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Paradoxical Conditioning of the Plasma Copper and Corticosterone Responses to Bacterial Endotoxin

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EXTON, M. S., D. F. BULL, M. G. KING AND A. J. HUSBAND. *Paradoxical conditioning of plasma copper and corticosterone responses to bacterial endotoxin.* PHARMACOL BIOCHEM BEHAV **52(2)** 347–354, 1995.—The cascade of physiologic mechanisms in response to infection, the acute phase response, is recognized as having a major role in host defense. Two such responses are an increase in plasma copper and activation of the hypothalamic-pituitary-adrenal axis, which are consistently reported to occur during bacterial infection. We aimed to determine whether the alterations in plasma copper and corticosterone were conditionable using the conditioned taste aversion paradigm. The regime involved the pairing of a novel-tasting saccharine solution (the conditioned stimulus) with lipopolysaccharide (the unconditioned stimulus). Seven days after the initial pairing of these stimuli (the test day), the saccharine solution was represented. Animals exposed to this condition displayed a significant decrease in plasma copper levels. In addition, these rats experienced a reduction in plasma corticosterone that was time dependent. Paradoxically, the conditioned response of both these variables were in a direction contrary to that reported during bacterial infection. These results suggest that some acute phase responses may condition as a rebound response, or in an opposing trend to that occurring as the initial reaction.

Conditioned taste aversion Endotoxin Lipopolysaccharide Copper Corticosterone
Hypothalamic-pituitary-adrenal axis Acute phase response

THE ACUTE phase response is a primary host defense mechanism composed of immunologic, endocrine, neurologic, metabolic, and behavioral modifications (27). Each of these mechanisms is not an isolated event but an overall host response designed to optimize immunocompetence. Research has demonstrated that certain acute phase parameters such as fever (6,7,20), sleep (20), anorexia (18), and plasma iron alterations (19) are conditionable using a conditioned taste aversion (CTA) paradigm. The present report examined the conditionability of two acute-phase responses, plasma copper and corticosterone alterations.

Bacterial infection has consistently been demonstrated to alter plasma levels of various trace metals. Administration of endotoxin produces a dramatic increase in plasma copper in vertebrates (5,56). The alteration of plasma trace metals is postulated to be an integral mechanism in the host response to infection. Copper deficiency produces impairment in various immunologic parameters (31–33,43,44), as well as an increased susceptibility to infection (23,42). In addition, increased copper has an inhibitory effect on bacterial growth (1,11).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is also consistently induced by bacterial infection. The infusion of endotoxin stimulates the HPA axis, producing an increase in the production of adrenocorticotropic hormone (ACTH) and corticosterone (41,47,52,55,62). The production of these hormones appears paradoxical to the acute phase response, because of their well known immunosuppressive actions (10). However, HPA axis activation may form an integral negative feedback loop (2). Thus, the endocrine changes during infection are posited to effect the downregulation of acute phase mechanisms, such as interleukin 1 (IL-1) production. Thus, the toxic effects of IL-1 overproduction (21) would be negated.

Akin to other acute-phase responses, endotoxin-induced plasma copper alterations and HPA axis activation are most likely mediated by IL-1 (2,4,12,36,39,40,46,47,57,59). In ad-

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dition, like other conditionable acute phase responses, plasma copper and corticosterone are mediated within the hypothalamus (5,24,38,45,50). Also, in accordance with acute-phase response production, HPA axis activation appears to be moderated by prostaglandins of the E series (PGE_2) (25,37). However, it must be recognized that both plasma copper and corticosterone alterations may be moderated by cytokines other than IL-1, such as tumor necrosis factor (14,51) and IL-6 (14,48,51).

Thus, as a result of the similar mode of functioning of acute-phase mechanisms and the known conditionability of some of these responses (6,7,18–20), this report aimed to condition plasma copper and corticosterone concentrations in response to bacterial endotoxin. In addition, reports on the conditionability of the HPA axis are conflicting. Plasma corticosterone has shown to be behaviorally conditionable using IL-1β as the UCS when administered peripherally (16), but not using central infusion (22). Thus, this report examined the conditionability of an inducer of IL-1, lipopolysaccharide (LPS).

