Hydrazine Sulfate Protects D-Galactosamine-sensitized Mice against Endotoxin and Tumor Necrosis Factor/Cachectin Lethality: Evidence of a Role for the Pituitary

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Summary

In previously published studies, we had demonstrated that hydrazine sulfate pretreatment protected mice against the lethal effects of endotoxin and that this protection was accompanied by a sustained increase in hepatic phosphoenolpyruvate carboxykinase activity (Silverstein, R., C.A. Christoffersen, and D.C. Morrison. 1989. Infect. Immun. 57:2072). The same hydrazine sulfate pretreatment has now been found to protect mice against endotoxin in the D-galactosamine model with an increase in the endotoxin LD_{50} of approximately four orders of magnitude. Elimination of the pretreatment period, or administration of an additional dose of D-galactosamine at the time of hydrazine sulfate pretreatment, renders the mice refractory to the protection. Given the sensitivity of phosphoenolpyruvate carboxykinase regulation to several hormones, we investigated the possibility that protection may have been hormone mediated. In addition to determining the effect of hydrazine sulfate on the plasma levels of phosphoenolpyruvate carboxykinase regulating hormones, we have investigated the effects of hydrazine sulfate on endotoxin lethality in mice whose capacity to respond hormonally to external stimuli has been compromised by hypophysectomy. Our results show a significant enhancement in circulating levels of plasma corticosterone 30 min after hydrazine sulfate injection. Moreover, hypophysectomy results in a marked increase in sensitivity of mice to endotoxin challenge as well as an abrogation of the protection against endotoxin lethality mediated by hydrazine sulfate. Although hydrazine sulfate protection distinguishes between sensitivity brought on, individually, by D-galactosamine and by hypophysectomy, mice sensitized by both hypophysectomy and D-galactosamine are not protected against endotoxin lethality by hydrazine sulfate. We conclude that hydrazine sulfate protection against endotoxin lethality is endocrine dependent, with the available evidence implicating a pituitary/adrenal axis, with glucocorticoid involvement. In as much as D-galactosamine is known to act directly in the liver in disrupting protein synthesis, it is proposed that events in the liver are critical to the hydrazine sulfate-mediated protection against endotoxin and are possibly the target of the endocrine involvement. Hydrazine sulfate pretreatment also protects D-galactosamine-sensitized mice against the lethal effects of injected tumor necrosis factor/cachectin.

Bacterial endotoxins have been implicated as a major factor in the pathogenesis of Gram-negative septic shock, a clinical problem with significant morbidity and mortality (1-3). Many experimental animal models of endotoxin lethality have been established, with the objectives of understanding not only the pathogenic mechanisms involved, but also potential therapeutic approaches to reverse the deleterious pathophysiologic effects of this potent bacterial product. A number of agents have been examined in terms of addressing the acute lethal effects of endotoxin, including glucocorticoids (4), an-

tibody to endotoxin (LPS) (5), and antibody to TNF/cachectin (6, 7). Our laboratory has recently reported that a dose of 30-80 mg/kg hydrazine sulfate given 5 h before challenge will protect these animals against the lethal effects of an LD_{50-80} dose of endotoxin (8). In that study, it was also established that hydrazine sulfate pretreatment had the effect of maintaining hepatic phosphoenolpyruvate carboxykinase (EC4.1.1.32) (PEPCK)[1] activity after endotoxin challenge.

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[1] *Abbreviation used in this paper:* PEPCK, phosphoenolpyruvate carboxykinase.

In the absence of hydrazine sulfate pretreatment, as expected, the levels of hepatic PEPCK were significantly depressed (8), consistent with the well-established observations of the late Professor Berry and colleagues (9, 10). Indeed, our interest in hydrazine sulfate was initially based on these findings coupled with an earlier observation that hydrazine sulfate injection of normal rats led to an increase in hepatic PEPCK upon isolation (11). As to the relationship of these observations to possible mechanisms of hydrazine sulfate protection against endotoxin, two possibilities were discussed in our previous report: (a) that the increase in PEPCK might act against the hypoglycemia associated with endotoxin shock; and (b) that the elevation of PEPCK might have been hormonally induced (8). Although protecting doses of hydrazine sulfate were found to have little effect on plasma glucose (8), the possibility remained that hydrazine sulfate elevation of hepatic PEPCK might have been hormonally induced. In this regard, it is well established that PEPCK transcription is subject to regulation by glucocorticoid, insulin, glucagon, and thyroid hormone (12–15). If the hydrazine sulfate–stimulated increase in PEPCK was hormonally induced, then the observed protection might also have been hormonally induced. We therefore began the present study by examining for possible effects of hydrazine sulfate effects on plasma corticosterone, triiodothyronine, insulin, and glucagon. This, in turn, led to a comparison of the effects of hydrazine sulfate on mice sensitized to the lethal effects of endotoxin through hypophysectomy and/or D-galactosamine. The latter is increasingly being applied as a model of endotoxin and TNF/cachectin sensitization and of liver injury (16–21). Additional experiments then focused on the properties of the hydrazine sulfate pretreatment period, and on hydrazine sulfate protection against challenge from injected TNF/cachectin.

Materials and Methods

Animals. CF₁ females were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). They were of the same age and housed under the same conditions as in our previous report (8). In some experiments, as specified, C3H/He (C3He/BK) female mice were used. These were obtained from Bantin and Kingman Labs (Freemont, CA). They are a derivative of the C3H/HeJ strain manifesting the same phenotypic hyporesponsiveness to endotoxin. The C3H/He mice used in these experiments express the endotoxin nonresponder phenotype and are therefore essentially equivalent to the C3H/HeJ strain provided by The Jackson Laboratories (Bar Harbor, ME). Due to the fire at The Jackson Laboratories last year, C3H/HeJ mice were not available at the time these studies were conducted, and an alternate vendor was used.

