Stress Response and Drug Metabolism in Mice

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A modified stress model was studied to investigate the relationship between the stress response and drug metabolism in mice. Stress was induced in male CD-1 mice by a daily ip injection of hypertonic (1.5 M) saline for up to 3 days, whereas control animals received isotonic (0.15 M) saline. Two hours after receiving the saline injection on the first, second, or third day, animals were euthanized, and serum corticosterone (CORT) and liver aminopyrine N-demethylase (AD) and aniline hydroxylase (AH) activities were determined. To detect any effect of osmotic stimulation, a second control group was given 1.5 M saline as drinking water. There was no difference in CORT levels, AD activity, or AH activity between untreated animals and 0.15 M saline treatment. Intraperitoneal injection of 1.5 M saline markedly increased serum CORT concentrations compared to 0.15 M saline regardless of the duration of the treatment. Injection of 1.5 M saline also decreased both hepatic enzyme activities at each time point. Osmotic stimulation alone by hypertonic drinking water had no significant effect on CORT levels, AD activity, or AH activity. In another series of experiments, intact, sham-operated, and adrenalectomized mice were exposed to the stress model. Injected hypertonic saline decreased AD and AH activities in intact and sham-operated animals compared to isotonic saline-treated animals but both enzyme activities were reduced after adrenalectomy regardless of saline treatment used. In conclusion, a suitable model was established to study the interactions between the stress response and the hepatic drug metabolism in mice.

Stress is defined in terms of changes in resting biology. Some of these changes include several hormonal responses such as the secretion of catecholamines from the adrenal medulla, adrenocorticotropic hormone from the anterior pituitary, and corticosteroids from the adrenal cortex (Axelrod and Reisine, 1984). The biological response to a specific chemical can be modified in animals exposed to a variety of physical, psychological, and environmental stressors (Vogel, 1987). Rodents exposed to several stressful conditions, such as overcrowding, isolation, noise, and exposure to cold environment, show a decreased rate of oxidative metabolism of xenobiotics (Pollack et al., 1991), which could alter the toxicity of these compounds. Thus, apparent toxic effects in animals receiving a relatively high dose of a chemical under study may well be mediated by stress (Miller, 1992).

In order to investigate interactions between the stress response and altered rates of drug metabolism, it is necessary to determine and define a suitable model for stress. A number of experimental paradigms of stress, such as restraint, isolation, overcrowding, swimming, cold exposure, exposure to flashing light, and placing an animal cage on a linear oscillating shaker, have been used to study physiological responses (Husain et al., 1979; Katz et al., 1981; Lang et al., 1983; Pollard, 1984; Gibbs, 1985; Armario et al., 1986; Iwai et al., 1986; Wilbold et al., 1986; Vogel, 1987; Harbuz and Lightman, 1989). We chose to investigate a modified physical stress model used in rats by Lightman and Young (1987).

The procedure is based on the administration of intraperitoneal hypertonic saline. Such a stimulus results in a predominant activation of peritoneal afferent nerves with minimal animal handling and discomfort (Harbuz and Lightman, 1989). These authors found that corticotropin-releasing factor (CRF) mRNA was significantly increased in the group exposed to hypertonic saline. The time course of the changes in CRF mRNA showed that an increase in message occurred as early as 2 hr after the stressful stimulus and the same pattern of change was also seen after 8 hr. The response of hypotalamic CRF to any type of stress is clearly a balance between the rate of synthesis and the rate of release at the median eminence.

The objectives of the present studies were to (1) establish a suitable stress model in mice to characterize the effects of stress on standard hepatic drug metabolism enzymes and (2) characterize the effects of adrenalectomy and pharmacological inhibition of steroidogenesis on stress-induced alterations of hepatic drug metabolism.