METHOD

Subjects

Forty-five (copper analysis), 58 (corticosterone analysis), 28 (2-h corticosterone control analysis), 21 (copper control study), and 28 (corticosterone control study) experimentally naive male rats, aged between 110 and 120 days at the beginning of the experiment, were used. All rats were of the Australian Albino Wistar inbred strain, and were obtained from the University of Newcastle Psychology Department animal house, from AAEC stock. Animals with a mean weight of 389 ± 16 g were used, and were individually housed in standard wire laboratory cages (18 × 23 × 15 cm). Cages were kept in an air-conditioned holding room at an ambient temperature of 22.0 ± 1.0°C. The rats had access to standard laboratory food pellets and tapwater ad lib, except during the water deprivation phase of the appropriate experiments. A 12 L : 12 D cycle, with lights on at 0800 h, was maintained throughout the experiments.

Apparatus

The conditioned stimulus (CS) used was a 1% saccharine solution. LPS derived from *Escherichia coli* endotoxin (Difco 026 : B6, Sydney, Australia), at a dosage of 100 μg/kg, was employed as the unconditioned stimulus (UCS). Pyrogen-free 0.1% saline solution (Newcastle Veterinary Supplies, Newcastle, Australia) was used for the control injections. All syringes, needles, and consumables used were pyrogen free. A Perkin-Elmer 4100 Zeeman atomic absorbtion spectrometer (Sydney, Australia) was used for analysis of plasma copper concentrations. A direct-reading fluorimeter (Perkin-Elmar) was also implemented for plasma corticosterone analysis.

TABLE 1

TREATMENT GROUPS

	Conditioning Day		
Group	CS	UCS	Test Day (CS)
SAC/LPS	Saccharine	LPS	Saccharine*
WAT/LPS	Water	LPS	Water*
SAC/SAL	Saccharine	Saline	Saccharine*
WAT/SAL	Water	Saline	Water*

*Saline control injection, all animals.

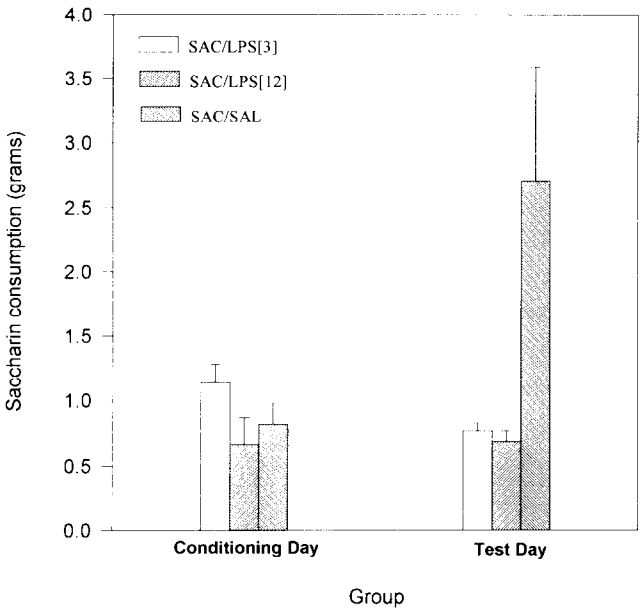

FIG. 1. Saccharine consumption on the conditioning and test days for the copper experiment.

Procedure

For 6 days before experimentation, animals were handled gently to minimize stress throughout the entire experimental paradigm. Animals were then assigned to one of four groups (Table 1). In the analysis of plasma corticosterone concentration, three groups were administered the experimental condition of a saccharine/LPS pairing. One group was decapitated 6 h post-CS representation [SAC/LPS(6)], another 2 h after CS representation [SAC/LPS(2)]; one group had blood collected 1 h post-CS representation [SAC/LPS(1)]. This paradigm was similar to that employed for the copper analysis; however, one group had blood collected 12 h [SAC/LPS(12)] and the other 3 h [SAC/LPS(3)] after CS representation. Blood was collected 2 h after CS representation for all groups in the third experiment.