Plasma Hormone and Glucose Levels. Mice were decapitated and blood was drained into heparin- and aprotinin-containing tubes. Plasma was obtained as previously described (8). For each datum, blood was taken from 12 mice into four pools, each from three individual mice. This allowed for sufficient plasma to reliably assay for glucagon, insulin, and corticosterone using commercial RIA kits. The glucagon, triiodothyronine, and insulin kits were from Cambridge Medical Technologies (Cambridge, MA), and the one for corticosterone was from ICN Biomedicals (Carson, CA). Rat insulin standard was used in the insulin RIA and was obtained from

Ronald Chance, Lilly Research Labs (Indianapolis, IN). Significance of the triiodothyronine data was confirmed with RIAAID (Cambridge Medical Technologies). Plasma glucose was determined by the coupled enzyme assay method as previously described (8).

Hypophysectomy and Recovery. Before surgery, mice were anesthetized with 350 mg/kg Avertin. After swabbing with 70% ethanol, a 1-cm incision was made in the skin of the ventral neck region, and the salivary glands were retracted. The sternohyoideus muscle was penetrated with a crossacting curved forceps and the basisphenoid bone exposed. A hole (no. 6 burr) was drilled at the junction between the occipital and the basisphenoid bones and the underlying anterior pituitary was removed by negative pressure. The wound was closed by a 9-mm would clip. Sham-operated mice underwent the same procedure except the hole in the bone was not drilled. After surgery, the animals were placed back on feed, and were given water supplemented with 5% dextrose. By 48 h, plasma corticosterone levels were undetectable. At the conclusion of experiments, see below, and after death of the animal by cervical dislocation, the skull was opened and completeness of hypophysectomy confirmed by use of 4× magnification. Animals with pituitary remnants were excluded from the study. Failure rate did not exceed 5%.

Lethality Studies. Mice were taken off feed at 10 pm, and then challenged with endotoxin at 1 pm the next afternoon, at which time they were also placed back on feed. Mice that had been hypophysectomized or sham operated were treated similarly and were allowed 60 h to recover from surgery before lethality study. Dextrose supplementation for these mice was withdrawn from the drinking water at the start of the fasting period and was resumed at the time of challenge and refeeding. For the lethality studies with D-galactosamine-treated mice, the same fasting/challenge times were used as above, with D-galactosamine given along with endotoxin or TNF/cachectin at the time of challenge. Lethality was monitored for 48 h.

Chemicals. Hydrazine sulfate and D-galactosamine were obtained from Sigma Chemical Co. (St. Louis, MO). The D-galactosamine was added with LPS (sonicated) or TNF/cachectin as a solution of 45 mg/ml D-galactosamine in freshly prepared (from the solid sodium phosphates) PBS, such that each mouse received 18 mg (per 25 g body weight) D-galactosamine in 0.4 ml, as prescribed by Lehmann et al. (16). The LPS from *Salmonella enteritidis*, extracted by aqueous phenol (22), was purchased from Difco Laboratories (Detroit, MI). Human recombinant TNF/cachectin was generously provided by Chiron Corp. (Emeryville, CA) as purified by Dr. George Kuo.

Statistics. χ^2 contingency tables (23) were used to assess the significance of hydrazine sulfate protection on endotoxin lethality. The plasma hormone and glucose data are presented as plus or minus the SEM, with p values determined by Student's t test (24).

Results

The animal model that we have developed to study hydrazine sulfate protection against septic shock in normal mice has been described previously (8). In these studies, we established that injection of hydrazine sulfate before endotoxin challenge was necessary to observe protective effects, with a 5-h pretreatment period being used in most experiments. To assess potential hydrazine sulfate–mediated hormonal effects during the critical pretreatment period between hydrazine sulfate administration and endotoxin challenge, we have monitored the plasma levels of corticosterone, triiodothyronine, insulin, and
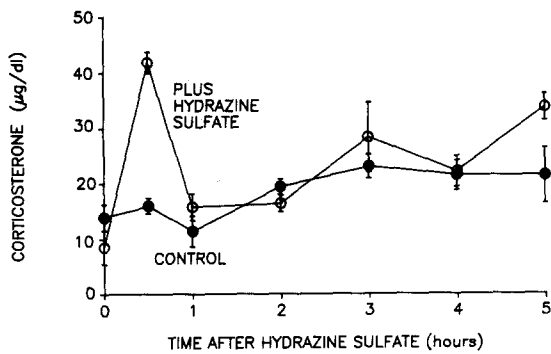
Figure 1. Effect of hydrazine sulfate on plasma corticosterone levels during the pretreatment period. Plus hydrazine sulfate, 80 mg/kg hydrazine sulfate; control, saline vehicle. Four mice (pools) per datum, see Materials and Methods, ± SEM; 30 min ($p < 0.005$).

glucagon in normal fasted CF_1 mice, at various times after injection of the hydrazine sulfate. Fasting and injection times simulated those of the lethality studies. All determinations were performed on the same blood samples, as described in Materials and Methods. Significant differences were seen in plasma corticosterone and triiodothyronine levels, during different time periods, and in opposite directions. At 30 min, the corticosterone level of the hydrazine sulfate–treated mice was increased to 41.8 ± 1.9 μg/dl compared with 16.0 ± 0.1 μg/dl for saline-treated controls ($p < .005$) (Fig. 1). The level of triiodothyronine was unaffected for 2 h, and then, unexpectedly, began to decrease. At 4 h, it was 1.86 ± 0.36 μg/dl vs. 3.80 ± 0.50 μg/dl for controls ($p < .05$), and again at 5 h, it was 1.81 ± 0.51 μg/dl vs. 3.92 ± 0.37 μg/dl for controls ($p < .05$) (Fig. 2). The differences in glucagon and insulin at each of the time points throughout the pretreatment period were <30%, random in direction, and, in each instance, statistically insignificant ($p > 0.5$). These results indicate that hydrazine sulfate can, in fact, significantly influence circulating levels of plasma hormones during the critical 5-h period before endotoxin challenge.