MATERIALS AND METHODS

Animal use. Male CD-1 mice (Charles River Laboratories), weighing 30 to 35 g, were used in the present experiments. The mice were housed five per cage in a AAALAC-accredited facility, and were fed commercially.
obtained rodent chow. Food and water were supplied ad libitum (in selected experiments, adrenalectomized animals were given a 1% NaCl solution as drinking water). Following arrival, animals were acclimated for about 10 days prior to being randomized to treatment groups based on body weight. All animals rooms were on a defined light cycle (typically 14 hr light/10 hr dark). All animal care and handling procedures were in accordance with the requirements of the AAALAC and applicable NIH guidelines.

**Corticosterone determinations.** For serum corticosterone measurements, trunk blood samples were collected and maintained at room temperature for at least 1 hr. Following centrifugation (1000g, 15 min), serum was transferred to small capped vials and stored frozen (−30°C) until analyzed. Prior to the assay, 10 µl of serum was diluted with 2 ml of steroid diluent (phosphosaline gelatin buffer, pH 7.0 ± 0.1). Corticosterone levels were measured using a double antibody corticosterone RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA). The interassay coefficients of variation for corticosterone measured during these experiments was 15% for a low standard (25 ng/ml) and 8% for a high standard (1000 ng/ml) concentration, respectively. Sensitivity of this assay, examined by the statistical procedure described by McCann et al. (1983), was 4 ng/ml (buffer control value ±95% confidence interval). Since corticosterone concentrations in mice vary according to handling techniques and sample collection methods, laboratory baseline values were determined. The baseline range for this laboratory and for samples collected in the morning was 58–110 ng/ml.

**Metabolic enzyme determinations.** At necropsy, the liver was removed for standard marker determination of xenobiotic oxidative metabolism (mono-oxygenase-mediated aminopyrine N-demethylation and aniline hydroxylase activities). The liver was blotted dry and weighed, and a 20% homogenate in ice-cold 0.1 M phosphate buffer (pH 7.4) was prepared. The resulting homogenate was centrifuged at 10,000g for 15 min at 4°C; 0.5 ml of the supernatant was used in the reaction mixture. Reaction mixtures of 2 ml total volume were incubated in 25-ml Erlenmeyer flasks in a shaking incubator (−120 oscillations/min) at 37°C with air as a gaseous phase. Each flask (3 flasks/sample) contained the following: nicotinamide (50 µmol), glucose 6-phosphate (10 µmol), magnesium chloride (25 µmol), and NADP (0.5 µmol). Forty-five micromoles of semicarbazide hydrochloride was added to the reaction mixture when the N-demethylation of aminopyrine was measured. The appropriate substrate was then added to the incubation mixture: either aminopyrine (5 µmol) or aniline (10 µmol). The reaction was started by adding the supernatant to the reaction mixture and was stopped 30 min later by the addition of either 15% zinc sulfate (aminopyrine N-demethylase) or 20% trichloroacetic acid (aniline hydroxylase). Aminopyrine N-demethylase and aniline hydroxylase activities were determined as described by Gibson and Skett (1986). In both assays, product formation was linear with respect to time and protein concentration. Protein was quantified as described by Smith et al. (1985) using bovine serum albumin as a standard.

**Effects of stress on standard hepatic drug metabolism enzymes.** Stress was induced by a daily ip injection of hypertonic (1.5 M) saline (for 1, 2, or 3 days, 0.01 ml/g of body weight; dose levels were based on a pilot study), whereas control animals received an equivalent volume of isotonic (0.15 M) saline. Two hours after receiving the injection on the first, second (26 hr), or third (50 hr) day, groups of mice were decapitated; a blood sample was collected for corticosterone level determinations, and the liver was removed for determination of aminopyrine N-demethylase and aniline hydroxylase activities. A second control group, used to detect any effect of osmotic stimulation, was given hypertonic saline as drinking water for 7 days. The animals were euthanized on Day 8 and the same parameters were measured. To further examine the temporal relationships of serum corticosterone elevations and liver enzyme activities, another experiment was performed. Stress was induced by a single ip injection of 1.5 M saline (0.01 ml/g of body weight), whereas control animals received an equivalent volume of 0.15 M saline. Fifteen minutes, 30 min, or 2 hr after each injection, groups of mice were decapitated and the same parameters were measured.

