PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 177, 42–46 (1984)

Effect of Tyrosine Modification on the Biological and Immunological Properties of Equine Chorionic Gonadotropin (41909)

H. PAPKOFF,[1] H. M. S. MURTHY, AND J. F. ROSER

Hormone Research Laboratory, University of California, San Francisco, California 94143

Abstract. The tyrosine residues of equine chorionic gonadotropin have been nitrated with tetranitromethane and the resulting effects on the biological and immunological activities of the hormone studied. All of the tyrosine residues in equine chorionic gonadotropin were found to react with tetranitromethane when a 100-fold molar excess of reagent was used or with an 8.6 molar excess in the presence of 5 *M* guanidine hydrochloride. Complete nitration abolished the biological activities and decreased the immunological activity of the hormone. The nitration of one tyrosine residue resulted in the loss of 70% of the LH activity of equine chorionic gonadotropin; the FSH activity declined in a similar fashion. Maximal nitration resulted in the loss of about 50% of the immunological activity of the native hormone. Nitrated derivatives of equine chorionic gonadotropin were unable to compete with the native hormone in the rat Leydig cell assay for LH. The results indicate that the tyrosine residues of equine chorionic gonadotropin play an important role in the manifestation of both the FSH and LH activity of the hormone.

Equine chorionic gonadotropin (eCG)[2] is a glycoprotein hormone produced during pregnancy in the mare by the endometrial cups of the placenta. It is similar to the pituitary gonadotropins and consists of two nonidentical subunits (1, 2). It is noteworthy for its high carbohydrate content (40–45%), its great stability, and the fact that it possesses both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) activity as a function of the same molecule (3). As with the pituitary gonadotropins, the biological activities of eCG are a function of the β subunit (3). It is not known, however, whether the same or different structural areas of the eCG β subunit are responsible for the LH and FSH activity. Previous studies on this problem from this laboratory have assessed the role of sialic acid (4) and histidine residues (5). In the present work we examine the effects of nitrating the tyrosine residues in eCG with tetranitromethane (TNM).

Tetranitromethane is a specific, mild reagent for the nitration of tyrosine residues in proteins and has been used extensively for structure and function studies of proteins (6–8). Previous reports from this laboratory and others have shown that glycoprotein hormones are sensitive to tyrosine modification by tetranitromethane. Ovine LH (9–11), bovine LH (12), and hCG (13, 14) have been subjected to nitration of tyrosine by tetranitromethane to elucidate the role of tyrosine in the structure and activity of these hormones. In the present study the tyrosine residues of eCG have been modified with TNM and the resultant effects on the biological and immunological activities of the hormone examined.

Materials and Methods. Highly purified eCG (15,000 IU/mg) was used in these studies and was prepared by methods described previously (15). Tetranitromethane (Aldrich Chemical Co.) and guanidine hydrochloride (Eastman) were used without further purification.

The nitration of eCG was carried out essentially as described by Sairam *et al.* (9) and Ma *et al.* (16). Reactions were carried out at room temperature (23°C) in 0.05 *M* Tris–HCl buffer, pH 8.0, containing, 1 *M* NaCl, and, in one case, with 5 *M* guanidine·HCl. Aliquots of TNM in absolute

[1] To whom correspondence should be sent.

[2] Abbreviations used: eCG, equine chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; TNM, tetranitromethane; V_e/V_0, gel filtration ratio of elution volume of protein peak to elution volume of Blue 2000 Dextran.

0037-9727/84 $1.50

ethanol were added to 1 ml solution containing 2 mg of eCG. The kinetics of nitration was monitored by measuring the absorbance of the reaction mixture at 428 nm in a Beckman DB spectrophotometer at various intervals. For calculating the formation of nitrotyrosine a value of 4100 for the molar extinction of nitrotyrosine at 428 nm was used (6). The molecular weight of eCG was taken to be 56,000 as inferred from the data of Moore and Ward (17). Nitrated derivations of eCG were recovered by desalting reaction mixtures on columns of Sephadex G-25 in 0.05 *M* NH_4HCO_3 followed by lyophilization. Amino acid analyses were by the method of Spackman *et al.* (18) in a Beckman Model 119C amino acid analyzer.

