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Central Corticotropin-Releasing Hormone Activates the Sympathetic Nervous System and Reduces Immune Function: Increased Responsivity of the Aged Rat*

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ABSTRACT

CRH acts within the brain to activate the sympathetic nervous system and reduce cellular immune function. To determine the effects of age on CRH-induced elevations of sympathetic activity and suppression of immunity, we examined the responses of plasma catecholamines, neuropeptide-Y (NPY), corticosterone, and splenic natural killer (NK) activity after microinjection of rat CRH (200 pmol) into the lateral ventricle of aged (24-month-old) Fischer 344 (F344) rats compared to those in young (4-month-old) F344 rats. Basal concentrations of plasma norepinephrine and NPY were higher in the aged than in the young animals. In addition, CRH produced a greater elevation of plasma levels of catecholamines and NPY, which persisted for a longer period of time in the aged rats compared to responses in the young animals. Splenic NK activity showed an age-related decrement at baseline, and CRH induced a further significant ($P < 0.01$) reduction of lytic activity in the aged rats, but did not alter cytotoxicity in the young rats. Corticosterone basal levels and responses were similar in the aged and young rats. These results show an age-related increase in autonomic outflow and suppression of NK activity after central CRH administration. In aged animals, the central nervous system may have a role in abnormal regulation of sympathetic activity and suppression of natural cytotoxicity *in vivo*. (*Endocrinology* **131:** 1047–1053, 1992)

AGING has been found be associated with a decline of natural killer (NK) cell activity (1–6), a subpopulation of lymphocytes considered important in host defense against such viruses as herpes simplex and cytomegalovirus (7–10). However, there are few data that have evaluated the role of the central nervous system in the neural modulation of NK cytotoxicity during aging, even though substantial evidence has demonstrated that changes in noradrenergic innervation of lymphoid tissue occur with aging (11–13), plasma catecholamine levels after stress are increased in aged rats (14–16), and central activation of the sympathetic nervous system has a role in mediating the suppression of NK activity *in vivo* (17–19).

CRH has been implicated as a neurotransmitter in the central nervous system that coordinates neuroendocrine (20) and autonomic outflow (21–23), producing changes in visceral function, such as immune responses (24–27). Indeed, the release of endogenous CRH after stress has been demonstrated to induce elevations in plasma levels of norepinephrine and epinephrine (21), which mediate the suppression of NK activity independent of the activation of the pituitary-adrenal axis (17, 24).

An age-related dysregulation of CRH and other physiological systems has been proposed (28), and hypothalamic CRH might be hypersecreted in aged animals. For example, CRH release from hypothalamic fragments of aged rats is increased at rest and after acetylcholine stimulation *in vitro* (29), the anterior pituitary response to CRH *in vivo* is dampened in aged animals (30), and an age-related decrease in hypothalamic and anterior pituitary CRH receptors has been found (31, 31a).

Based on the key role of CRH in the regulation of both the autonomic nervous system and NK cells and the possibility of age-related changes in central CRH systems, the present investigation was designed to examine further CRH-induced activation of the sympathetic nervous system and suppression of immunity and to determine the effects of aging on elevation of plasma levels of catecholamines and neuropeptide-Y (NPY) and reduction of NK activity after exogenous CRH treatment. Since the levels of plasma catecholamines after stress are increased in aged rats (16), we hypothesize that CRH will also induce an exaggerated release of epinephrine, norepinephrine, and NPY in the aged animals. Furthermore, this increased sympathetic response to CRH will be associated with a further decrement of NK activity in aged rats compared to young animals independent of adrenocortical responses. To test these predictions, splenic NK activity was assayed, and basal levels and responses of epinephrine, norepinephrine, NPY, and corticosterone were determined after the central administration of CRH in aged and young rats.

Materials and Methods

Animals

Male nonbreeder Fischer 344 (F-344) rats (n = 109), aged 4 months (n = 58) and 24 months (n = 51; NIA colony from Harlan Sprague

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Dawley Inc., (Indianapolis, IN) were housed in groups of two before experimentation in constant room temperature (22 C) animal facilities (12 h of light, 12 h of darkness; lights on at 0630 h). They had continuous access to water and food. Animals were handled daily for a 5-min period for 2 weeks to allow for sufficient habituation to manipulation of intracerebral cannula needed for injection of CRH or the saline vehicle.