**Characterization of the effects of adrenalectomy on stress-induced alterations of hepatic drug metabolism.** Adrenalectomized and sham-adrenalectomized animals were obtained from Charles River Laboratories. Upon arrival, the animals had free access to isotonic (0.15 M) saline and drinking water, respectively, and were included in the experiments 10 days after surgery. Intact, sham-operated, and adrenalectomized mice (n = 5/group) were exposed to the stress model. Half of the animals in each group were given a single ip injection of hypertonic saline (0.01 ml/g of body weight), whereas the other half received an equivalent volume of isotonic saline. Two hours after treatment, groups of mice were euthanized, a blood sample was collected for serum corticosterone levels, and the liver was removed for determination of aminopyrine N-demethylase and aniline hydroxylase activities.

To determine the possible role of corticosterone in mediating hepatic drug metabolism, adrenalectomized and sham-operated mice were injected subcutaneously with 0, 0.5, 1, or 2 mg/kg of corticosterone (n = 5/group; 0.01 ml/g of body weight) in propylene glycol and exposed 2 hr later to the same stress model. The dose levels were based on reports of subcutaneous administration regimes known to mimic the stress profile of corticosterone release (Peeters et al., 1992) and from a series of range-finding studies in which blood samples were collected from stressed or corticosterone-treated adrenalectomized animals and analyzed for serum corticosterone concentrations by radioimmunoassay. Two hours after the saline injection, groups of mice were euthanized and the same liver parameters were measured.

**Characterization of the effects of pharmacological inhibition of steriodogenesis on stress-induced alterations of hepatic drug metabolism.** Intact mice were injected subcutaneously with 0, 2, 6, or 16 mg/kg of aminoglutethimide (Sigma, Chemical Co., St. Louis, MO), an inhibitor of 17alpha-hydroxylase (Sigma, Chemical Co., St. Louis, MO), an inhibitor of cytochrome P450 side chain cleavage enzyme and of steroid biosynthesis (n = 5/group; 0.01 ml/g of body weight), and exposed 2 hr later to the stress model. Two hours after the saline injection, groups of mice were euthanized and the same parameters as before were measured. In a separate experiment, intact mice were injected ip with 0, 50, 75, or 100 mg/kg of metyrapone (Sigma, Chemical Co.), an inhibitor of corticosterone synthesis (n = 5/group; 0.01 ml/g of body weight), and exposed 2 hr later to the same stress model described above. Two hours after the saline injection, the same parameters were measured.

**Statistical analysis.** In every experiment, results from 5 animals per treatment group (stress model included 10 animals, half of which were given hypertonic saline, whereas the other half received isotonic saline) are presented as means ± SD. One-way or two-way analysis of variance methods were used as appropriate. If a significant effect occurred (p ≤ 0.05), differences among groups were determined using the Student Newman–Keuls multiple comparison test. Student’s t test was used to detect any difference between groups receiving either hypertonic saline as drinking water or plain water for 7 days.

**RESULTS**

**Effects of stress on standard hepatic drug metabolism enzymes.** There were no differences in serum corticosterone (Fig. 1a), hepatic aminopyrine N-demethylation (Fig. 1b), or hepatic aniline hydroxylase (Fig. 1c) levels between baseline values (untreated animals) and isotonic saline administration. Intrapertitoneal injection of 1.5 M saline markedly increased serum corticosterone concentrations compared to 0.15 M saline injection regardless of the duration of the treatment (Fig. 2). However, increased corticosterone levels in 1.5 M saline-treated animals did not differ among the different days of treatment (Fig. 2a). Hypertonic saline decreased both hepatic enzyme activities at each time point.
markedly increased serum corticosterone levels compared to isotonic saline injection at 30 min and 2 hr, but not at 15 min (Fig. 3a). Hypertonic saline also decreased both hepatic

(Figs. 2b and 2c). Osmotic stimulation alone after 7 days of hypertonic saline in place of drinking water had no significant effect on either serum corticosterone levels, aminopyrine N-demethylase activity, or aniline hydroxylase activity (data not shown). Intraperitoneal injection of 1.5 M saline

FIG. 1. (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in untreated animals vs animals exposed to isotonic (0.15 M) saline solution. All values are means ± SD for n = 5 mice.