LH bioactivity of nitrated eCG preparations was determined by measuring testosterone production *in vitro* from a suspension of rat testis interstitial cells (19). FSH activity was determined by the *in vitro* production of cAMP using a suspension of immature rat seminiferous tubule cells (20). Immunological activity of nitrated eCG preparations was assessed by a homologous double-antibody radioimmunoassay for eCG (21) using purified eCG as standard and as radioligand. Comparison of eCG with pituitary LH and FSH in the above assays has been previously reported (1, 2, 21).

Results and Discussion. *Nitration of tyrosine residues in eCG.* The rate of nitration of tyrosine residues in eCG under various conditions is shown in Figs. 1 and 2. The rate and degree of nitration of eCG were dependent on the molar excess of TNM present in the reaction mixture. In 2 hr with an 8.6 molar excess of TNM (based on molar tyrosine content of eCG) two residues of the seven present in eCG were nitrated. In an experiment with ovine LH under identical conditions performed to validate the experimental conditions, five of the seven tyrosine residues of ovine LH were nitrated (Fig. 2), confirming results obtained previously (9). Complete nitration of all seven of the tyrosine residues in eCG was approached with a 100-fold excess of TNM. Estimation of nitrotyrosine content spectrophotometrically has been previously shown to be in good agreement with amino acid analysis (9). The content of other amino acids in nitrated eCG

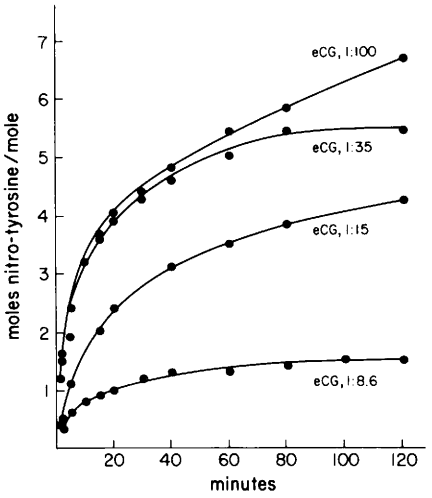

FIG. 1. Rate of nitration of equine chorionic gonadotropin in 0.05 *M* Tris buffer, pH 8.0–1 *M* NaCl, 23°C, with varying concentrations of tetranitromethane ranging from 1:8.6 to 1:100 (molar ratio of tyrosine reagent).

derivatives was in good agreement with that of the native hormone. The finding that all seven tyrosine residues in eCG can be nitrated suggests a greater accessibility of the tyrosine to the reagent than in the case of ovine LH where only five of the seven tyrosine residues can be nitrated under similar conditions (9). All of the tyrosines in ovine LH can be nitrated if the reaction is performed in the presence of 5 *M* guanidine hydrochloride. In a similar experiment with eCG (Fig. 2) it is seen that all seven tyrosines are still nitrated but the rate of nitration is much more rapid than in the absence of the guanidine hydrochloride.

The products of the nitration reaction were analyzed by gel filtration on a Sephacryl S-200 column in 0.05 *M* NH_4HCO_3. The elution profile of eCG with two nitrated tyrosines is shown in Fig. 3 and indicates the presence of high-molecular-weight polymers. The yield of the monomeric fraction was 45–50% and eluted from the column with a V_e/V_0 of 1.28 (Peak B) which is close to the elution volume of native eCG (V_e/V_0 = 1.38). The degree of polymerization of nitrated hormones has been shown to increase progressively with an increase in the extent of nitration (22) and may be due to crosslinking induced by tetranitromethane.

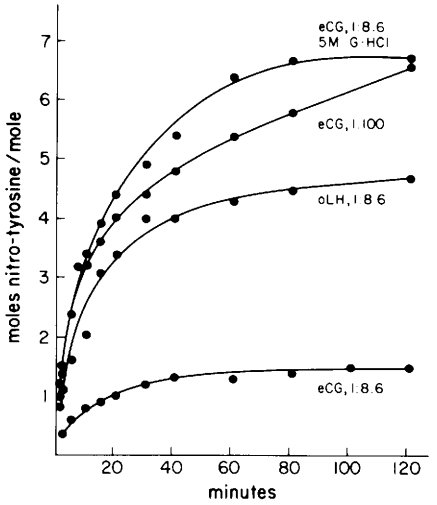

FIG. 2. Rate of nitration of equine chorionic gonadotropin with and without 5 M guanidine hydrochloride (5 *M* G·HCl) and compared to ovine LH. Ratios of tyrosine:reagent indicated at each curve. 0.05 *M* Tris, pH 8.0–1 *M* NaCl.