Given these differences in corticosterone and triiodothyronine plasma levels between hydrazine sulfate– and saline-pretreated mice, experiments were then carried out to assess
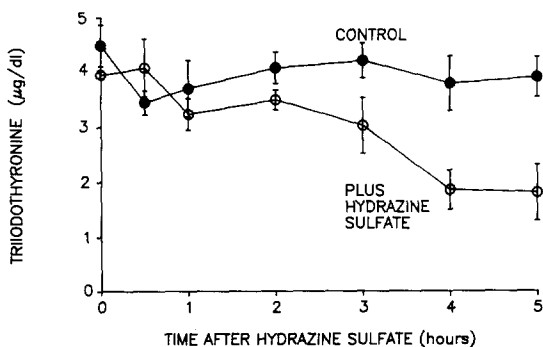

Figure 2. Effect of hydrazine sulfate on plasma triiodothyronine levels during the pretreatment period. Same plasma samples as in Fig. 1, ± SEM; 4 h ($p < 0.05$), 5 h ($p < 0.05$).
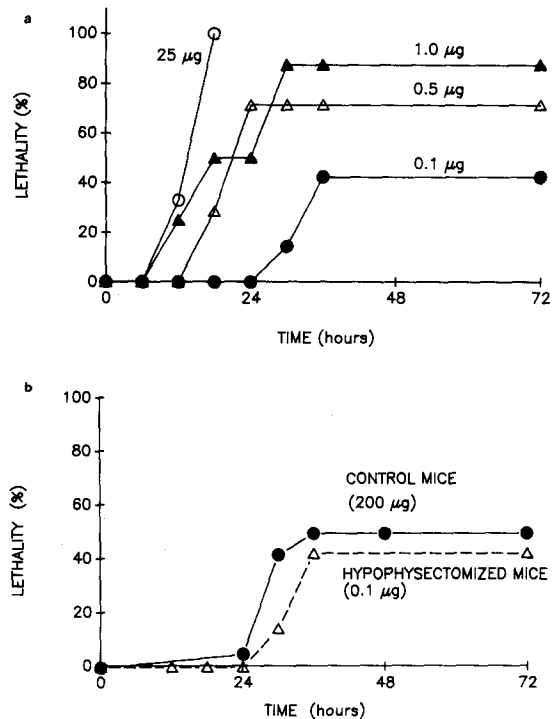

Figure 3. (A) Endotoxin lethal dose response for hypophysectomized mice. (O) 25 μg; (▲) 1.0; (△) 0.5 μg; (●) 0.1 μg. Seven mice per dose. (b) Effect of endotoxin dose, in parentheses, on lethality kinetics. Control mice, intact. Data from reference 8. Hypophysectomized mice, from a.

the effects of removing the anterior pituitary on the murine response to endotoxin, and then the potential capacity of hydrazine sulfate to modulate that response. The response of hypophysectomized mice to challenge with various doses of endotoxin is shown in Fig. 3 a and establishes an LD_{50} of ∼0.2 μg. This contrasts with the normal LD_{50} of ∼200 μg (8). When the temporal course of the lethality response of hypophysectomized mice to endotoxin is compared with the equivalent responses for normal mice (Fig. 3 b) a three-order of magnitude decrease in the LD_{50} can be readily appreciated, even though, as shown in Fig. 3 b, the crisis period for lethality at or near the LD_{50} remains 24–36 h after endotoxin challenge for both groups of animals. Thus, hypophysectomized mice, as had earlier been shown for adrenalectomized mice (25, 26), are significantly increased in sensitivity to endotoxin.

We next wished to assess the effects of hydrazine sulfate in preventing endotoxin lethality in hypophysectomized mice. An important concern before administration of hydrazine sulfate to hypophysectomized mice is that of potential hydrazine toxicity. In particular, hydrazine sulfate in sufficient dose is capable of producing marked hypoglycemia beginning soon after its administration, most likely due to its well-recognized effects on PEPCK (11). The hypoglycemia is then either rapidly reversed or results in the death of the animal. Although we have not earlier observed significant effects of hydrazine sulfate on glucose levels in intact mice given protecting doses of hydrazine sulfate, it does not necessarily follow that these
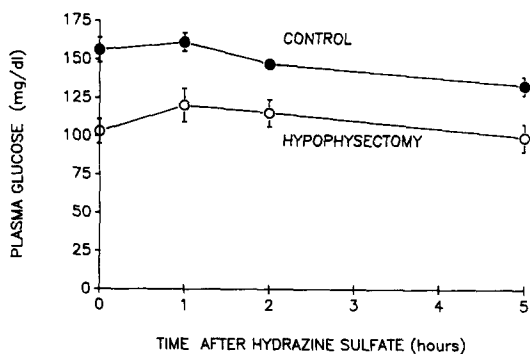
Figure 4. Effect of 50 mg/kg hydrazine sulfate on plasma glucose during the pretreatment period. Control, sham operated. Five mice per datum, ± SEM.

same doses of hydrazine sulfate may not adversely affect plasma glucose levels in hypophysectomized mice. Accordingly, plasma was obtained from hypophysectomized mice both before and at various times after injection with hydrazine sulfate under conditions simulating the pretreatment phase of the earlier described hydrazine sulfate protection studies. The mice had been hypophysectomized and then allowed to recover for 60 h. They were then fasted overnight and through the 5 h after the hydrazine sulfate injection exactly as described earlier. As shown in Fig. 4, the effect of removing the pituitary is to bring about a not unexpected reduction in plasma glucose in the fasted mice, from 156 ± 8 mg/dl to 103 ± 8 mg/dl. These differences are reflected in the time zero (pre-hydrazine sulfate treatment) data. Further, it is evident that injection of 50 mg/kg hydrazine sulfate does not result in any additional decrease in plasma glucose, nor are these combined treatments lethal to the mice. All hypophysectomized- and hydrazine sulfate–treated mice survived (10/10) without noticeably adverse effect for 2 wk after hydrazine sulfate, at which time they were killed.