FIG. 2. (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in isotonic (0.15 M) vs hypertonic (1.5 M) saline-treated animals at 1, 2, or 3 days after treatment. All values are means ± SD for n = 5 mice. Asterisks indicate statistical difference between isotonic vs hypertonic saline treatment within the same day (p < 0.05).
neal injection of 1.5 M saline markedly increased serum corticosterone levels compared to isotonic saline injection in intact and sham-operated animals (Fig. 4a). Increases in enzyme levels after 2 hr, but not at 15 or 30 min posttreatment (Figs. 3b and 3c).

**Characterization of the effects of adrenalectomy on stress-induced alterations of hepatic drug metabolism.** Intraperito-

**FIG. 3.** (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in isotonic (0.15 M) vs hypertonic (1.5 M) saline-treated animals at 15, 30, or 120 min after treatment. All values are means ± SD for *n* = 5 mice. Asterisks indicate statistical difference between isotonic vs hypertonic saline treatment within the same time point (*p* < 0.05).

**FIG. 4.** (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in isotonic (0.15 M) vs hypertonic (1.5 M) saline-treated intact, sham-operated (sham), or adrenalectomized (adrenal X) animals. All values are means ± SD for *n* = 5 mice. Asterisks indicate statistical difference between isotonic vs hypertonic saline treatment within the same surgical condition (*p* < 0.05).
corticosterone levels were significantly higher in sham-operated than in intact animals. Serum corticosterone levels were not completely abolished in adrenalectomized mice. Hypertonic saline significantly decreased aminopyrine N-demethylase and aniline hydroxylase activities in intact and sham-operated animals compared to respective isotonic saline-treated animals. Monooxygenase activities were reduced in adrenalectomized animals compared to sham-operated animals, but neither activity was significantly altered by hypertonic saline treatment (Figs. 4b and 4c). However, hepatic aminopyrine N-demethylase activity was not different among corticosterone-treated groups in adrenalectomized mice (Fig. 5a) despite the fact that in preliminary range-finding studies the highest dose of corticosterone given to these animals resulted in plasma concentrations that closely matched values resulted from stress (≈ 400–600 ng/ml). Likewise, hypertonic saline decreased hepatic aniline hydroxylase activity in sham-operated animals, but enzyme activity was not different among corticosterone-treated groups in adrenalectomized mice (Fig. 5b).

Characterization of the effects of pharmacological inhibition of steroidogenesis on stress-induced alterations of hepatic drug metabolism. Aminoglutethimide (6 and 16 mg/kg) inhibited the serum corticosterone response to ip injection of 1.5 M saline (Fig. 6a). Similarly, these doses of aminoglutethimide blocked the reduction in hepatic enzyme activities following hypertonic saline treatment (Figs. 6b and 6c). Metyrapone treatment resulted in a dose-related inhibition in the serum corticosterone response to ip injection of 1.5 M saline, but failed to alter hepatic enzyme activities (Figs. 7a–7c).

DISCUSSION

At present, little is known about the biochemical mechanisms by which stress-induced changes affect the actions of chemicals. Stress research has been primarily focused on the neuroendocrine response at the hypothalamic–pituitary–adrenal axis level, but the diversity of physiological changes generated by stress can produce distant effects (Axelrod and Reisine, 1984). Several studies have demonstrated the effects of stress on the metabolism of xenobiotics (Bousquet et al., 1965; Furner and Stitzel, 1968; Capel et al., 1980; Pollack et al., 1991). Furner and Stitzel (1968) found that in rats exposed to cold stress for 4 days the rate of metabolism of ethylmorphine and aniline was elevated, and the rate of metabolism of hexobarbital was decreased. Furthermore, studies using different forms of stress showed either an increased metabolic clearance (Bousquet et al., 1965) or a decreased metabolic rate (Pollack et al., 1991). These findings suggest that the rate of metabolism of xenobiotics in response to stress varies depending upon which enzyme system is studied and what type of stress is examined.

