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Physiologic Concentrations of Cortisol Suppress Cell-Mediated Immune Events in the Domestic Pig[1] (41926)

HOLLIE J. WESTLY AND KEITH W. KELLEY[2]

College of Agriculture, Washington State University, Pullman, Washington 99164

Abstract. Mild restraint of the domestic pig (*Sus scrofa*) caused activation of the hypothalamic–pituitary–adrenal axis, atrophy of the thymus gland, and a significant reduction in cell-mediated, delayed-type hypersensitivity reactions. Physiologic concentrations of cortisol suppressed, *in vitro*, phytohemagglutinin- and concanavalin A-induced proliferation of porcine thymocytes, splenocytes, and peripheral blood mononuclear cells, even though cortisol was only mildly cytolytic. These results extend the concept of an endocrine influence on regulation of cell-mediated immune events to species other than humans and rodents.

Endocrine regulation of immune events has added a new level of understanding of how lymphoid cells interact with one another, and represents a concept that was accurately predicted in 1977 by Besedovsky and Sorkin (1). Several recent results support an endocrine–immune system network (2–5). New findings have even shown that a secretory product of activated T cells can increase plasma cortisol and affect turnover rate of hypothalamic catecholamines (6, 7), thus establishing a direct, afferent link between activated T cells and the brain.

Another line of evidence supporting an endocrine–immune system link is the effect of adversive environmental stimuli on the immune system (8–12). According to this concept, stress causes the release of a wide variety of hormones which alter regulation of immune cells (13–17). For example, ACTH, β-endorphin, cortisol, and norepinephrine are all released in response to acute stress, and all of these hormones have been demonstrated to affect a variety of immune events (18–25). In certain cases, the stress-induced change in immune function can be abrogated by endocrine gland ablation (12).

There are two important problems with many of the above studies: (a) pharmacologic concentrations of hormones were often used *in vitro*, and (b) almost all of the relevant experiments were conducted with either rodents or humans. To firmly establish an endocrine–immune system network, physiologic concentrations of hormones must be used, the phenomenon must be shown to occur *in vivo* and a comparative approach should be used to ensure this concept is valid in species other than humans and rodents.

In this report, we used the domestic pig (*Sus scrofa*) to demonstrate that a mild, short-term period of restraint sufficient to activate the hypothalamic–pituitary–adrenal axis can reduce the size of the thymus gland and suppress an *in vivo* cell-mediated immune event. Cortisol, which is the major adrenocortical hormone in the pig, is known to be immunosuppressive at pharmacologic levels. We found that physiologic concentrations of cortisol reduced mitogen-induced proliferation of lymphoid cells *in vitro*. These results with the domestic pig are consistent with the hypothesis that hormones have regulatory effects on immune cells.

Experimental Procedure. *Lymphoid cell preparation.* Thymus, spleen, and heparinized peripheral blood were collected from 6 to 12 adult female pigs. Thymus and spleen cells were prepared by repeated flushings of lymphoid organs with sterile RPMI 1640 tissue culture medium (GIBCO, Grand Island, N.Y.), pH 7.4, enriched with 100 units · ml^{-1} penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin, 0.25 $\mu g \cdot ml^{-1}$ fungizone, 24 m*M* sodium bicar-

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[2] To whom correspondence should be addressed: 126 ASL, 1207 West Gregory Drive, University of Illinois, Urbana-Champaign, Ill. 61801.

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bonate, and 20 m*M* Hepes buffer. Blood was diluted 1:2 with RPMI 1640. Thymus, spleen, and diluted blood suspensions were layered over 3.6 ml Histopaque 1077 (Sigma, St. Louis, Mo.) in 16 × 125-mm sterile glass tubes with fitted caps. Samples were centrifuged at 400*g* for 40 min at room temperature. Cells at the plasma interface were collected and washed at 150*g* for 10 min at room temperature with 10 ml of 0.87% ammonium chloride to lyse contaminating erythrocytes. The mononuclear cell pellet was washed two times with RPMI 1640 and concentration of cells was determined using a Coulter ZBI counter with a 100-μl aperture tube. All cell suspensions were adjusted to 6×10^6 cells · ml^{-1} with enriched RPMI 1640 that contained 10% heat-inactivated fetal bovine serum. Cell viability was determined with 0.16% trypan blue and was >95% for all cell suspensions.