Table 1. *Effect of Hydrazine Sulfate on Endotoxin Lethality in Hypophysectomized Mice*

Hydrazine Sulfate	Lethality (deaths/total)			
	Exp. 1	Exp. 2	Exp. 3	Total
mg/kg				
–	2/6	6/11	5/10	13/27
5	ND	ND	5/11	5/11
10	0/6	4/10	ND	4/16
20	ND	ND	8/11	8/11
50	3/6	6/11	ND	9/17
160	6/6	ND	ND	6/6

Mice were hypophysectomized and allowed to recover, and then fasted, as described in Materials and Methods. Pretreatment with hydrazine sulfate, 5 h. Endotoxin, 0.2 μg. Controls were pretreated with the saline vehicle.

Having established that hypophysectomized mice are markedly increased in sensitivity to endotoxin, and that hydrazine sulfate treatment of such mice at a dose of 50 mg/kg does not compound the hypoglycemia associated with hypophysectomy, we next investigated whether hydrazine sulfate would reduce lethality due to endotoxin in these hypophysectomized mice, otherwise using the same experimental conditions as in our previous model for protection. As is clear from the results of the several experiments, which have been summarized in Table 1, hydrazine sulfate at a variety of doses is unable to provide appreciable protection to hypophysectomized mice against an LD50 dose of endotoxin. This is in marked contrast to our earlier published data with normal mice, in which hydrazine sulfate provided complete protection against an endotoxin LD50 over a dose range of 30–80 mg/kg hydrazine sulfate (8).

While these results are consistent with hydrazine sulfate protection being hormone mediated, the possibility exists that sensitization of mice to endotoxin might abrogate the protective effects of hydrazine sulfate. In that regard, D-galactosamine is well recognized for its capacity to sensitize mice to endotoxin to an even greater extent than does hypophysectomy. Moreover, its immediate effects are hepatospecific (27) and result from uridine nucleotide depletion (28). In preliminary experiments, we confirmed that D-galactosamine given at the time of endotoxin challenge (and in the absence of hydrazine sulfate treatment) lowers the LPS LD50 by more than four orders of magnitude (28). We then explored the effect of hydrazine sulfate, given 5 h before challenge, and at a dose of 80 mg/kg. The results showed not only that the protection was not abrogated in these D-galactosamine-treated mice, but that the LD50 was raised by approximately four orders of magnitude, approaching the LD50 seen in normal mice (Fig. 5). Further, the doses of hydrazine sulfate in which protection first became apparent are similar to those for hydrazine sulfate protection of normal, unsensitized mice (8). A comparison of this hydrazine sulfate dose-response profile to that for hypophysectomized mice (Fig. 6) shows dramatically the differences in response of the mice, depending on the mode of endotoxin sensitization. It should be noted
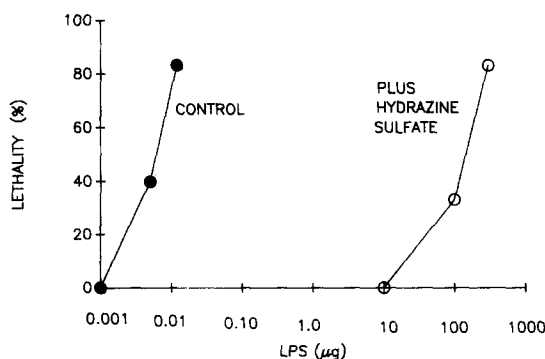

Figure 5. Effect of 5-h, 80 mg/kg hydrazine sulfate pretreatment on endotoxin lethality as seen from the LPS dose response profile. 18 mg D-galactosamine was given at the time of challenge. Control, pretreated with saline vehicle in place of hydrazine sulfate. Six mice per datum. Not shown are data showing full protection with 0.012, 0.1, and 1.0 μg LPS.
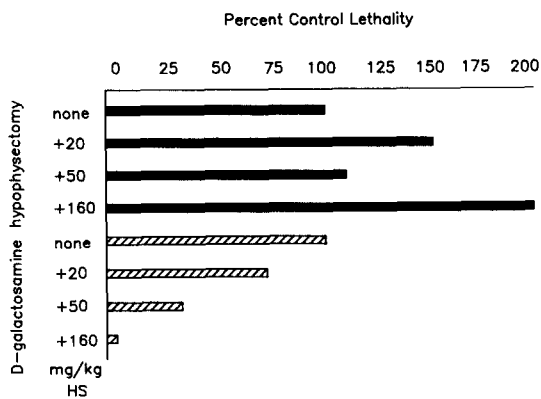
Percent Control Lethality

