interactions at different sites on the same receptor or via completely different pathways. Interestingly, Gschwendt (7) observed a biphasic dose-response relationship for the accumulation of estrogen-receptor in chick liver chromatin after antiestrogen treatment. This result suggests that the biphasic character of the apo II and vitellogenin responses to tamoxifen results from initial or early events at the level of the receptor.

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