Lymphocyte mitogenesis. Sixty microliters of the adjusted lymphocyte suspensions was dispensed into sterile 96-well flat-bottomed microtiter plates (Linbro, McLean, Va.). All lymphocyte preparations were assayed in triplicate cultures. Initial studies were conducted with dose response curves to determine optimal concentrations for stimulation of each cell type with the mitogens phytohemagglutinin (PHA, Sigma) and concanavalin A (ConA, Sigma). Both mitogens were diluted in the same medium used for lymphoid cells, and one optimal and one suboptimal initial concentration of each mitogen were tested: 0.0025 and 0.0125 mg · ml^{-1} for ConA and 0.0125 and 0.0625 mg · ml^{-1} for PHA. A total of 40 μl of these mitogens was added to appropriate culture wells to yield final amounts of 0.1 and 0.5 μg of ConA per culture (final concentration of 0.5 and 2.5 μg · ml^{-1}) and 0.5 and 2.5 μg of PHA per culture (final concentration of 2.5 and 12.5 μg · ml^{-1}).

Hydrocortisone-21-sodium succinate was prepared by dissolving 7.20 mg in 100 ml of enriched RPMI 1640, in the absence of fetal bovine serum. One milliliter of this solution was diluted to 10 ml to form the working solution of 5387 ng · ml^{-1} cortisol and passed through a 0.45-μm filter. Further dilutions were made such that, after adding 100 μl of cortisol to each cell culture, the final concentrations of steroid were 13, 27, 135, 270, and 2694 ng · ml^{-1} with a final concentration of 5% fetal bovine serum in the culture medium. One hundred microliters of medium without fetal bovine serum was substituted for cortisol in control cultures. After 72 hr incubation at 38°C, 5% CO_2, cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine, sp act 6.7 Ci/mmole (New England Nuclear, Boston, Mass.). Cultures were incubated for an additional 16 hr and cells were harvested on glass filter paper disks using a semiautomatic cell harvester.

Filter disks were dried and placed in scintillation vials with 7 ml scintillation fluid. Vials were counted for 2 min in a Packard Tri-Carb beta scintillation counter. Mitogen stimulated assays were expressed as the mean counts per minute (cpm) of triplicate cultures or as Δ cpm, the mean cpm of stimulated cultures minus the mean cpm of control cultures containing no mitogen (background).

Lymphocyte cytotoxicity. To determine whether cortisol was directly cytotoxic toward lymphoid cells, 200 μl of 6×10^6 viable cells per milliliter was incubated in duplicate with 200 μl of various concentrations of hydrocortisone-21-sodium succinate in sterile 12 × 75-mm culture tubes with caps. Cells were stained with a 50% dilution of 0.16% trypan blue solution to determine dead cells and counted under phase contrast microscopy at 18 and 36 hr of incubation.

In vivo studies. In the first experiment, twenty-four 8-week-old pigs were closely confined to determine if this procedure was sufficient to activate the hypothalamic–pituitary–adrenal axis and affect delayed-type hypersensitivity reactions. Animals were assigned to a control or restraint treatment on the basis of litter. Control pigs remained in their home pen, while restrained pigs were placed in a 13 × 33-cm expanded metal box of adjustable length for 2 hr. The confinement box permitted the pigs to stand, sit, or lie down but not to turn around. Blood was collected by jugular venipuncture into vacutainers containing EDTA immediately prior to and after the 2-hr restraint period. This restraint procedure was repeated at 0800 hr for 3 consecutive days. Plasma was separated by centrifugation at 1650*g* at 4°C and stored at −20°C until analysis. Plasma cortisol was assayed by validated radioimmunoassay (26).