Figure 6. Comparative effects of hydrazine sulfate (HS) 5-h pretreatment on endotoxin lethality in mice sensitized by hypophysectomy or with D-galactosamine. Hydrazine sulfate dosage as shown, mg/kg HS. Endotoxin dose (LD50): hypophysectomy, 0.2 μg endotoxin; D-galactosamine, 0.01 μg endotoxin. Hypophysectomy data are taken from Table 1.
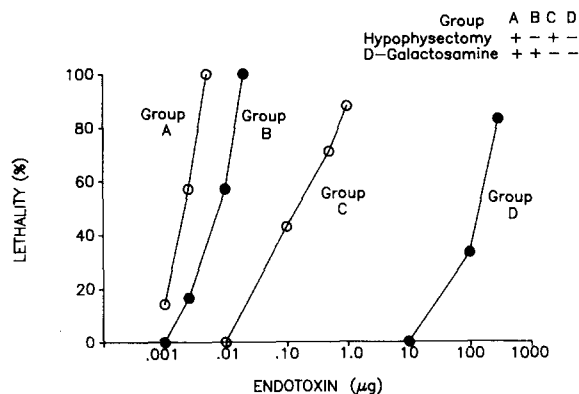

Figure 7. Effects of hypophysectomy on endotoxin sensitivity in normal and D-galactosamine-treated mice. Group A, D-galactosamine treated and hypophysectomized; group B, D-galactosamine treated and sham operated; group C, hypophysectomized; group D, sham operated. Seven mice per datum.

Figure 8. Effect of 5-h hydrazine sulfate pretreatment on endotoxin lethality in mice sensitized by hypophysectomy and by treatment with D-galactosamine. Control, pretreated with saline vehicle. Plus hydrazine sulfate, 80 mg/kg. Seven mice per datum.

overlap in the endotoxin sensitization states produced, notwithstanding the differences in the means of sensitization.

Given that the physiological states are so closely overlapping in terms of endotoxin sensitivity, why was hydrazine sulfate able to confer protection against one sensitization state, but not against the other? Conceivably, hydrazine might protect by a pituitary-dependent mechanism, involving subsequent impact on the liver. If so, then hypophysectomy might be expected to abrogate the protection whether or not mice were treated with D-galactosamine at the time of endotoxin challenge. If, however, hydrazine sulfate protection against D-galactosamine sensitization were not dependent on the pituitary, then an increase in the endotoxin LD50 might result from hydrazine sulfate pretreatment of mice that had been both hypophysectomized and treated with D-galactosamine at the time of challenge. Under such circumstances, the LD50 might be expected to rise toward that seen in the absence of D-galactosamine treatment, i.e., 0.2 μg (Fig. 3 a), an increase of ∼100-fold. As seen in Fig. 8, hydrazine sulfate does not protect hypophysectomized mice from D-galactos-

that the observed increase in lethality seen with the hypophysectomized mice given 20 mg/kg hydrazine sulfate (Fig. 6) is probably not statistically significant, and is not manifest at the increased hydrazine sulfate dose of 50 mg/kg. The highest dose of hydrazine sulfate may, however, be toxic to the hypophysectomized mice.

Given this marked difference in response to hydrazine sulfate depending on the mode of sensitization, the question arises as to whether the two physiological states with respect to endotoxin sensitivity are themselves completely distinct. If so, the increased sensitivity to endotoxin elicited by hypophysectomy, ∼10³, and that elicited by D-galactosamine treatment, ∼10⁴, together should elicit an increased sensitivity to endotoxin on the order of 10⁷. As shown by the data in Fig. 7, the observed LD50 for the hypophysectomized and D-galactosamine-treated mice is ∼0.002 μg, or only four-fold lower than that for intact mice treated with D-galactosamine. Thus, it would appear that there is considerable
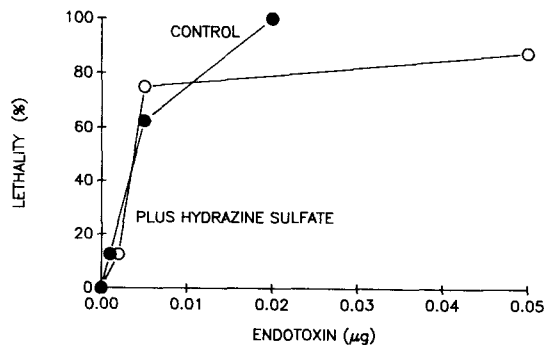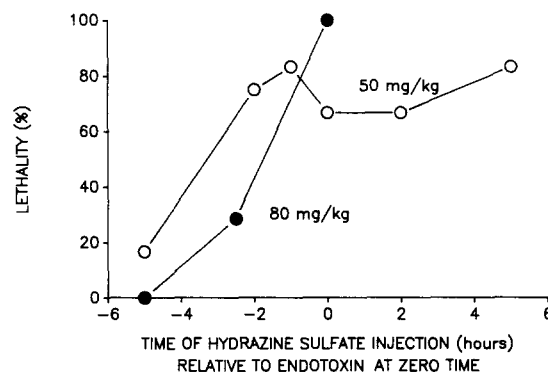

Figure 9. Effect of hydrazine sulfate pretreatment time on endotoxin lethality. 18 mg D-galactosamine given at the time of challenge. (O) 50 mg/kg hydrazine sulfate profile, 0.01 μg endotoxin (10/12 deaths, saline vehicle). 12 mice per datum (two experiments, six mice each). (●) 80 mg/kg hydrazine sulfate profile, 0.015 μg endotoxin (10/10 deaths, saline vehicle). Seven mice per datum.

Table 2. *Effect of D-Galactosamine Pretreatment on Hydrazine Sulfate Protection against LPS*

		Lethality (deaths/total)		
Pretreatment	Challenge	Exp. 1	Exp. 2	Total
D-GalN	D-GalN	0/5	0/10	0/15
D-GalN	LPS + D-GalN	8/10	3/5	11/15
HS	LPS + D-GalN	1/5	1/10	2/15*
HS + D-GalN	LPS + D-GalN	8/10	3/5	11/15*

D-GalN, D-galactosamine; HS, hydrazine sulfate. Pretreatment, 5 h before challenge. Hydrazine sulfate, 80 mg/kg; D-GalN, 18 mg; LPS, 0.012 µg. Lethality, 48 h.
* $p < 0.005$.

amine sensitization, as the hydrazine sulfate and control mice exhibit superimposable LPS dose lethality profiles. Thus, the ability of hydrazine sulfate to protect against endotoxin lethality in both normal and D-galactosamine-sensitized mice is pituitary dependent.