On the initial day of the experiment, rats were placed on a water deprivation regime. This schedule allowed the animals 15 min of drinking at 0900 h each day. This time period was the only opportunity the animals had to consume fluid. A two-bottle system was employed, with a bottle presented on both the right and left sides of the cage. For the water training section of the experiment, both bottles contained tapwater. On the conditioning and test days, however, one bottle contained water while the other contained the relevant CS. This allowed particular experimental groups the option of drinking either water or saccharine on these days.

Seven days following the commencement of water deprivation, the conditioning day procedure was employed. Each rat was supplied with the relevant CS during the allotted drinking time. Immediately following this period, each rat received an intraperitoneal (IP) injection of the appropriate UCS in a dose

volume of 1.0 ml. After a further 7 days, animals were again presented with the germane CS during the drinking session. All rats were subsequently administered a control IP injection of 0.1% pyrogen-free saline.

Twelve hours (copper experiment), 6 h (corticosterone experiment), 3 h [SAC/LPS(3) group in the copper experiment], 2 and 1 h [SAC/LPS(2) and SAC/LPS(1), respectively, in the corticosterone experiment] following CS representation, rats were decapitated and trunk blood was collected in microcentrifuge tubes. Serum samples were then centrifuged at 3000 rpm for 5 min. Plasma was subsequently pipetted into aliquots and immediately stored at −80°C until the analysis of plasma copper and corticosterone. Plasma copper levels were analysed using graphite furnace atomic absorption spectroscopy, using the methodology described by Williams et al. (60). Corticosterone concentration was measured using fluorimetric analysis, implementing the method reported by Mattingly (35).

Statistical Analysis

Plasma copper and corticosterone, as well as saccharine, data were analysed using one-way analysis of variance (ANOVA). Post hoc simple effects analyses (Tukey's HSD test) were implemented for significant effects, with Bonferroni α adjustments made to avoid experimenter error.

RESULTS

Conditioning of Plasma Copper

Figure 1 shows saccharine consumption of the SAC/LPS and SAC/SAL groups. On the conditioning day, no differences were found in saccharine consumption among the groups [$F(2, 26) = 2.675$, $p > 0.05$]. However, on the test day, there was a significant difference among the groups in saccharine consumed [$F(2, 26) = 6.655$, $p < 0.01$]. Post hoc analysis revealed no significant difference between the two SAC/LPS groups on the test day; however, significant taste aversion was displayed by both of these groups when compared to the SAC/SAL control animals ($p < 0.01$).

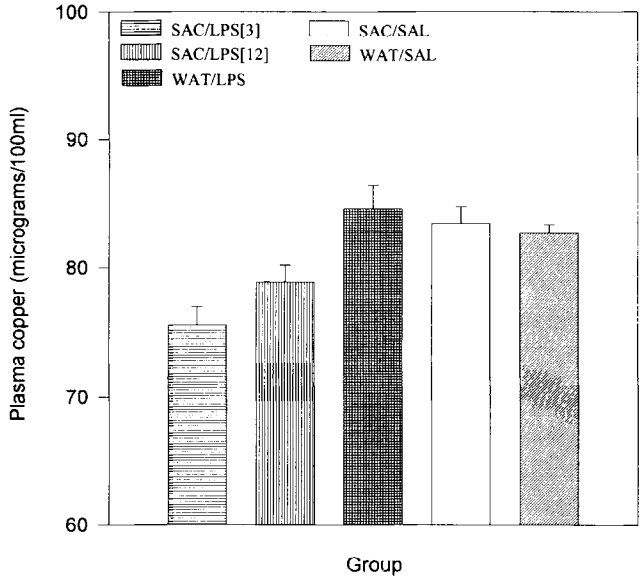

FIG. 2. Plasma copper levels on the test day. Two groups underwent the saccharine/LPS pairing. One group had blood collected 3 h after CS representation (SAC/LPS[3]), and the other 12 h after reexposure (SAC/LPS[12]).