Surgical methods

Two weeks before the experiments involving central injection, intracerebroventricular (icv) cannulae (24 gauge) were placed stereotaxically over the lateral cerebral ventricle, as previously described (25, 32).

Animals were anesthetized with isoflurane, and Silastic-tipped PE-50 cannulas were inserted through the right jugular vein to the right atrium 1 day before the plasma epinephrine, norepinephrine, and NPY sampling experiments. These indwelling venous catheters were used for serial blood sampling on the day of the experiment.

Treatments

The treatment groups used were: 1) young (4-month-old) rats icv infused with saline, 2) young rats icv infused with CRH, 3) aged (24-month-old) rats icv infused with saline, and 4) aged rats icv infused with CRH.

For icv infusion on the day of the experiment, the infusion needle (30 gauge) was extended 1 mm beyond the tip of the guide cannula into the lateral ventricle, and 200 pmol CRH (J. Rivier, Peptide Biology Laboratory, Salk Institute) dissolved in 2 μl 0.9% saline were infused by Hamilton syringe (Reno, NV) over a 30-sec period. Previous studies in our laboratory have demonstrated that CRH produces a dose-dependent reduction of NK activity in which the 200-pmol dose of icv CRH significantly elevates plasma catecholamine levels and reduces splenic NK activity (17). The flow of CRH or saline during central infusion was readily observed in all animals used in the experiments, and placement of the ventricular cannulae was verified after the experiment by injecting 10 μl trypan blue into the cannulae. To ensure that icv infusions of saline yield values of NK activity and catecholamines comparable to those in home cage controls that do not receive such infusions, handling habituation procedures were employed before the day of the experiment, as previously described (25).

Determination of plasma hormones

For the experiments involving serial determination of catecholamines, NPY, and corticosterone, blood (0.3–0.5 ml) was collected through the jugular venous catheter at the times described for each experiment. Immediately after blood sampling, an infusion of an equal volume of heparinized saline was administered. The maximum amount of blood collected for each animal was 1.5 ml. Each rat was used only once, and all experiments were repeated at least twice.

Blood for catecholamine, NPY, and corticosterone determinations was collected on ice in tubes containing 50 mg/ml EDTA and 500 kallikrein inhibitor units aprotinin (Sigma Chemical Co., St. Louis, MO). Samples were immediately centrifuged, and decanted plasma was snap-frozen and stored at −70 C until assay of catecholamines, NPY, or corticosterone.

Plasma concentrations of epinephrine and norepinephrine were measured using radioenzymatic methods modified from the assay previously described (33, 34). Briefly, this assay is based on the conversion of the catecholamines to their respective methyl derivatives using the enzyme catechol-methyltransferase in the presence of S-adenosylmethionine, serving as an *methyl*-^{3}H donor. After the enzymatic reaction, unreacted S-adenosyl-L-[*methyl*-^{3}H]methionine was removed by extraction, and the products were separated using TLC on silica plates. The areas corresponding to ^{3}H-methylated derivatives were cut out and placed in scintillation vials, eluted with aqueous solvent, extracted into nonpolar scintillation cocktail, and counted by liquid scintillation spectrometry.

Measurement of plasma concentrations of NPY was performed using RIA procedures (34). Antibodies against NPY were prepared by coupling the peptide to human globulin (Research Plus Laboratories, Inc., Denville, NJ) and immunizing rabbits using previously described procedures (34). Before RIA, plasma samples were extracted using octadecyl (C-18) Bond Elut columns (Analytichem International Harbor City, CA). Columns were prewashed with 1 vol 2.5 ml methanol (HPLC grade), then 2 vol triethylammonimum formate (TEAF). A maximum of 1 ml plasma was applied by gravity, and the columns were washed again with 2 vol TEAF. NPY was eluted with 2 ml 75% acetonitrile-25% TEAF and lyophilized in a Speed-Vac (Savant, Hicksville, NY). Samples were reconstituted in RIA buffer before assay.