The possibility that a pituitary effect acting on the liver might involve changes in hepatic protein levels, coupled with the fact that D-galactosamine rapidly decreases hepatic protein synthesis (27), led us to explore the possibility that D-galactosamine sensitization at the time of challenge and hydrazine sulfate desensitization during the pretreatment period might reflect closely related phenomena. Conceivably, hydrazine sulfate might effect certain changes during the pretreatment period that not only might be helpful to host defense against endotoxin, but that might otherwise not occur under the influence of simultaneous D-galactosamine treatment. Accordingly, we ascertained whether or not hydrazine sulfate pretreatment was, in fact, necessary for the protection against D-galactosamine sensitization, and whether or not injection of D-galactosamine not only at the time of endo-

toxin challenge but also at the start of the pretreatment period might impact on that protection. Accordingly, hydrazine sulfate was given to mice at different times before challenge with endotoxin. The effect of hydrazine sulfate given at the same time or after the time of endotoxin challenge was also assessed. The data, shown in Fig. 9, confirm that a period of hydrazine sulfate pretreatment is necessary for protection, and document a requirement for pretreatment of the order of several hours. Not shown in Fig. 9 are data with 80 mg/kg hydrazine sulfate given at 10 or 15 h before challenge. Protection was still evident after these extended pretreatment times, with a corresponding lethality of 0/7 and 1/7, respectively. Evidence that the need for an extended time was not primarily a reflection of time needed for hydrazine clearance from the circulation was suggested by determination of hydrazine concentration in plasma after intraperitoneal injection of 80 mg/kg hydrazine sulfate. By 30 min after injection, plasma hydrazine concentration had peaked, and by 1 h, >75% of the hydrazine had been cleared from the circulation (unpublished data).

Having established that hydrazine sulfate pretreatment is necessary to the protection against D-galactosamine sensitization, we then investigated the effect of D-galactosamine being present at the start of the pretreatment period. D-Galactosamine was given in two equal doses, each 18 mg. One dose was given, as previously, at the time of challenge. The other, 5 h before, was at the time of hydrazine sulfate pretreatment. As shown by the cumulative results of two experiments, administration of D-galactosamine at the start of the 5-h pretreatment period completely abrogated the protective effects of hydrazine sulfate (Table 2). Control experiments have established that the lethality observed is not simply the result of the mice receiving two doses of D-galactosamine alone, since 0/15 mice died under such conditions in the absence of endotoxin. When this experiment was carried out under conditions in which hydrazine sulfate was given 10 h before challenge, and 5 h before the first of the two doses of D-galactosamine, protection was again readily apparent. This latter

Table 3. *Effect of Hydrazine Sulfate on TNF/Cachectin: Endotoxin Lethality*

		Lethality (deaths/total)				
Hydrazine Sulfate	Challenge	Exp. 1	Exp. 2	Exp. 3	Total	
mg/kg						
–	None	0/5	0/6	0/6	0/17	
50	None	0/6	0/6	ND	0/11	
–	TNF	ND	5/8	2/2	7/10	$p < 0.005$
50	TNF	ND	0/8	0/2	0/10	
–	Endotoxin	4/4	5/8	8/8	17/20	$p < 0.005$
50	Endotoxin	2/5	2/8	0/7	4/20	

CF1 mice were treated with either 50 mg/kg hydrazine sulfate or saline vehicle 5 h before challenge with 0.5 µg TNF/cachectin (Chiron Corp.) or 0.04 µg endotoxin. Mice were sensitized with 18 mg D-galactosamine given at the time of challenge. Lethality, 48 h.

result eliminates the possibility that the two doses of D-galactosamine per se abrogated the protection, rather than their relationship to the times of hydrazine sulfate and endotoxin administration.

We then examined the possibility that since TNF/cachectin has been implicated as a major contributing factor in the pathological effects of endotoxin (6, 7), and D-galactosamine also sensitizes mice to the lethal effects of TNF/cachectin (16), hydrazine sulfate protection against endotoxin in D-galactosamine-treated mice might also extend to protection against TNF/cachectin itself. As shown in Table 3, hydrazine sulfate pretreatment (50 mg/kg, -5 h) provided significant protection of mice against the lethal effects of both endotoxin and TNF/cachectin. We also tested the ability of hydrazine sulfate to protect against TNF/cachectin in an endotoxin-resistant strain. The C3H/HeJ endotoxin hyporesponsive mouse has been reported to be at least 100-fold more resistant than normal mice to the lethal effects of endotoxin in the D-galactosamine model, while exhibiting no significant difference in sensitivity to the lethal effects of TNF/cachectin (16). We have confirmed these results with the C3H/HeBK strain used in our own studies. Whereas the LD_{50} for TNF/cachectin in D-galactosamine-sensitized mice was 0.5 μg both for CF1 and C3H/HeBK mice, the LD_{50} for LPS in D-galactosamine-sensitized mice was $\sim$0.005 μg for CF1 mice and $\sim$0.8 μg for the C3H/HeBK strain. We then queried whether hydrazine sulfate pretreatment (-5 h, 160 mg/kg) would protect these LPS-hyporesponsive mice against challenge from an LD_{80-90} dose of either endotoxin or TNF/cachectin. In both instances, significant ($p < 0.005$) protection was evident: against TNF/cachectin (0.6 μg), 9/10 vs. 0/6 deaths; and against LPS (1.2 μg), 5/6 vs. 0/6 deaths.