To evaluate cell-mediated immunity *in*

vivo, we employed a standard T-lymphocyte-mediated skin test that has been used in a variety of species (27–32). Prior to restraint, all pigs received an intradermal injection in the rear flank of 250 μg PHA in sterile saline. Size of the mononuclear cell infiltration was recorded by measuring double skinfold thickness with a constant tension micrometer immediately prior to and after the 2-hr restraint period. Amount of skin swelling was expressed as the absolute skin thickness prior to injection minus the absolute skin thickness at the subsequent times. Hematoxylin- and eosin-stained histological preparations of these injection sites revealed a mononuclear cell inflammatory response at 24 and 48 hr which extended focally through all areas of the dermis and was usually associated with blood vessels. A few focal accumulations were present at the dermal–epidermal junction. Some edema was present. Absence of a significant number of neutrophils indicated that this response was not merely an acute inflammatory reaction. These results are in agreement with earlier findings in pigs (32).

In another experiment with twenty-four 6-week-old pigs, pigs were either left in their home cage or restrained as outlined above. After the third restraint period, all pigs were weighed and sacrificed. Spleen, thymus, mesenteric lymph nodes, and adrenal glands were excised and weighed immediately.

Statistical analysis. All experiments were analyzed as a randomized complete block design. Differences between treatment means were tested by planned orthogonal comparisons for the *in vitro* experiments and differences between control and restraint groups were determined by Student's *t* test.

Results. *Mitogenesis in vitro.* Hydrocortisone-21-sodium succinate caused a linear ($P < .01$) reduction in mitogen-induced activation of all types of porcine lymphoid cells that were tested (Fig. 1). Linear trends were similar for the two doses of ConA and PHA, so values for the two mitogen doses were averaged. A cortisol concentration of 135 $ng \cdot ml^{-1}$ was chosen as near the maximal physiologic dose that can be achieved in the domestic pig after an injection of ACTH (33, 34). At this concentration, there was a 44% reduction ($P < 0.001$) in ConA- and PHA-induced activation of porcine splenocytes. Interestingly, even at a concentration which is below normal basal blood cortisol levels in the pig (13 $ng \cdot ml^{-1}$), there was also a significant reduction in both ConA and PHA activation of splenocytes.

Results with thymocytes and PBMC are also shown in Fig. 1. At 135 $ng \cdot ml^{-1}$, there was a 47% reduction ($P < .001$) in ConA activation of thymocytes when compared to control cultures containing no added cortisol. However, the reduction in mitogenesis of PHA-stimulated thymocytes at this concentration of cortisol did not reach a statistically acceptable level of significance ($P > 0.05$). As with thymocytes, 135 $ng \cdot ml^{-1}$ of cortisol caused a reduction ($P < 0.001$) in ConA-induced activation of PBMC. There was no significant reduction in PHA activation of PBMC at any concentration of cortisol that was used in this study.

Cytotoxicity in vitro. Since high concentra-

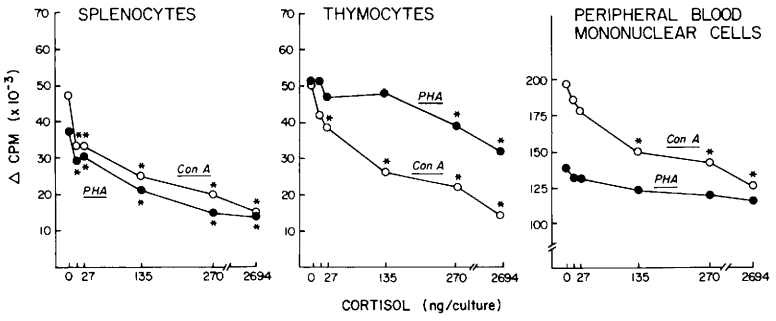

FIG. 1. Effect of cortisol on ConA- and PHA-induced proliferation of splenocytes, thymocytes, and PBMC. *Difference ($P < 0.05$) from zero cortisol values. Background and pooled standard deviations of ConA- and PHA-stimulated cultures were, respectively, splenocytes: 2278, 10916, and 7471; thymocytes: 1708, 1443, and 1708; and PBMC: 4709, 44713, and 37596.