Plasma levels of corticosterone were measured by RIA procedures in unextracted samples using an antibody produced against corticosterone 21-hemisuccinate-BSA (ICN Biomedicals, Inc., Costa Mesa, CA).

Assay of NK cytotoxicity

For experiments involving assay of NK activity, animals were removed from the cages and killed by decapitation 1 h after icv infusion of either saline or CRH. NK activity was assessed 1 h after CRH infusion, since CRH induces a maximal reduction of splenic cytotoxicity within 20 min, and this reduction of NK activity significantly persists for up to 1 h (35).

To measure splenic NK cytotoxicity, the spleen was surgically removed and dissociated into a single cell suspension (36). Examination of the spleens from the aged animals revealed splenomegaly in 3 of the 15 aged animals. These diseased aged animals were excluded from further analysis, consistent with the procedures of Ackerman and colleagues in the study of β-receptor cell density of lymphocytes in aged rats (11). Splenocytes were centrifuged in 50-ml tubes at 400 × *g* at room temperature for 30 min over 12 ml Ficoll Paque (Pharmacia, Piscataway, NJ) to yield mononuclear cells. Cells at the interface were collected and washed twice with PBS. The cytotoxicity of isolated splenic lymphocytes was measured in a standard 3-h chromium release assay performed in 0.2-ml volumes in U-bottom microplates. Effector cells were titrated in triplicate against 1×10^4 ^{51}chromium-labeled YAC-1 murine lymphoma target cells across the effector to target cells ratios of 100:1, 50:1, 25:1, and 12.5:1. Incubation of target cells with 0.1 N HCl yielded the value for the total release of ^{51}chromium. Spontaneous release was the amount of radioactivity released in medium alone; it averaged 10% of the total amount released in the presence of 1.0 N HCl. The percentage of specific cytotoxicity was calculated using the formula (experimental release − spontaneous release)/(total release − spontaneous release) × 100 (36).

Statistical analysis

To evaluate whether basal levels or responses of plasma epinephrine, norepinephrine, NPY, or corticosterone differed after icv CRH administration, repeated measures analysis of variance (ANOVA) was used. The repeated measures ANOVA tested for differences in the four groups (group effect), change in dependent variables over time (time effect), and group differences in response of dependent variables over time (group × time interaction). For significant group by time interactions that indicated difference in the responses of the four groups to icv CRH, planned comparisons were used to determine differences between each of the groups at individual time points. Group differences in NK activity were tested using a repeated measures ANOVA, in which the effector to target cell ratio across the four dilutions was treated as a within-subjects repeated measure consistent with previously described analyses of NK data (17–19, 24). In comparison, the use of a single effector to target cell ratio does not take into account group differences in the slope of the effector to target cell dilution curve and is not adequate for quantitative comparisons of activities.

Results

To determine the effects of aging on CRH-induced activation of the autonomic nervous system, basal levels and responses of epinephrine, norepinephrine, and NPY after central CRH administration were compared between aged

and young animals. Figures 1-3 illustrate, respectively, differences in epinephrine, norepinephrine, and NPY between the groups.

For epinephrine, basal levels were similar in the aged and young animals (Fig. 1). However, CRH induced an elevation of epinephrine in the aged rats that occurred more rapidly, reached a higher peak value, and was sustained throughout the blood-sampling period compared to responses in the young rats.

In contrast, with similar basal levels of epinephrine in the aged and young rats, basal levels of norepinephrine were significantly ($P < 0.001$) higher in the aged rats than in the young animals (Fig. 2). The responses of plasma norepinephrine in the aged animals after CRH infusion again occurred more rapidly, reached higher peak values, and were sustained throughout the experimental sampling period, whereas the young Fischer rats showed only a modest increase in norepinephrine 15 min after CRH, which returned to baseline at 60 min.

Basal plasma levels of NPY were also elevated in the aged animals compared to those in the young rats, similar to the age-related increase in plasma norepinephrine (Fig. 3). In addition, CRH induced a transient increase in circulating concentrations of NPY in the aged animals, but not in the young rats.