Discussion

Earlier studies from our laboratory established that pretreatment of mice with hydrazine sulfate provided significant protection of these animals against subsequent challenge with lethal doses of endotoxin. It was also shown that hydrazine sulfate pretreatment acted to reverse the decrease in hepatic PEPCK activity that follows endotoxin challenge, suggesting a possible hormonal response to hydrazine sulfate that might contribute to the observed protection (8). In this report, we have shown that hydrazine sulfate administration stimulates an early increase in plasma corticosterone followed by a decrease in circulating triiodothyronine. Both of these hormones are pituitary dependent and contribute to PEPCK regulation. We then found that hypophysectomy abrogated the protection against endotoxin, providing strong evidence for the concept that the protection was pituitary dependent. We then determined that hydrazine sulfate protection was nevertheless not abrogated when mice were made sensitive to endotoxin upon treatment with the hepatotoxin D-galactosamine. On the contrary, the endotoxin LD_{50} was raised by approximately four orders of magnitude, almost completely reversing the D-galactosamine sensitization effect. Given the sharp difference in the capacity of hydrazine sulfate to protect depending on the mode of sensitization, we explored to what extent

the endotoxin sensitization states brought about by D-galactosamine treatment and by hypophysectomy are mutually independent. Lethality studies in which mice were D-galactosamine treated, hypophysectomized, or both, revealed strong overlap on the endotoxin LD_{50} between the two modes of sensitization. Further investigation of hydrazine sulfate protection of D-galactosamine-treated mice revealed that protection was abrogated after hypophysectomy, as shown also with normal mice. Further, that the protection required several hours of pretreatment, with a requirement that D-galactosamine not be present during the pretreatment period.

These collective results are consistent with an hypothesis in which hydrazine sulfate, either directly or indirectly, stimulates increased pituitary/adrenal hormonal release. Key cellular targets for these hormones are suggested to be hepatocytes and/or macrophages. Corticosteroid stimulation of macrophages result in their decreased sensitivity to subsequent endotoxin stimulation (29, 30). Corticosteroids also have the effect of stimulating the hepatocyte in response to endotoxin (31).

Our results confirm and extend the results of several earlier investigators who have examined hormonal effects on endotoxin sensitivity in mice. Thus, as shown by Bertini et al. (25), adrenalectomized mice are rendered highly susceptible to the lethal effects of endotoxin, with an LD_{50} comparable with that reported here for hypophysectomized mice. Similarly, as shown by Berry and Smythe (4), corticosteroids will protect against endotoxin, if given before endotoxin challenge.

Our data of hydrazine-induced changes in circulating hormone levels, abrogation of hydrazine protection against endotoxin by hypophysectomy, and the marked protection conferred by hydrazine sulfate against the endotoxin sensitization arising from the hepatospecific toxin, D-galactosamine, suggest a possible liver/pituitary link with respect to hydrazine sulfate protection against endotoxin. It is also possible that hydrazine may interfere, directly or indirectly, with endotoxin stimulation of the macrophage. In either case, the fact that hypophysectomized/D-galactosamine-treated mice remain totally refractory to hydrazine sulfate pretreatment indicates that hydrazine sulfate-mediated modulation of pituitary/adrenal function is most likely to be a critical factor in the manifestation of the observed protection against endotoxin.

There is little question but that mononuclear phagocytes play a critical role in mediating the lethal effects of endotoxin. In this regard, Galanos and colleagues (32) have adoptively transferred normal peritoneal macrophages into D-galactosamine-treated C3H/HeJ endotoxin-hyporesponsive mice and observed sensitivity to endotoxin indistinguishable from control mice. Further, it has been established that macrophage-derived TNF/cachectin is essential in mediating endotoxin lethality (6, 7). In this respect, therefore, it is perhaps noteworthy that hydrazine sulfate pretreatment also has been found to protect D-galactosamine-sensitized mice against the lethal effects of TNF/cachectin. Since hepatocytes appear to be the selective target for D-galactosamine, as discussed above, and since pituitary/adrenal-derived hormonal factors from hydrazine sulfate-injected mice appear to reverse D-galactosamine sensitization, we have speculated that hepatocytes may be the

pivotal target cell for any prophylactic endocrine effect induced by hydrazine sulfate pretreatment. If so, glucocorticoid involvement in this regard, and perhaps with hepatic synthesis of acute phase proteins (31), could provide a potentially satisfying explanation for the protection observed by us and others.

On the other hand, it is known that glucocorticoid pretreatment of murine macrophages decreases their sensitivity to endotoxin as measured by TNF/cachectin production. As shown by Beutler et al. (29), glucocorticoid regulation of TNF/cachectin secretion is manifest both at the level of transcription and translation. Moreover, consistent with the results reported here and elsewhere, regulation of macrophage TNF/cachectin production requires that cells be pretreated with corticosteroids before LPS stimulation.

Whatever the precise mechanisms of the protection effects observed with hydrazine sulfate, our data, as well as those of other investigators, suggest that: (a) pituitary/adrenal–derived hormonal factors are key participants in the host response to endotoxin; (b) these hormonal factors act by regulating hepatocyte cellular function and/or secretion; (c) up- or downregulation of hormone levels acts to regulate host responses to endotoxin, and finally; (d) liver-derived factors/cytokines serve to regulate the mononuclear phagocyte response to endotoxin. It is likely that each of these other influences in addition to that defined in d may be regulated by the presence of endotoxin, in part giving rise to the multifactorial effects of this complex bacterial product in vivo.

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References

1. Kreger, B.E., D.E. Craven, P.C. Carling, and W.R. McCabe. 1980. Gram-negative bacteremia. III. Reassessment of etiology, epidemiology, and ecology in 612 patients. *Am. J. Med.* 68:332.

