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THE EFFECTS OF BACTERIAL LIPOPOLYSACCHARIDE ON PLASMA CORTICOSTERONE CONCENTRATIONS AND BODY TEMPERATURES OF NEW ZEALAND RABBITS (*ORYCTOLAGUS CUNICULUS*)

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ABSTRACT

Twelve New Zealand rabbits were injected with *Salmonella typhosa* endotoxin, 10 ng/kg b.w., via an auricular marginal vein and the effects of the pyrogen on the rectal temperatures and plasma corticosterone concentrations of these animals were observed.

Our data showed significant increases of the core temperatures from the normal 39.3 +/- 0.18 to 40.9 +/- 0.43 C ($p < 0.001$). Radioimmunoassay results of the plasma corticosterone levels were 5.76 +/- 3.7 ug/100 ml in the pre-injection blood samples and 9.02 +/- 3.7 ug/100 ml in the plasmas obtained from the animals, one hour after the pyrogen was administered. The increase of corticosterone was significant ($p < 0.05$).

INTRODUCTION

Lipopolysaccharides or bacterial endotoxins are found to be useful to test the functions of the hypothalamo hypophyseal adrenal axis. In such tests the pyrogenic substance is administered to laboratory animals or human subjects and concentrations of plasma corticotropin (ACTH) or glucocorticoid are measured. A correlation between endotoxin and cortisol was observed by Wolff (1973) who stated that this is a convenient way of testing the pituitary adrenal function.

Lipopolysaccharides are derived from the cell wall of gram negative bacteria. Commercial preparations have been produced from *Escherichia coli, Pseudomonas aeruginosa, Salmonella abortus equi, Salmonella typhosa,* and *Pasteurella multocida.* The minimum amount of the pyrogen that is required to induce fever and other types of biological responses in animals or humans varied according to the species or strain of the microbe from which the substance was derived. Wolff (1973) reported that rabbits required 5 ng/kg of endotoxin extracted from *S. abortus equi* compared to 50 ng/kg of the pyrogen obtained from *S. typhosa*. Elin *et al.*, (1981) used a purified lipopolysaccharide prepared from *E. coli* 0113 which is the national reference bacterial endotoxin of the Bureau of Biologics of the Food and Drug Administration, Bethesda, Maryland. The rabbits in their study developed fever with 0.23 to 0.7 ng/kg doses of this product.

Several methods are proposed by investigators for quantitative measurements of the pituitary response to various stressful stimuli. However, a standardized test to measure this response is not available to date. Bacterial pyrogen is being used on the pituitary adrenal axis as a stressing agent. It is reported that both animals and human beings experience a rise in plasma corticosteroids when lipopolysaccharide is administered to them. (Melby *et al.*, 1960; Farmer *et al.*, 1961; Toth and Krueger, 1990). Methods of measuring plasma concentrations of glucocorticoid differ from laboratory to laboratory and the values obtained by these methods differ greatly. The plasma corticosterone in normal rats was measured by the competitive protein binding technique and fluorometry. The results of the two different methods were 4.3 +/- 0.8 and 8.0 +/- 0.8 ug/100 ml respectively, Stark *et al.*, (1973/1974).

The purpose of this study was to determine the minimal pyrogenic dose of the *S. typhosa* endotoxin for the rabbits in our laboratory and to observe the effect of this minimal dose on the concentrations of plasma corticosterone measured by radioimmunoassay.

MATERIALS AND METHODS

New Zealand white rabbits, *Oryctolagus cuniculus* (Myrtle's Rabbit Farm, 4678 Bethesda Road, Thompson Station, Tennessee 37179) were used in this study. The animals were housed in individual cages in the air conditioned animal facilities at Arkansas State University. The room temperature was maintained at approximately 21.4 C. Purina rabbit chow and tap water were available *ad libitum* to the rabbits. These animals were acclimated to the housing conditions during a period of seven or ten days before the experiment was started.

At the beginning of the experiment, the rabbits were randomly separated into two groups, the controls and the experimentals. The body weights of the animals were determined with a digital, small animal weight scale (Henry Schein, Co., 5 Harbor Park Drive, Fort Washington, New York 11050) and were recorded to the nearest gram. The rectal temperatures of these animals were measured with a Unisonic Digital Thermometer (1115 Broadway, New York, New York 10010) and were recorded to the nearest tenth of a degree Celsius.