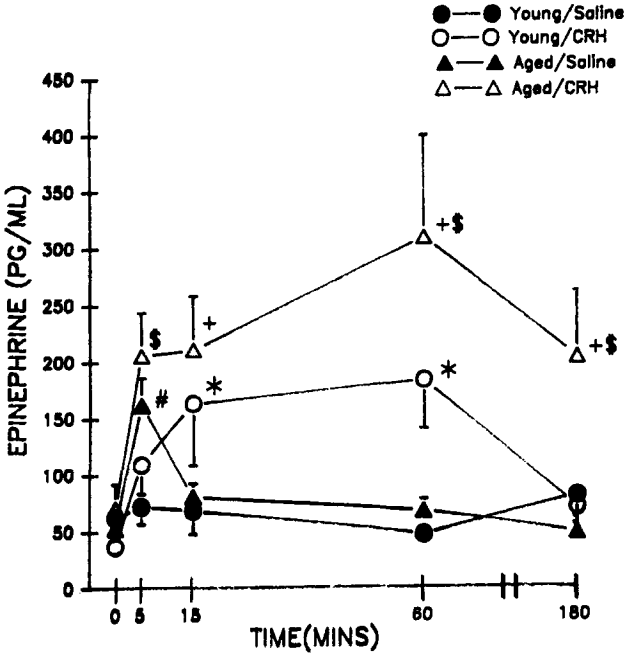

FIG. 1. Effects of age on CRH (200 pmol, icv)-induced elevation in plasma epinephrine concentrations. Results were obtained in 10–12 animals/group and are depicted as the mean ± SEM. A repeated measures ANOVA over time demonstrated a significant group effect ($F = 4.8$; df = 3,39; $P < 0.01$), a significant time effect ($F = 10.6$; df = 4,156; $P < 0.001$), and a significant group by time interaction ($F = 4.6$; df = 12,156; $P < 0.001$). Planned comparisons tested for group differences at the five times of measurement and comparisons that were significantly ($P < 0.05$) different at the individual time points are illustated as follows: *, young/saline *vs.* young/CRH; +, aged/saline *vs.* aged/CRH; #, young/saline *vs.* aged/saline; and $, young/CRH and aged/CRH. If no symbol is illustrated for a group comparison at an individual time point, then the values were similar.

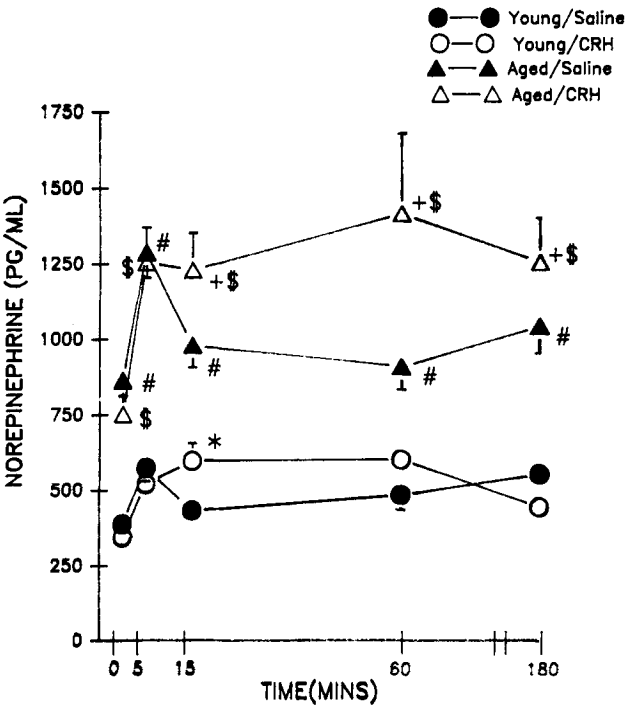

FIG. 2. Effects of age on CRH (200 pmol, icv)-induced elevation in plasma norepinephrine concentrations. Results were obtained in 9–12 animals/group and are depicted as the mean ± SEM. A repeated measures ANOVA over time demonstrated a significant group effect ($F = 28.0$; df = 3,38; $P < 0.001$), a significant time effect ($F = 14.9$; df = 4,152; $P < 0.001$), and a significant group by time interaction ($F = 3.8$; df = 12,1526; $P < 0.001$). Planned comparisons tested for group differences at the five times of measurement and comparisons that were significantly ($P < 0.05$) different at the individual time points are illustated as follows: *, young/saline *vs.* young/CRH; +, aged/saline *vs.* aged/CRH; #, young/saline *vs.* aged/saline; and $, young/CRH and aged/CRH. If no symbol is illustrated for a group comparison at an individual time point, then the values were similar.