Effect on LH activity. The nitration of the tyrosine residues in eCG resulted in the loss of LH activity. The LH activity of various nitrated derivatives as determined *in vitro* by testosterone production by rat Leydig cells is shown in Fig. 4. Nitration of one tyrosine residue caused a 75–80% loss of LH activity. Ninety-five percent of the LH activity was lost by the nitration of two tyrosine residues, and greater nitration resulted in almost complete loss of LH activity. Ovine LH loses its activity in a similar fashion upon nitration (9–11), but prolactin, interestingly, retains full biological potency after total nitration of all the tyrosines in the hormone. Deglycosylated derivatives of ovine LH (23) and hCG (24, 25) have been found to bind to their target receptors but are unable to stimulate either cAMP production or steroidogenesis. Indeed, such derivatives are able to competitively inhibit the action of the native hormones. The loss of LH activity in eCG in which the tyrosine residues have been modified by nitration could be a result of a similar mechanism as that described above for deglycosylated ovine LH or hCG. In this study, however, it was found that a 50-fold excess of eCG derivatives with two or more nitrated tyrosines had no effect on the action of native eCG in the rat Leydig cell assay for LH, suggesting that nitration of tyrosine residues affects the ability of the derivative to bind to the receptor.

Effect on FSH activity. The effect of nitration of the tyrosine residues in eCG on FSH activity was also examined. These results, determined by the ability of eCG and nitrated derivatives to stimulate *in vitro* cAMP production by immature rat seminiferous tubule preparations are summarized in Table I. The results show that the nitration of one tyrosine residue resulted in an 85% loss of FSH

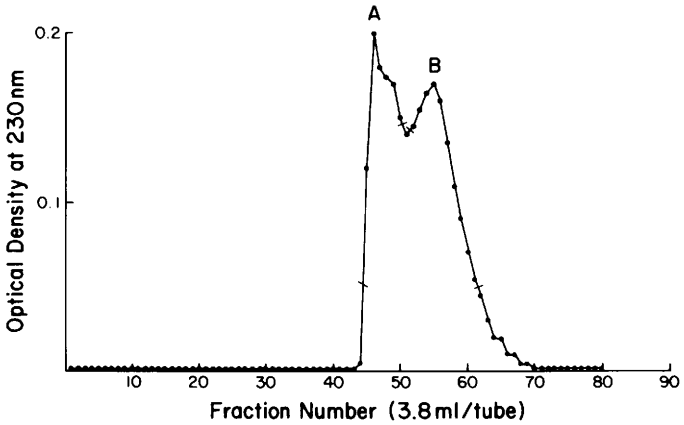

FIG. 3. Elution profile of 3.2 mg equine chorionic gonadotropin with two tyrosines nitrated on a 3.5 × 100-cm column of Sephacryl S-200 in 0.05 M NH_4HCO_3; 3.8-ml fractions. Peak B represents the monomer fraction.