tions of glucocorticoids are lympholytic in some species, we asked whether the reduction in mitogenesis could be explained by lysis of lymphoid cells. Because trypan blue staining would not detect cells that had been previously lysed, we analyzed data on the basis of total viable cells recovered and percent viable cells at the time of counting (Table I). Even pharmacologic concentrations of cortisol caused a maximal lysis of only 21% of any cell type at 18 hr (calculated as total viable cells recovered). Based on percentage viable cells, pharmacologic concentrations of cortisol caused a maximal lysis of only 10%. At a maximum physiologic concentration of 135 $ng \cdot ml^{-1}$, cortisol caused a reduction ($P < 0.05$) in total viable thymocytes at 18 and 36 hr, a reduction in splenocytes at 18 hr but not at 36 hr, and no statistical change in total percentage viable PBMC at 18 or 36 hr of incubation.

Cortisol in restrained pigs. Average, basal levels of plasma cortisol in control pigs ranged from 18 to 28 $ng \cdot ml^{-1}$ (Table II), which is in agreement with previously published reports in pigs (33, 34). On Days 1 and 2 of restraint, initial plasma cortisol of pigs to be restrained did not differ from home-cage controls. After the end of the 2-hr restraint period, plasma cortisol increased to 60 ($P < 0.05$) and 37 ($P < 0.05$) $ng \cdot ml^{-1}$, respectively, on these 2 days. On Day 3, plasma cortisol was significantly higher before initiation of restraint in the control pigs. However, after the restraint session, cortisol was significantly higher than in nonrestrained controls.

PHA skin reactions. Substantial swelling was elicited by an intradermal injection of PHA, and this swelling peaked at about 12 mm at 24 hr (Table III). This swelling after PHA injection compared to only 0.12 mm after injection of saline (Table III, footnotes),

TABLE I. CYTOLYTIC EFFECTS OF CORTISOL ON THYMOCYTES, SPLENOCYTES, AND PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER 18 AND 36 hr INCUBATION

	18 hr		36 hr	
	Total viable cells ($\times 10^6$)	Percent viable cells	Total viable cells ($\times 10^6$)	Percent viable cells
Thymocyte				
0	0.576	87	0.410	64
13	0.534	81*	0.376	62
27	0.538	83*	0.372	60*
135	0.518*	82*	0.358*	59*
270	0.500*	80*	0.324*	57*
2694	0.476*	77*	0.326*	54*
SD	0.03	4.1	0.02	5.5
Splenocyte				
0	0.458	71	0.300	50
13	0.408*	65*	0.288*	48
27	0.350*	66*	0.292	48
135	0.380*	65*	0.286	49
270	0.380*	64*	0.298	48
2694	0.388*	66*	0.268	45*
SD	0.027	4.9	0.02	5.3
PBMC				
0	0.578	77	0.384	64
13	0.560	76	0.388	67
27	0.536	77	0.400	66
135	0.612	78	0.414	66
270	0.554	79	0.434	70*
2694	0.600	80*	0.382	70*
SD	0.042	3.6	0.025	5.1

Note. Total viable cells at time zero was 1.2×10^6 per culture. For thymocytes, $N = 15$, for splenocytes, $N = 14$, and for PBMC, $N = 14$. Data are expressed as means, and the * indicates the mean is different from control cultures at $P < .05$.

TABLE II. PLASMA CORTISOL IN RESTRAINED PIGS

	Day 1		Day 2		Day 3	
Treatment	Before	After	Before	After	Before	After
Control	25.7 ± 2	25.0 ± 5	23.7 ± 3	18.5 ± 4	28.2 ± 3	20.9 ± 2
Restraint	24.7 ± 3	60.1 ± 7*	24.5 ± 2	37.1 ± 5*	19.0 ± 2*	39.1 ± 6*

Note. Data are expressed as ng · ml^{-1}, means ± SE; *difference from control at $P < 0.05$.

which amounted to approximately a 100-fold increase caused by PHA injection. Restrained pigs always had lower PHA-induced skin swelling responses than control pigs, and this reduction was significant before initiation of restraint on both Days 2 (27%) and 3 (25%).