2. Wolff, S.M., and J.V. Bennett. 1974. Gram-negative rod bacteremia (editorial). *N. Engl. J. Med.* 291:733.

3. Morrison, D.C., and J.L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* 38:417.

4. Berry, L.J., and D.S. Smythe. 1964. Effects of bacterial endotoxins on metabolism VII. Enzyme induction and cortisone protection. *J. Exp. Med.* 120:721.

5. Ziegler, E.J., J.A. McCutchan, J. Fierer, M.P. Glauser, J.C. Sadoff, H. Douglas, and A.I. Braude. 1982. Treatment of Gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. *N. Engl. J. Med.* 307:1225.

6. Beutler, B., I.W. Milsark, and A.C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science (Wash. DC).* 229:869.

7. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.).* 330:662.

8. Silverstein, R., C.A. Christoffersen, and D.C. Morrison. 1989. Modulation of endotoxin lethality in mice by hydrazine sulfate. *Infect. Immun.* 57:2072.

9. Rippe, D.F., and J.L. Berry. 1972. Study of inhibition of induction of phosphoenolpyruvate carboxykinase by endotoxin with radial immunodiffusion. *Infect. Immun.* 6:766.

10. McCallum, R.E., T.W. Seale, and R.D. Stith. 1983. Influence of endotoxin treatment on dexamethasone induction of hepatic phosphoenolpyruvate carboxykinase. *Infect. Immun.* 39:213.

11. Ray, P.D., R.L. Hanson, and H.A. Lardy. 1970. Inhibition by hydrazine of gluconeogenesis in the rat. *J. Biol. Chem.* 245:690.

12. Müller, M.J., A. Thomsen, W. Sibrowski, and H.J. Seitz. 1982. 3,5,3'-Triiodothyronine-induced synthesis of rat liver phosphoenolpyruvate carboxykinase. *Endocrinology.* 111:1469.

13. Cimbala, M.A., W.H. Lamers, K. Nelson, Y. Monahan, H. Yoo-Warren, and R.W. Hanson. 1982. Rapid changes in the concentration of phosphoenolpyruvate carboxykinase mRNA in rat liver and kidney. *J. Biol. Chem.* 257:7629.

14. Lamers, W.H., R.W. Hanson, and H.M. Meisner. 1982. cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in rat liver nuclei. *Proc. Natl. Acad. Sci. USA.* 79:5137.

15. Beale, E.G., N.B. Chrapkiewicz, H.A. Scoble, R.J. Metz, D.P. Quick, R.L. Noble, J.E. Donelson, K. Biemann, and D.K. Granner. 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP): structures of the protein, messenger RNA, and gene. *J. Biol. Chem.* 260:10748.

16. Lehmann, V., M.A. Freudenberg, and C. Galanos. 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine treated mice. *J. Exp. Med.* 165:657.

17. Schade, U.F., I. Burmeister, R. Engel, M. Reinke, and D.T. Wolter. 1989. Lipoxygenase inhibitors suppress formation of tumor necrosis factor *in vitro* and *in vivo*. *Lymphokine Res.* 8:245.

18. Tiegs, G., M. Wolter, and A. Wendel. 1989. Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. *Biochem. Pharmacol.* 38:627.

19. Clawson, G.A., J. Sesno, K. Milam, Y.F. Wang, and C. Gabriel. 1990. The hepatocyte protein synthesis defect induced by galactosamine involves hypomethylation of ribosomal RNA. *Hepa-*

tology. 11:428.

20. Wallach, D., H. Holtmann, H. Engelmann, and Y. Nophar. 1988. Sensitization and desensitization to lethal effects of tumor necrosis factor and IL-1. *J. Immunol.* 140:2994.

21. Wu, G.Y., V. Keegan-Rogers, S. Franklin, S. Midford, and C.H. Wu. 1988. Targeted antagonism of galactosamine toxicity in normal rat hepatocytes *in vitro. J. Biol. Chem.* 263:4719.

22. Westphal, O., O. Luderitz, and F. Bister. 1952. Uber die extraktion von bakterien mit phenol-wasser. *Z. Naturforsch.* 7b:148.

23. Bennett, C.A., and N.L. Franklin. 1954. Statistical Analysis in Chemistry and the Chemical Industry. John Wiley & Sons, New York. 620–626, 694–695.

24. Croxton, F.E. 1953. Elementary Statistics with Applications in Medicine. Prentice-Hall, Inc., Englewood Cliffs, NJ. 235–239, 326–327.

25. Bertini, R., M. Bianchi, and P. Ghezzi. 1988. Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 167:1708.

26. Abernathy, R.S., F. Halberg, and W.W. Spink. 1957. Studies on the mechanism of chlorpromazine protection against Bru-

cella endotoxin in mice. *J. Lab. Clin. Med.* 49:708.

27. Decker, K., and D. Keppler. 1974. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev. Physiol. Biochem. Pharmacol.* 71:77.

28. Galanos, C., M.A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc. Natl. Acad. Sci. USA.* 76:5939.

29. Beutler, B., N. Krochin, I.W. Milsark, C. Luedke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science (Wash. DC).* 232:977.

30. Beutler, B., and A. Cerami. 1988. The history, properties, and biological effects of cachectin. *Biochemistry.* 27:7575.

31. Baumann, H., K.R. Prowse, S. Marinkovic, K.-A. Won, and G.P. Jahreis. 1989. Stimulation of acute phase response by cytokines and glucocorticoids. *Ann. NY Acad. Sci.* 557:280.

32. Freudenberg, M.A., D. Keppler, and C. Galanos. 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. *Infect. Immun.* 51:891.