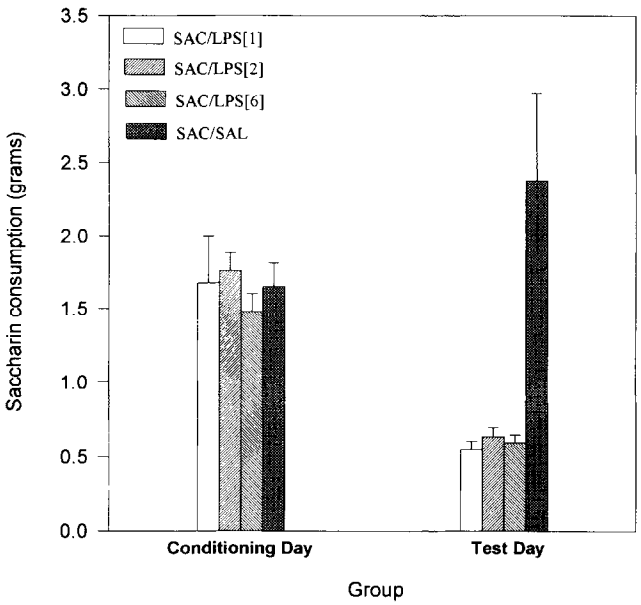

FIG. 3. Saccharine consumption on the conditioning and test days for the corticosterone experiment.

Figure 2 shows plasma copper on the test day. Upon CS representation, the SAC/LPS animals displayed a significant reduction in the plasma concentration of copper [$F(4, 40) = 9.747$, $p < 0.0001$]. Post hoc analysis revealed no difference between the two SAC/LPS groups or the remaining controls. However, both the SAC/LPS groups displayed significantly lower plasma copper than the SAC/SAL ($p < 0.015$), WAT/LPS ($p < 0.015$), and WAT/SAL ($p < 0.015$) animals.

Conditioning of Plasma Corticosterone

Saccharine consumption on the conditioning and test days is displayed in Fig. 3. On the conditioning day, the four groups showed no difference in saccharine consumption [$F(3, 38) = 0.76$, $p > 0.05$]. However, on the test day, the three SAC/LPS groups displayed a significant reduction in the amount of saccharine consumed [$F(3, 38) = 12.92$, $p < 0.001$]. The SAC/LPS groups did not differ significantly in their level of saccharine consumed ($p > 0.05$).

Figure 4 shows the level of plasma corticosterone on the test day. On the test day, the SAC/LPS animals displayed a decrease in plasma corticosterone. This reduction was not apparent for the SAC/LPS animals decapitated 1 or 6 h post-CS representation. Analysis of variance displayed significant differences between the groups [$F(4, 45) = 19.675$, $p < 0.0001$]. Post hoc analysis demonstrated that the SAC/LPS(2) animals experienced significantly lower plasma corticosterone than the remaining experimental groups (all $p < 0.001$).

Conditioning of Plasma Corticosterone (2-h Controls)

Because only the 2-h plasma sampling displaying reduced corticosterone levels, there was a possibility that this result was due to circadian variation in steroid levels. Thus, the conditioning paradigm was again implemented, with all four

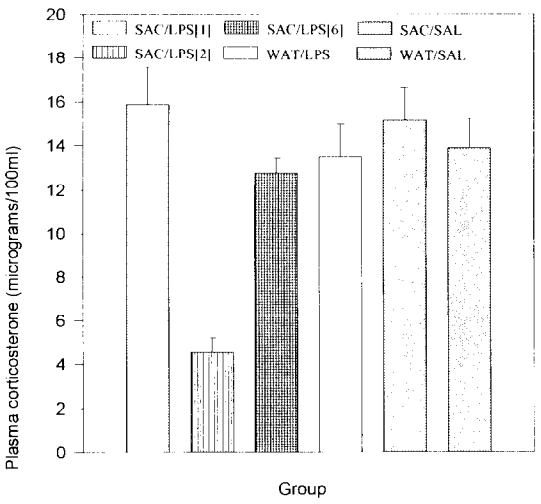

FIG. 4. Plasma corticosterone levels on the test day. One SAC/LPS group was decapitated 1 h after CS representation (SAC/LPS[1]) and another 2 h after representation (SAC/LPS[2]), whereas blood was collected from the other 6 h after saccharine reexposure (SAC/LPS[6]).

experimental groups (SAC/LPS, SAC/SAL, WAT/LPS, and WAT/SAL) having blood collected 2 h after CS representation. The amount of saccharine consumed by the two SAC groups is displayed in Fig. 5. On the conditioning day, no significant difference was observed between the groups in level of saccharine consumed [$F(1, 12) = 0.026$, $p > 0.05$]. However, on the test day the SAC/LPS group demonstrated a significant reduction in saccharine consumption [$F(1, 12) = 22.608$, $p < 0.0001$].