Organ weights. Wet weights of three types of lymphoid organs and the adrenal glands were recorded after the third restraint period and expressed as a percentage of body weight (Table IV). Restraint caused a 29% reduction ($P < 0.05$) in size of the thymus gland, but had no effect on size of the spleen, mesenteric lymph nodes, or adrenal glands.

Discussion. Recent research with humans and rodents supports the concept that immune function can be modified by the hormonal environment of lymphoid cells. Thymic hormones provide a good example of this phenomenon. More recently, an immunologic circuit between the primate thymus and hypophyseal–pituitary–adrenal system has even been proposed (3). In many animal systems, it is well known that an acute, physical stimulus is sufficient to cause activation of a wide variety of hormonal systems. In the present report, we confirmed that our paradigm of short-term restraint was sufficient to activate at least one of these hormonal systems, as evidenced by higher plasma cortisol after the restraint period. This stress-induced change in hormonal profiles was associated with significantly reduced PHA skin reactions and smaller thymus glands.

Although glucocorticoids are known to be immunosuppressive at pharmacologic concentrations, little emphasis has been devoted to their effects on a variety of lymphoid cells at normal, physiologic levels (25). Therefore, we incubated peripheral blood lymphocytes, thymocytes, and splenocytes with concentrations of cortisol that are normally found in the blood of pigs. Similar to other species, pharmacologic levels of glucocorticoids reduced mitogen-induced proliferation of lymphoid cells. More important, however, substantial immunosuppression was detected at the maximal physiologic level of cortisol (135 ng · ml^{-1}), and with splenocytes, concentrations as low as 13 ng · ml^{-1} were sufficient to significantly reduce both PHA and ConA activation of lymphoid cells. Furthermore, the total effect of cortisol may be of even greater magnitude than reported here because the fetal bovine serum used in the culture medium probably contained small, additional amounts of glucocorticoids (1 to 30 ng · ml^{-1}, HyClone technical bulletin) and may there-

TABLE III. PHA SKIN REACTIONS IN RESTRAINED PIGS

	Day 1		Day 2		Day 3	
Treatment	Before	After	Before	After	Before	After
Control	—	4.6 ± 0.6	13.8 ± 0.6	12.5 ± 0.8	7.7 ± 0.5	6.9 ± 0.5
Restraint	—	3.5 ± 0.5	10.0 ± 0.4*	10.5 ± 0.9	5.8 ± 0.6*	5.7 ± 0.6*

Note. Data are expressed as Δ mm swelling, means ± SE; *difference from control at $P < 0.05$. Initial thicknesses of PHA-injected control and restrained pigs were 2.33 ± 0.09 and 2.52 ± 0.09 mm, respectively (means ± SE). Initial thicknesses of saline-injected control and restrained pigs were 2.2 ± 0.09 and 2.4 ± 0.07 mm, respectively. There were no differences among treatments after saline injection, averaging increases of only 0.10, 0.12, and 0.09 mm on Days 1, 2, and 3, respectively.

TABLE IV. SELECTED ORGAN WEIGHTS IN RESTRAINED PIGS

Treatment	Organ			
	Thymus	Spleen	Mesenteric lymph nodes	Adrenal
Control	0.14 ± 0.010	0.17 ± 0.013	0.08 ± 0.008	0.012 ± 0.001
Restraint	0.10 ± 0.001*	0.16 ± 0.006	0.07 ± 0.006	0.010 ± 0

Note. Data are expressed as a percentage of body weight, means ± SE; and *difference from control at $P < 0.05$.

fore have been immunosuppressive even in control cultures. Therefore, lymphoid cells of the pig seem to be very sensitive to low concentrations of at least one hormone.

The reduction in thymidine uptake that was observed in these experiments occurred at relatively optimal doses of mitogens. However, even greater reductions in blastogenesis may have occurred if more suboptimal concentrations of mitogens had also been tested (35, 36). This reasoning probably also explains a discrepancy between our results and an earlier report relative to the effects of cortisol on PHA stimulation of PBMC. In that report (37), 362 $ng \cdot ml^{-1}$ of cortisol caused a 60% reduction in blastogenic responses of porcine PBMC to PHA, whereas in our experiments, PHA stimulation of PBMC was relatively resistant to suppressive effects of cortisol up to 2694 $ng \cdot ml^{-1}$. However, the previous workers stimulated lymphocytes with only 0.8 $\mu g \cdot ml^{-1}$ of PHA, whereas we used much higher final PHA concentrations of 2.5 and 12.5 $\mu g \cdot ml^{-1}$. Our explanation concurs with previous conclusions that cortisol is much more suppressive when low doses of mitogens are used to stimulate lymphoid cells.