As soon as the body weight and rectal temperature were recorded a 0.5 ml blood sample was collected from the middle auricular artery of each rabbit using a heparinized syringe fitted with a 1/2", 26G needle. The samples were transferred from the syringe to heparinized collection tubes, labeled and kept in ice during the collection period. These samples were centrifuged in a clinical centrifuge (IEC Clinical Centrifuge, International Equipment Co., A Division of Damon, 300 Second Needham Heights, Maryland 02194) and the separated plasmas were transferred to labeled vials and stored at -10 C until assayed.

Following the blood collection the rabbits in the control group were given 0.9% NaCl in distilled water at the rate of 0.1 ml/kg b.w. via one of the marginal ear veins. The experimental rabbits were administered a solution of lipopolysaccharide (Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178) which was at a concentration of 100 ng/ml in 0.9% saline. The rabbits required the pyrogen at 10 ng/kg b.w. in order to produce appreciable fever.

One hour after the injection the rectal temperature of each rabbit was recorded and approximately 0.5 ml of its blood was collected. The plasma was obtained and stored as previously described.

Corticosterone concentrations in the plasma samples were determined by radioimmunoassay. Chemicals for the assay were obtained from various sources as named: Labeled hormone, 1,2-^{3}H-Corticosterone (New

England Nuclear Corporation, 549 Albany Street, Boston, Massachusetts 02118); unlabeled corticosterone, bovine serum albumin - RIA grade Fraction V powder, and bovine gamma globulin (Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178); corticosterone antiserum No. B3-163 (Endocrine Sciences Products, 18418 Oxnard Street, Tarzana, California 91356); boric acid - Certified A.C.S., ammonium sulfate - Certified A.C.S., toluene - HPLC grade, 2,5-Dipenyloxazole (PPO) - scintanalyzed, sodium chloride Certified A.C.S., sodium hydroxide - Certified A.C.S., and sodium azide - purified (Fisher Scientific Company, Fair Lawn, New Jersey 07410); as well as methanol - glass distilled (Burdick and Jackson Laboratories Inc., Muskegon, Michigan 49442).

All glassware used in this assay were soaked in alconox overnight, rinsed with tap water and immersed in 2 N nitric acid for a minimum of two hours. After a final rinse with distilled water they were dried in inverted positions in a clean oven at 60 C.

The reagents such as 10 N sodium hydroxide, 0.9% sodium chloride, saturated ammonium sulfate and 0.05 M boric acid of pH 8 were prepared in distilled water and these solutions were stored at room temperature in labeled bottles. A solution of bovine serum albumin (10%) was made with distilled water. The bovine gamma globulin (2.5%) was dissolved in the saline reagent. These protein solutions were preserved with 0.1% sodium azide and stored at 4 C. Unlabeled corticosterone was dissolved in redistilled ethanol to a concentration of 1 ug/ml, and the labeled hormone was diluted with methanol to obtain 250 uc/ 5 ml. These preparations of the labeled and unlabeled corticosterones were stored at 4 C as stock solutions. The antiserum which was obtained as a dry powder was reconstituted with glass distilled water according to the supplier's specification. It was stored at -10 C. The scintillation fluid for this assay was mixed in the laboratory by adding 20 g of PPO to 4 1 of toluene containing 80 ml of glass distilled methanol.

For the radioimmunoassay of corticosterone in the rabbit plasma, 50 ul of sample was mixed with 950 ul of borate buffer containing 0.25 % BSA and the mixture was placed in a water bath at 60 C for thirty minutes. After the incubation, 50 ul aliquots of the diluted sample were placed in duplicate assay tubes which were conical centrifuge tubes of 2 ml capacity. From each sample dilution that was included in an assay about 50 ul was collected in a test tube to be used as a pool of samples. This was used as an indicator of the nonspecific binders of the labeled corticosterone. Aliquots of pooled samples were placed in duplicate assay tubes as similarly as the samples.

One milliliter of the stock unlabeled corticosterone solution was evaporated to dryness in a vacuum oven at 40 C at a pressure setting of 25. The residue was redissolved in methanol and was diluted to construct a standard curve of 0, 0.125, 0.25, 0.5, 0.75, and 1.0 ng/0.1 ml in methanol. One hundred ul of these standards were placed in duplicate assay tubes and the methanol was evaporated in the vacuum oven as described.