Figure 6 shows the level of plasma corticosterone on the test day. On the test day, a significant difference in plasma corticosterone was demonstrated among the groups [$F(3, 24) = 22.673$, $p < 0.0001$]. Post hoc analysis revealed the SAC/LPS animals to have significantly lower corticosterone than all other animals ($p < 0.001$).

Copper Control Study

Because of the paradoxical nature of the conditioned response, a control study was completed to ensure that the plasma copper response to LPS was the same in our laboratory as those reported elsewhere. Thus, three groups were employed: a group-administered saline, and two administered LPS. The LPS animals had blood collected postinjection at identical times to those in the conditioning study post-CS representation (3 and 12 h). Saline animals were decapitated 12 h after LPS administration. Plasma levels of copper are shown in Fig. 7. One-way ANOVA revealed a significant difference between the three groups [$F(2, 18) = 56.38$, $p < 0.001$]. Post hoc analysis demonstrated that the LPS animals decapitated 12 h after injection had significantly greater levels of plasma copper than the other groups ($p < 0.01$). In addition, the LPS group that had blood collected 3 h after injection showed significantly raised levels of plasma copper ($p < 0.05$).

Corticosterone Control Study

Corticosterone response to LPS was also investigated because of the paradoxical nature of the conditioned response. Animals in this study were again placed into groups concordant with the conditioning study. That is, three groups were administered LPS, with decapitation times varying across groups (1, 2, and 6 h postinjection). The remaining group was administered saline and was decapitated 2 h after endotoxin administration. Plasma corticosterone responses in the four groups are displayed in Fig. 8. One-way ANOVA displayed a

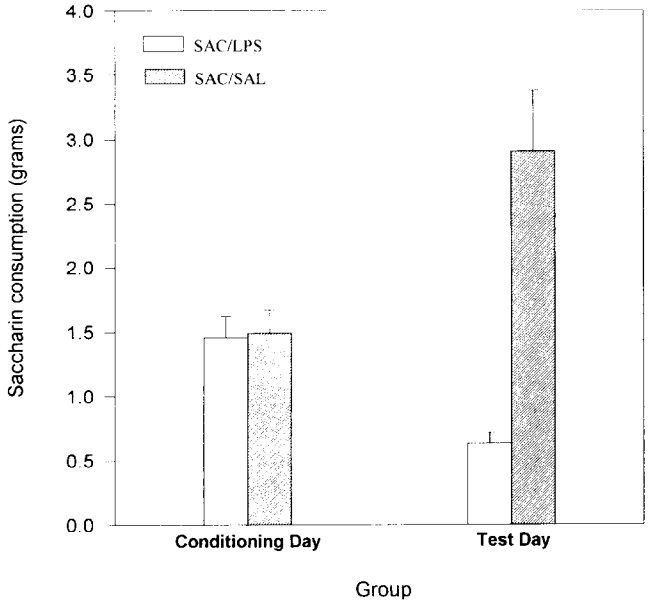

FIG. 5. Saccharine consumption on the conditioning and test days for the corticosterone 2-h control experiment.