These studies indicate that *in vitro* incubation of lymphoid cells with cortisol or a short restraint paradigm which caused an elevation in blood cortisol levels resulted in suppressed immunoresponsiveness. However, one must be cautious in comparing cortisol effects *in vitro* versus *in vivo*. In the *in vitro* system, pig lymphoid cells were exposed to cortisol continuously for 88 hr. However, the restraint paradigm *in vivo* caused elevated plasma cortisol for shorter periods of time. Therefore, exposure of lymphoid cells to elevated cortisol was less than in the *in vitro* system. Although this apparent correlation between *in vivo* and *in vitro* results is valuable, additional experiments will be needed with adrenalectomized animals to conclusively establish that adrenal steroids are responsible for restraint-induced immunosuppression. We have conducted these experiments and supported this interpretation using mice as the experimental model (12).

In several species, it is well accepted that pharmacologic concentrations of cortisol cause lysis of immature, cortical thymocytes, resulting in enrichment of the more mature, medullary thymocytes (38–40). In contrast to thymic involution caused by high doses of glucocorticoids, adrenalectomy results in thymic and lymph node hypertrophy (41). Thus, there appears to be an inhibitory influence of glucocorticoids even at basal levels. Indeed, susceptibility of lymphocytes to the lytic effects of pharmacologic concentrations of cortisol led to the classification of some animal species (42) as glucocorticoid resistant (man, monkey, guinea pig, ferret) and glucocorticoid susceptible (mouse, rat, hamster, rabbit). With pigs, the present report indicates that pharmacologic concentrations of cortisol caused significant but minimal cell death when compared to results of other species (43). Therefore, it is unlikely that the lytic effects of cortisol reduced cell numbers sufficiently to totally explain the reductions in mitogen-induced blastogenesis. These results also firmly establish the pig as a corticoid-resistant species (44).

Existence of soluble, chemical messengers within the immune system, such as the interferons, interleukin 1 (IL-1), interleukin 2 (IL-2) and colony-stimulating factor, may provide new insights into the mechanism of action of several hormones. New evidence with the primate, rodent, and bovine species has shown that even physiologic concentrations of glucocorticoids can inhibit T-cell-mediated cytotoxicity, suppress autologous and heterologous mixed lymphocyte reac-

tions, inhibit expression of Ia antigens and suppress T-lymphocyte-induced blastogenesis (45–51). Furthermore, physiologic levels of glucocorticoids suppress both IL-1 (48) and IL-2 (52) production, and deficits in autologous mixed lymphocyte reactions and T-cell-mediated cytotoxicity can be restored by addition of exogenous IL-2 (46, 53). Since activated lymphocytes display an increase in the number of glucocorticoid receptors (37, 54, 55), physiologic rather than pharmacologic levels of glucocorticoids that correspond to specific binding activity could have important regulatory effects *in vivo.* Thus, the present data obtained with a glucocorticoid-resistant species extend the idea that even low, physiologic concentrations of glucocorticoids have important, immunomodulatory effects.

Although we investigated effects of cortisol only *in vitro,* effects of other hormones that were released *in vivo* by the restraint paradigm are probably also important. For example, ACTH and β-endorphin are released simultaneously in response to acute stress (56), and both have recently been shown to affect regulation of certain components of the immune system (4, 8, 21, 23, 24). Both norepinephrine and epinephrine were probably increased in plasma after the restraint period, and these hormones have important regulatory effects on the immune system (22, 57, 58). Investigations into how these hormones affect immune function will lead to a better understanding of regulatory circuits within the immune system, and will also contribute to an important practical problem by enhancing our understanding of stress-associated infectious and neoplastic diseases.

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