The injection of isotonic saline, included as the control group for the hypertonic saline injection, could be considered a low level source of stress since no differences were seen in any of the measured parameters between baseline (untreated) animals and mice receiving isotonic saline. This may reflect the minimal level of stress to which these animals were exposed during usual handling. On the other hand, intraperitoneal injection of hypertonic saline markedly increased serum corticosterone concentrations compared to isotonic saline injection regardless of the duration of the treatment. Furthermore, hypertonic saline decreased hepatic monooxygenase activities at each time point, i.e., 1, 2, or 3 days.
FIG. 6. (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in isotonic (0.15 M) vs hypertonic (1.5 M) saline-treated animals pretreated with different doses of aminoglutethimide. All values are means ± SD for n = 5 mice. Asterisks indicate statistical difference between isotonic vs hypertonic saline treatment within the same aminoglutethimide dose level (p ≤ 0.05).

FIG. 7. (a) Serum corticosterone, (b) hepatic aminopyrine N-demethylase (AD), and (c) hepatic aniline hydroxylase (AH) levels in isotonic (0.15 M) vs hypertonic (1.5 M) saline-treated animals pretreated with different doses of metyrapone. All values are means ± SD for n = 5 mice. Asterisks indicate statistical difference between isotonic vs hypertonic saline treatment within the same metyrapone dose level (p ≤ 0.05).
Although the administration of hypertonic saline can have osmotic effects, experiments confirmed that the osmotic stimulus following replacement of drinking water with hypertonic saline for up to 7 days did not result in changes in either serum corticosterone levels or hepatic enzyme activities. Therefore, it is the stress rather than the osmotic stimulus that causes the increase in serum corticosterone levels and decreases in hepatic enzyme activities. The present experiments show that hypertonic saline injection produces a stress response similar to other stresses such as cold and swimming (Pollack et al., 1991; Harbuz and Lightman, 1989). The serum corticosterone response was elicited as early as 30 min after hypertonic saline injection, indicating activation of corticotrophin-releasing factor neurons of the hypothalamic paraventricular nucleus by possible stimulation of periventricular afferent nerves (Swanson et al., 1986).

The importance of the adrenal gland on hepatic drug metabolism has been known since the late 1960s. It was of interest, therefore, to compare the effects of this stress model on hepatic enzymes in adrenalectomized animals, i.e., mice lacking an intact pituitary–adrenal axis. Serum corticosterone production was not completely abolished in every experiment using adrenalectomized mice, but was at significantly lower levels. This might be related to the presence in some animals of accessory adrenal cortical nodules which would not be removed during adrenalectomy (Hummel, 1958). The in vitro metabolism rate of aminopyrine and aniline were significantly reduced following adrenalectomy. Exposure of adrenalectomized animals to hypertonic saline did not change this response. It has been known since the late 1960s that hepatic enzyme activities are reduced after adrenalectomy but the exact mechanism by which this occurs is still in debate. Corticosterone is not the only steroid hormone secreted by the adrenal gland. It could be argued that the effects of adrenalectomy on drug metabolism are the result of changes in adrenal production of mineralocorticoids, glucocorticoids, and adrenaline, the subsequent changes in pituitary outflow of hormones such as adrenocorticotropic hormone (ACTH) and/or other physiological changes induced by hypoadrenalism. One attractive explanation is the lack of negative feedback of the hypothalamic–pituitary–adrenal axis after adrenalectomy that results in changes in endocrine factors other than corticosterone (e.g., an increase in pituitary hormones and cytokines). In this regard, a direct effect of ACTH on drug metabolism has been reported (Gustafsson, 1980). Several authors have also shown that adrenalectomy not only resulted in depletion of corticosterone and elevation of plasma ACTH but also resulted in an increase in plasma interleukin 6 (Schobitz et al., 1993; Zhou et al., 1993). Additionally, interleukins may depress cytochrome P450 enzyme activity (Ghezzi et al., 1986; Bertini et al., 1989; Ferrari et al., 1993). Therefore, based on these studies, adrenalectomy provides more questions than answers in the elucidation of the effect of stress on drug metabolism.