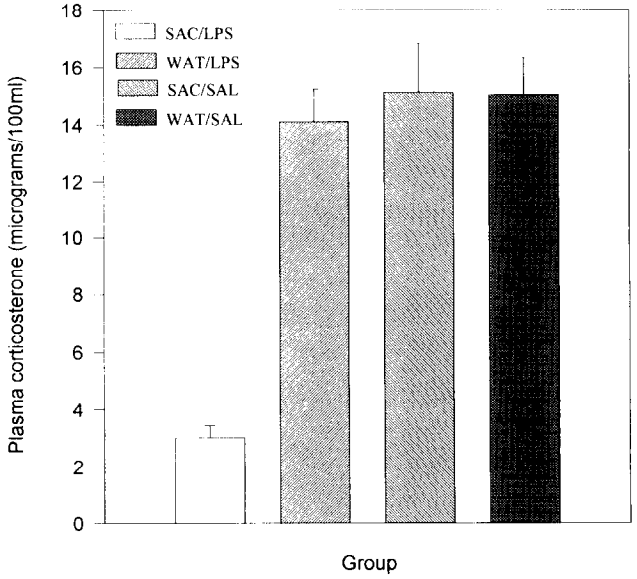

FIG. 6. Plasma corticosterone levels on the test day (2-h control experiment). All groups had blood collected 2 h after CS representation.

significant difference between the groups [$F(3, 24) = 43.68, p < 0.001$]. Post hoc analysis revealed that LPS elevated plasma corticosterone 2 ($p < 0.01$) and 6 ($p < 0.01$) h postinjection. However, corticosterone was not heightened 1 h after injection ($p > 0.05$).

DISCUSSION

The results of this report document a number of interesting findings. First, a significant conditioned effect was demonstrated with two acute-phase responses, plasma copper and corticosterone alterations. Second, this study reports that, contrary to previous conditioned acute-phase responses (6,7,18–20), the conditioned effects of these reactions were kinetically opposite to that which occur as an acute reaction to LPS. Third, the results demonstrate that for both copper and corticosterone, the conditioned concentration reduction is time dependent. For both responses, the conditioned effect occurred relatively early after CS representation and diminished with increasing time postexposure. In addition, the effect was demonstrated not to be due to circadian variation or to paradoxical effects of acute LPS administration.

The most dramatic result from the present study is the "paradoxical" conditioning of two acute-phase responses. This opposes previous reports from this laboratory (6,7,18–20) that demonstrated acute-phase responses to be conditionable in a kinetically identical manner to that occurring as the initial response to LPS. This result is surprising given the expanding body of literature indicating immune response conditioning (test day) to occur in a similar response to that on the conditioning day (28). An explanation for this may be that, contradictory to the current literature, the original LPS infusion (conditioning day) may have induced a response in the direction presently observed on the test day. This interpretation appears to be plausible, as electrical stimulation of hypothalamic regions can produce responses that are opposite to those

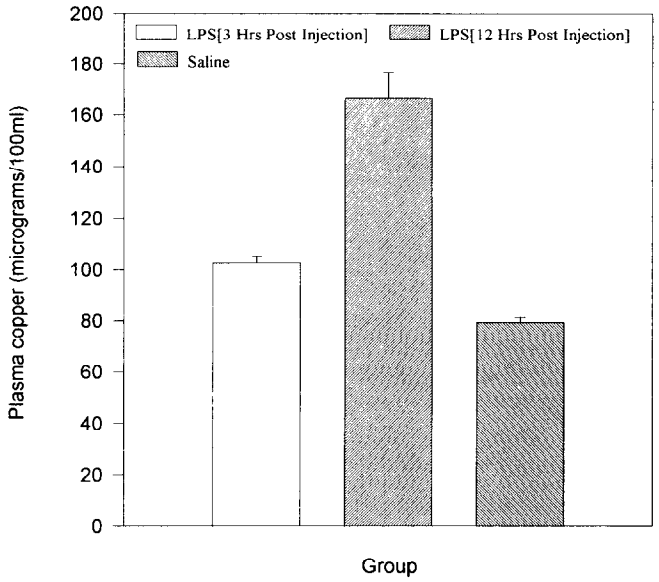

FIG. 7. Plasma copper for the copper control study (direct effects of LPS on plasma copper). Animals were injected with either saline or LPS. One LPS group had blood collected 3 h postinjection (LPS[3]); the other was 12 h after injection of LPS (LPS[12]).