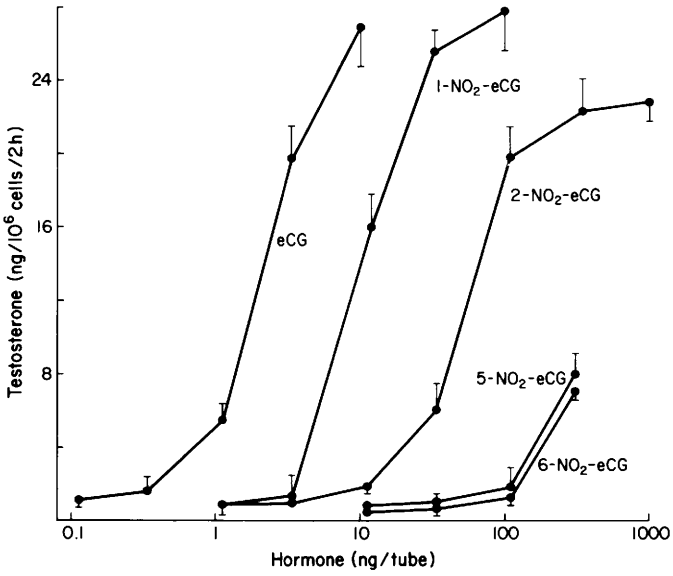

FIG. 4. LH activity of various nitrated derivatives of equine chorionic gonadotropin as measured by testosterone production in the rat Leydig cell assay.

activity. The nitration of two or more tyrosine residues caused almost total loss of activity. Thus, the results obtained for FSH activity are similar to those obtained for LH activity and suggest that the tyrosine residues of eCG are important for the manifestation of both the LH and FSH activity of eCG.

Effects on immunological activity. The immunological activity of various nitrated eCG derivatives was examined by a homologous radioimmunoassay for eCG. The results are seen in Fig. 5. All of the nitrated preparations of eCG exhibited parallel inhibition curves in the radioimmunoassay. The immunoreactivity, however, decreased somewhat with increasing degree of nitration. About 70% of the immunoreactivity was retained in derivatives with up to five tyrosine residues nitrated. The nitration of more than five residues caused a reduction of immunoreactivity to 50%. These results suggest that the immunoreactivity of eCG is more stable to tyrosine nitration than are the biological activities of the hormone.

TABLE I. EFFECT OF NITRATED DERIVATIVES OF eCG ON cAMP PRODUCTION IN IMMATURE RAT SEMINIFEROUS TUBULE PREPARATIONS

	Dose (μg)		
Derivative	10	33.3	100
	cAMP production (pmole/mg dry wt cells/10 min)[a]		
Native eCG	27.8 ± 7.2[b]	73.8 ± 10.5	98.5 ± 11.0
1-NO_2-eCG	10.5 ± 1.0	17.6 ± 3.0	39.0 ± 2.8
2-NO_2-eCG	10.5 ± 0.0	12.0 ± 2.4	16.4 ± 5.6
5-NO_2-eCG	10.5 ± 0.0	10.5 ± 0.8	14.6 ± 5.7
7-NO_2-eCG	10.5 ± 0.0	10.5 ± 0.0	13.4 ± 4.9

[a] Control values average 10.5 pmol/mg dry wt cells/10 min.

[b] ± SD.

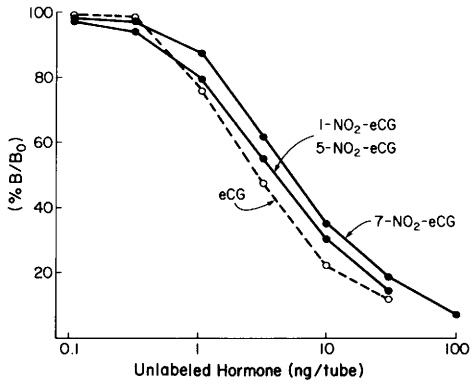

FIG. 5. Homologous eCG radioimmunoassay of various nitrated eCG derivatives.

In the present study no attempt was made to localize the tyrosine residues initially nitrated. Previous studies from this laboratory (9) with ovine LH indicated that nitration initially occurs on tyrosine residues in the α subunit. Even after complete nitration of ovine LH one tyrosine residue in each subunit remains unmodified. The nitration of these tyrosine residues is possible only after dissociation of the ovine LH. However, in the case of eCG, the tyrosine residues appear to be more accessible for nitration. Nonetheless, it is evident that limited nitration of eCG profoundly decreases the biological activities of eCG.

We thank J. D. Nelson and Beth Sabin for their technical assistance and Mrs. Marci Rohrer and Leslie Spector for help in preparing the manuscript. This work was supported in part by a grant, HD-05722, from the NICHD of the National Institutes of Health.

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Received March 26, 1984. P.S.E.B.M. 1984, Vol. 177.
Accepted May 16, 1984.