Figure 4 illustrates basal levels and corticosterone responses to CRH infusion in aged and young rats. Neither basal levels nor responses of corticosterone to CRH infusion differed between the aged and young rats. CRH induced a similar increase in plasma corticosterone, which was sustained in both age groups of animals throughout the testing interval.

NK activity was significantly ($P < 0.001$) different in the four groups 1 h after icv infusion (Fig. 5). The aged rats had significantly ($P < 0.001$) lower NK activity than the young animals. In addition, icv CRH produced a further significant ($P < 0.01$) reduction of splenic NK cytotoxicity in the aged animals, but did not alter lytic activity in the young animals.

Discussion

The present study investigated differences in the responses of the autonomic nervous system and NK cell activity after central administration of CRH in aged rats compared to young animals. First, an increase in resting sympathetic tone, as measured by elevated basal levels of plasma norepinephrine and NPY, was found in the aged rats. While an increased concentration of plasma norepinephrine has been previously

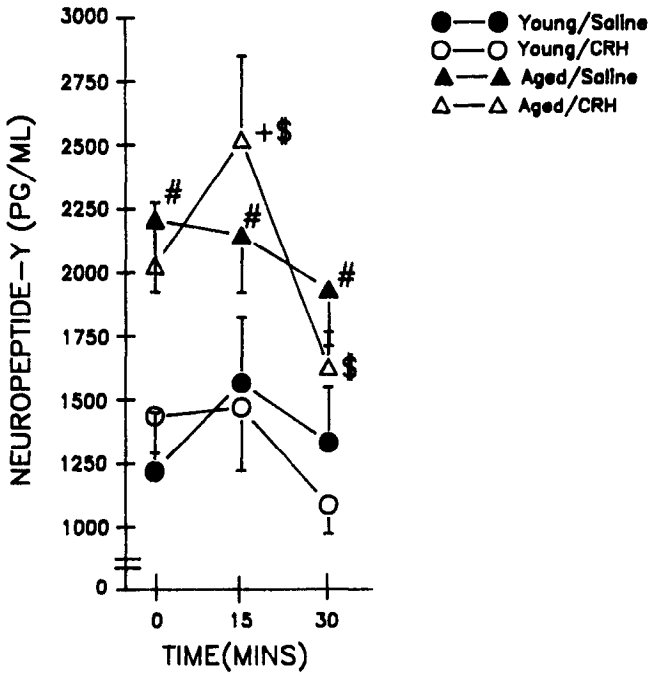

FIG. 3. Effects of age on CRH (200 pmol, icv)-induced elevation in plasma NPY concentrations. Results were obtained in 8–11 animals/group and are depicted as the mean ± SEM. A repeated measures ANOVA over time demonstrated a significant group effect ($F = 4.9$; df = 3,32; $P < 0.01$) and a significant time effect ($F = 6.6$; df = 2,64; $P < 0.01$), but no group by time interaction ($F = 1.3$; df = 6,64; $P < 0.29$). Planned comparisons tested for group differences at the five times of measurement and comparisons that were significantly ($P < 0.05$) different at the individual time points are illustated as follows: *, young/saline *vs.* young/CRH; +, aged/saline *vs.* aged/CRH; #, young/saline *vs.* aged/saline; and $, young/CRH and aged/CRH. If no symbol is illustrated for a group comparison at an individual time point, then the values were similar.