A mixture of the antiserum and labeled corticosterone was made afresh for the assay by adding 20 ml of the borate buffer, 5 ul of the stock tritiated corticosterone, 0.4 ml of 10% BSA, 0.4 ml of 2.5% BGG and 0.4 ml of the stock antiserum solution. This mixture was aliquoted at 250 ul into each of the assay tubes containing the knowns and the unknowns. A similar mixture without the antiserum was prepared and it was added to the assay tubes containing the pooled plasma samples in order to detect the total amount of the nonspecific binders. The contents of the reaction vessels were thoroughly mixed in a vortex mixture and the tubes were placed in a water bath at 37 C for 45 minutes and at room temperature for the following 2 hours. The reactions were terminated by the addition of 250 ul of saturated ammonium sulfate per assay tube. The tubes were vortexed and were centrifuged at 3000 rpm for 10 minutes in a clinical centrifuge. Four hundred ul of the supernatant from each tube was transferred to a corresponding counting vial and 10 ml of the scintilation fluid was added. The radioactivities of the samples were determined by a LS 100, Beckman, liquid scintillation counter.

A standard curve was plotted as shown in Figure 1. The concentrations of the unknowns were extrapolated from the curve. In the assay the non-specific binding was about 15 percent. The sensitivity of the assay was about 12.5 ng/100 ml of plasma. Based on the available data, statistical inferences were derived by use of one-way analysis of variance or the student t-test. The body weights, rectal temperatures, and the plasma corticosterone concentrations of the control rabbits were compared to similar data obtained from the experimental animals. In addition, the matched pair t-test was performed to determine the significance of the differences between the pre-injection and the post-injection values of the rectal temperatures and of the plasma corticosterone levels in the experimental rabbits.

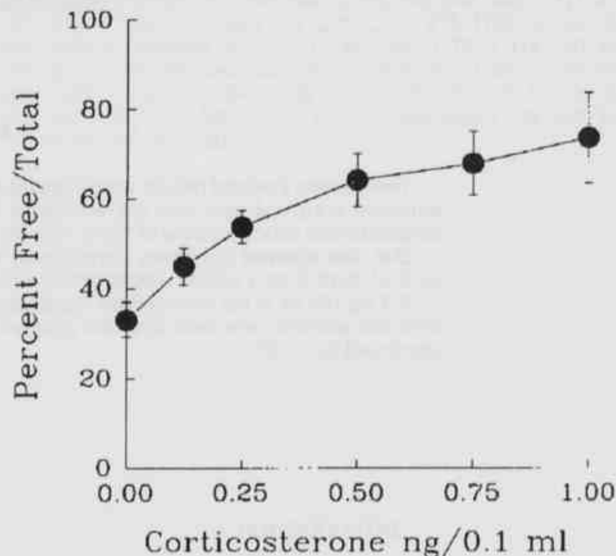

Figure 1. Cortiscosterone standard curve.

RESULTS AND DISCUSSION

Rabbits having body weights 1.5 to 4.3 kg were used in this study. The average body weights of the control group and the experimental group were similar (Table 1).

The rectal temperatures of the rabbits at the start of the experiment were 38.7 to 39.7 C. The mean rectal temperature of the control rabbits was similar to that of the experimental rabbits (Table 2).

Table 1. Body Weights of Rabbits.

Group of Animals	Number of Animals (N)	Body Weight (Kilograms) mean +/- s.d
Controls	12	2.72 +/- 0.93
Experimental	12	2.79 +/- 0.92

Table 2. Rectal Temperature of the rabbits.

Groups of Animals	Number of Animals (N)	Rectal Temperature (Celsius) (mean +/- s.d.) Pre-injection	Post-injection
Controls	12	39.1 +/- 0.25	39.2 +/- 0.28
Experimentals	12	39.3 +/- 0.18	40.9 +/- 0.43

The rabbits given 0.9% NaCl injections showed no significant change of the mean rectal temperature one hour after the injection. The experimental rabbits received lipopolysaccharide at the rate of 10 ng/kg body weight and showed a significant rise of the rectal temperatures one hour post-injections ($p < 0.001$). The amount of pyrogen used in this study is bigger than the amount of lipopolysaccharide administered by Elin *et al.*, (1981) to their rabbits. They preferred smaller doses which were between 0.23 and 0.7 ng/kg body weight for the animals. The endotoxin used by these investigators was prepared from *E. coli*. It is the national reference bacterial endotoxin of the Bureau of Biologics of the Food and Drug Administration, Bethesda, Maryland. A similar preparation of the reference bacterial endotoxin was administered to rabbits by Dinarello *et al.*, (1978) at the rate of 100 ng/kg and they reported that the rabbits consistently developed biphasic fever. Endotoxin derived from *S. typhosa* used by Wolff (1973) was not as potent as the pyrogen obtained from *E. coli*. Wolff's rabbits required this extract in doses as high as 50 µg/kg b.w., in order to develop fever. According to Greisman and Hornick (1969), the threshold pyrogenic dose of *S. typhosa* endotoxin was between 0.1 ng and 1.4 ng/kg b.w., that of *E. coli* endotoxin was about 1.0 ng/kg b.w. and that of *Pseudomonas* endotoxin was 50 to 70 ng/kg b.w. These data suggest that the endotoxic potency of lipopolysaccharides varies according to the species or strain of the microbes and the method of extraction.