Although adrenalectomy is frequently used in animal experiments to deplete adrenocortical hormones, it removes both cortex and medulla, generates metabolic deficits (Sakakura et al., 1981), changes the concentration of corticosteroid-binding globulin (Levin et al., 1987) and modifies the density of corticosteroid receptors (Kiss et al., 1988; Lowy, 1989; Luttge and Rupp, 1989). The use of pharmacological inhibition of steroid biosynthesis in the adrenal gland avoids many of the confounding variables associated with adrenalectomy (Roberts et al., 1993). Aminogluthimide, an inhibitor of the cytochrome P450 side chain cleavage enzyme (P450_50), responsible for the conversion of cholesterol to pregnenolone in steroid biosynthesis, was therefore used in selected experiments. This compound inhibited the serum corticosterone response and the reduction in hepatic enzyme activity to hypertonic saline challenge. However, corticosterone administration (0.5–2 mg/kg) did not alter hepatic enzyme activities in hypertonic vs isotonic saline-treated adrenalectomized mice. The steroid inhibitor metyrapone has been shown to be an inhibitor of P450 enzymes by attaching to the type II binding site (Netter, 1980). However, as can be seen in Fig. 7, metyrapone, at a dose that inhibited the serum corticosterone response to ip injection of 1.5 m saline, failed to alter the differences in hepatic enzyme activities between isotonic and hypertonic saline. Moreover, there seems to be no dose–response relationship, unlike the corticosterone response, in the hepatic enzyme activities. Therefore, under the present experimental conditions, there appears to be no effect of metyrapone on aniline hydroxylase and aminopyrine N-demethylease activities.

These experiments indicate that a steroid other than corticosterone may have been involved, either directly or indirectly, in the inhibition of the activity of hepatic aminopyrine N-demethylease and aniline hydroxylase. Possible candidates are the early precursors in the steroidogenic pathway of the adrenal cortex, such as progesterone and 17α-hydroxyprogesterone of the pregnane group or dehydroepiandrosterone and androstenedione of the androstan group.

It has been previously shown that rats had a significant reduction in the activity and content of cytochrome P450 after running (Day and Weiner, 1991). Studies on the metabolism of selected drugs confirmed that exercise affects the kinetics of these agents (Somani et al., 1990). Day et al. (1992) recently studied the effects of chronic running exercise on components of the hepatic drug-metabolizing enzymes in middle-aged and aged rats. They found a 34% decline in microsomal cytochrome P450 content and a 21% decrease of aniline hydroxylase activity after exposure to treadmill exercise in both age groups. In comparison to the inhibitory effects of chronic exercise, studies by Kasperek et al. (1982) showed similar results with acute treadmill
exercise. These authors found a 25% decrease in total hepatic cytochrome P450 content and a 29% decrease in aminopyrine N-demethylation activity. Therefore, based on our present results with aniline hydroxylase and aminopyrine N-demethylation activities, both findings (chronic and acute exercise) might well be the result of stress produced by the exercise rather than the activity itself. Since hepatic enzyme activities were altered in response to this stress model, Western immunoblotting techniques will be used in future experiments to determine the effect of the stress model on specific cytochrome P450 levels.

In conclusion, the studies reported herein demonstrate that exposure to hypertonic saline induces a stress response, and that hormones associated with this response interfere with hepatic drug metabolism. Further studies are needed to elucidate the stress-related mechanisms involved in this inhibition. Knowledge of these mechanisms will aid in understanding apparent drug toxicity which is secondary to stress.

REFERENCES