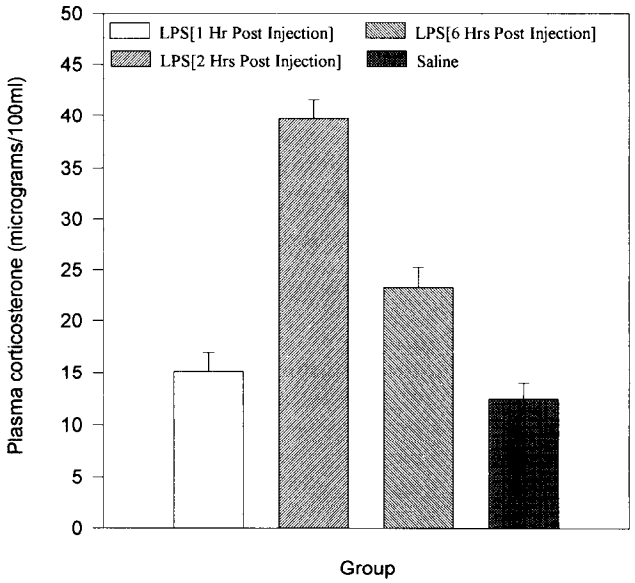

FIG. 8. Plasma corticosterone for the corticosterone control study (direct effects of LPS on plasma corticosterone). Animals were injected with either saline or LPS.

observed in the acute-phase response (49). However, this appears unlikely, as the present control studies indicated that LPS injection resulted in a significantly increased concentration of plasma copper (3 and 12 h postinjection) and corticosterone (2 and 6 h postinjection). In both of the present conditioning experiments, control data were below levels displayed in acute LPS-injected animals (control studies). This suggests that control groups were experiencing baseline levels of these molecules on the test day.

Paradoxical conditioning has been reported in other drug-induced changes. Both body temperature (9,29,34) and blood glucose (53,61) have been demonstrated to have conditioned responses opposing the direct effect of drug administration. Eikelboom and Stewart (17) posited that the effects may be due to the drugs acting on the efferent arm of the response, causing the "controller" of such responses to act in an opposite way so as to reinstate homeostasis. Thus, upon CS representation, the controlling physiologic mechanism acts in a similar mode as on the conditioning day, producing an effect that is opposite to the direct drug response. In the case of the present acute-phase responses the controller is thought to be the hypothalamus (5,24,38,45,50), which appears to regulate many of the responses to infection (3). It is probable that, in opposition to the theory posited by Eikelboom and Stewart (17), both the increase in plasma copper and corticosterone in response to endotoxin are initially mediated by direct effects on the hypothalamus (13,36,38,46). This would result in the drug-induced change not acting on the final effectors of these responses, such as the adrenals (corticosterone) and the liver (copper). Thus, these responses are induced by molecules acting on the afferent arm of the responsible physiologic mechanism. It therefore appears that the paradoxical conditioning observed in this study is not due to the drug's acting on the efferent arm of the acute-phase response, as was thought to be the case in other paradoxical conditioning of drug effects (17).

Another explanation for the observed paradoxical condi-

tioning is that the conditioned responses may have undergone a biphasic reaction. That is, the expected conditioned response may have a short latency after CS representation, and the reported results may have been a compensatory fall in the plasma copper and corticosterone. Although feasible, results from other conditioned acute-phase responses render this interpretation unlikely. Thus far, conditioned acute-phase responses have been demonstrated to follow a similar time course and have analogous kinetics to those observed in response to the unconditioned stimulus (6,7,18–20). The time of decapitation in this study coincides with the reported time of optimum copper and corticosterone responses to LPS (14, 36,41,47,55). Thus, if the present responses were to be consistent with previous findings, it would be expected that the results would show a conditioned effect opposite to that presently reported. Nevertheless, it may be that these acute-phase parameters are indeed conditionable in a similar direction to the UCR, with the timing of reaction being incompatible with previous investigations.