Graener and Werner (1986) injected (0.1 µg/kg b.w.) *S. typhosa* endotoxin, a product which is similar to the pyrogen used in this study, into an ear vein in rabbits and observed core temperature increases in the rabbits. The fever in these rabbits was described as biphasic due to the occurrences of two peaks in the time lapsed temperature graph. Greisman and Hornick (1969) had reported that small pyrogenic doses of endotoxin evoked febrile responses in rabbits, and the fever peaked at approximately 1.5 hours. Larger doses evoked a second peak attained within three hours. Atkins and Wood (1955a & b) recorded the features of the febrile response to typhoid vaccine by rabbits as (a) the short latent period (less than 10 minutes), (b) the abrupt monophasic response with a peak in 50-60 minutes and (c) the rapid defervescence to normal within 2 to 2 1/2 hours. The pyrogen that was used in this study at 10 ng/kg was sufficient to induce fever within an hour in the rabbits. The dose was insufficient to prolong the febrile state till three hours and to produce the second peak. The body temperatures of the rabbits in this study returned to normal within three hours.

The plasma corticosterone concentrations of the rabbits are presented in Table 3. The initial blood samples (pre-injection bloods) of the control rabbits and those of the experimental rabbits contained similar amounts of corticosterone. One hour after the administration of 0.9% saline the bloods of the control rabbits did not show marked changes in the amounts of the steroid. There were significant increases of corticosterone in the bloods of the experimental rabbits one hour after they received the lipopolysaccharide ($p < 0.05$).

Table 3. Plasma Corticosterone Concentrations of the rabbits.

Groups of Animals	Number of Animals (N)	Plasma Corticosterone Concentrations (microgram/100 ml) mean +/- s.d. Pre-injection	Post-injection
Controls	12	5.73 +/- 4.2	6.44 +/- 4.2
Experimentals	12	5.76 +/- 3.7	9.02 +/- 3.7

Endotoxins of gram-negative bacteria when injected into man and animals are known to stimulate adrenal secretory activity. Administration of 0.25 ug of a lipopolysaccharide derived from *S. abortus equi* resulted in a two fold increase of plasma cortisol concentrations in 5 healthy humans (Melby 1959). Farmer *et al.*, (1961) found in humans that plasma corticosterone concentrations increased from 16.4 ug to 44.2 ug per 100 ml in four hours when injected with 0.5 ug of *Salmonella* lipopolysaccharide. Carroll *et al.*, (1969) observed a rise of 8 ug/100 ml above the base level of the cortisol in the plasmas of human volunteers who received intravenous injections of *Salmonella* pyrogen.

Elin et al., (1981) observed in humans that the reference endotoxin when given in doses greater than 1 ng/kg b.w. interrupted the diurnal variations of serum cortisol and significantly elevated this steroid's levels. Melby *et al.*, (1960) reported increase of cortisol secretion in dogs which received a lethal dose of *E. coli* endotoxin intravenously. The increased secretory rate of the hormone in these animals was observed in bloods collected 30 minutes and 120 minutes after the administration of the pyrogen. Makara *et al.*, (1971) observed circulating corticosterone rising from basal levels of 12 to 15 ug/100 ml to higher levels of 45 to 60 ug/100 ml in rats which were given *E. coli* endotoxin intraperitoneally. Toth and Krueger (1990) stated that cortisol increased in the bloods of the rabbits which were given *P. mulocida* endotoxin. These findings are similar to the results of the present study.

CONCLUSIONS

The results of this study indicated that the rabbits in our laboratory required 10 ng/kg of *S. typhosa* lipopolysaccharide in order to produce a febrile response. Concomitant with the increase of body temperatures there were increases of the circulating amounts of corticosterone in the rabbits. The radioimmunoassay was deemed reliable because the hormone concentrations determined by this method, were in a wide range of 0.75 to 15.75 ug/100 ml.

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