The paradoxical conditioning of plasma copper may be due to an inappropriate time of blood sampling. Although in the present control experiments plasma copper was elevated 12 h after LPS injection, some studies have demonstrated that plasma copper can remain increased up to 7 days postinfection with *E. coli* (55). However, it must be noted that this study employed avian species and an extremely high dose of infective agent. However, it is possible that the observed decrease in plasma copper may have been a precursor to a conditioned elevation of this trace metal. Nevertheless, this appears unlikely, because most experimenters reported no significant changes in plasma copper proceeding 24 h postinjection (4,36,49,59). Indeed, Blatteis et al. (4) found that elevations of plasma copper 8 h after LPS administration were absent 24 h postinfusion.

The decrease in plasma copper reported here may have been due to an increased binding of copper with ceruloplasmin (58), which is a source of copper for peripheral cells and tissues (8). Thus, the decreased plasma copper may have been hepatically redistributed to organs that absorb this molecule, of which the liver and kidney are the primary storage sites (30). The present results demonstrated an alteration in plasma copper that occurred 3 h after CS representation. Most studies have found that in response to pyrogenic challenge, plasma copper is elevated between 8 and 24 h later. Thus, the speed of the present results appear to be anomolous. This might be because of a mechanism other than ceruloplasmin which is influential in the redistribution of copper. Acute phase proteins other than ceruloplasmin are synthesized in retort to infection (26). Thus, the rapid alteration in plasma copper in response to CS representation may be due to copper transport by albumin and transcuprein, which convey lower quantities of copper but have been shown to become rapidly involved after copper infusion (59). The plasma copper alterations observed in this study may result from two separate mechanisms: one involving ceruloplasmin and one comprising another transport system.

The conditioning of corticosterone demonstrated a reduced effect beyond 2 h post-CS representation. The significant conditioning at 2 h post-CS was shown not to be due to circadian rhythm variation. This course (although an opposite effect was observed) is similar to that reported in previous studies. That is, the time of nadir corticosterone levels in endotoxin-treated rats and mice is 2 h postinjection (14,41,55), with these levels returning to control levels after 8 h of recording time (41,55) Thus, the conditioned corticosterone kinetics would appear to be similar to that reported for other conditioned acute-phase responses, as the conditioned effect is not as dynamic as the unconditioned response (6,7,18–20). However, the direction of the steroid reaction reported here is in opposition to such responses. This result adds further ambiguous evidence to the conditioning of corticosterone. This is due to three different results reported: the ability to condition peripherally administered IL-1–induced corticosterone secretion (16), the inability to reproduce results using intracerebroventricular infusion of IL-1 (22), and the current paradoxical conditioning using LPS. LPS induces the secretion of corticosterone via the induction of IL-1 (15,46). Thus, it may be expected that the conditioned response to LPS would be of longer latency than that to IL-1. As the experiment by Dyck et al. (16) also collected blood 2 h after CS representation, the present conditioned response may have bypassed the conditioned steroid increase. That is, a conditioned rise in corticosterone may have occurred between the sampling at 2 and 6 h after CS representation. However, to our knowledge, there has been no report of an initial decrease in corticosterone and a subsequent rise in response to LPS. In addition, 1-h sampling demonstrated no conditioned effect. Therefore, because of the growing evidence that conditioned acute-phase responses mimic the kinetics of the unconditioned response, the present result appears to be enigmatic.

This report also appears to conflict with the results of Smotherman et al. (54), who completed taste aversion experiments using lithium chloride as the UCS. These researchers showed that reexposure to a sweet CS resulted in conditioned activation of the pituitary–adrenal axis. However, in these experiments, a single-bottle method was employed. Thus, animals were forced to drink a substance which they associated with illness. These methods compelled animals into a situation where perceived control of an aversive event (illness) was low. This would have produced a stressful situation, producing the release of steroids. In comparison, the present results were not due to a perceived lack of control, as the animals had a choice of consuming or avoiding the CS. Thus, the present results appear to be due to a conditioned response, as opposed to perceived conflict.

Our study demonstrates that not all acute-phase responses are conditionable in a direction typical of this multifactorial reaction to infection. To fully elucidate the response of plasma copper and corticosterone to our conditioning procedures, an experiment using multiple blood sampling would allow an optimum description of the time-dependent changes in the variables. It may be that this is neccessary unequivocally to explain the present paradoxical results.

ACKNOWLEDGEMENTS

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