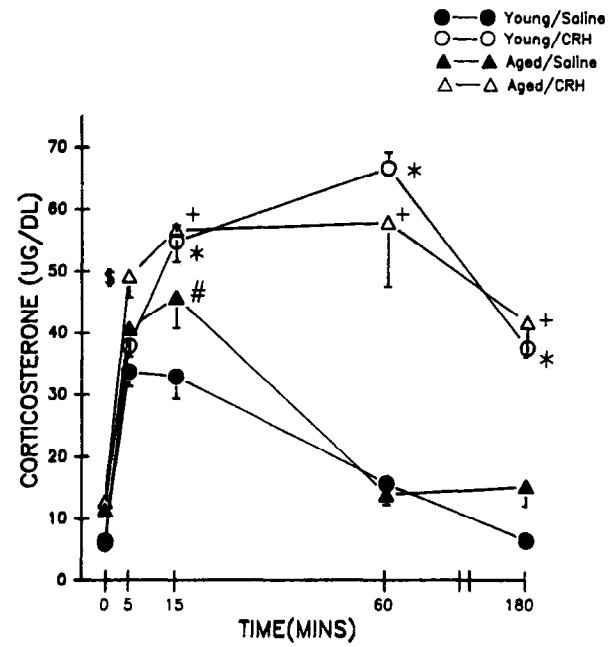

FIG. 4. Effects of age on CRH (200 pmol, icv)-induced elevation in plasma corticosterone concentrations. Results were obtained in 10–12 animals/group and are depicted as the mean ± SEM. A repeated measures ANOVA over time demonstrated a significant group effect ($F = 18.0$; df = 3,39; $P < 0.001$), a significant time effect ($F = 115.5$; df = 4,156; $P < 0.001$), and a significant group by time interaction ($F = 16.4$; df = 12,156; $P < 0.001$). Planned comparisons tested for group differences at the five times of measurement and comparisons that were significantly ($P < 0.05$) different at the individual time points are illustated as follows: *, young/saline *vs.* young/CRH; + aged/saline *vs.* aged/CRH; #, young/saline *vs.* aged/saline; and $, young/CRH and aged/CRH. If no symbol is illustrated for a group comparison at an individual time point, then the values were similar.

reported in some (37), but not all (16), studies of aged rats, measurement of plasma norepinephrine levels alone could reflect a diminished clearance of norepinephrine from the plasma, rather than an increase in sympathetic nervous activity. However, in humans the increase in plasma norepinephrine is due to a greater plasma norepinephrine appearance rate, rather than decreased norepinephrine clearance (38). The present findings that both norepinephrine and NPY are increased in the aged rat provide firm support for an increased level of sympathetic nervous system activity in aging, since norepinephrine and NPY are colocalized and are coreleased during sustained sympathetic nervous stimulation (39–42).

In addition to the age-related difference in basal levels of sympathetic activity, the present data demonstrate an increase in the response of the sympathetic-adrenal medullary system to central CRH in the aged rats. Peak responses of plasma epinephrine were greater in the aged rats than in the young animals, even though basal concentrations were similar. Likewise, in the aged rats, CRH-induced elevations of plasma concentrations of norepinephrine and NPY were greater than responses in the young animals. Finally, the duration of the responses of epinephrine and norepinephrine was prolonged in the aged animals. Age differences in the duration of activation of the sympathetic nervous system are not likely to be due to age-related differences in the central metabolism of CRH, since the magnitude and duration of corticosterone responses were similar in the aged and young animals. Rather, this hypersecretory profile of catecholamines after central CRH in the aged rat is consistent with the study of McCarty (16), in which acute cold stress induced a greater elevation of plasma levels of epinephrine and norepinephrine in aged animals, suggesting an age-related alteration in the regulation of the sympathetic nervous system.

Of unique and considerable interest in the present study is the age-related association between NK cytotoxicity and sympathetic activity, as measured by plasma levels of catecholamines and NPY both at rest and after central administration of CRH. For example, at rest, the aged rats showed increased basal levels of norepinephrine and NPY and a reduction of NK activity. Anatomical studies have revealed an extensive presence of noradrenergic fibers in both primary and secondary lymphoid organs (12, 13), in which noradrenergic neurons innervate both the vasculature and the parenchyma of the spleen and end in synaptic-like contacts with lymphocytes (12). Norepinephrine acts as a neurotransmitter that binds to lymphocyte β-adrenergic receptors and reduces cellular function *in vitro*, as measured by NK activity

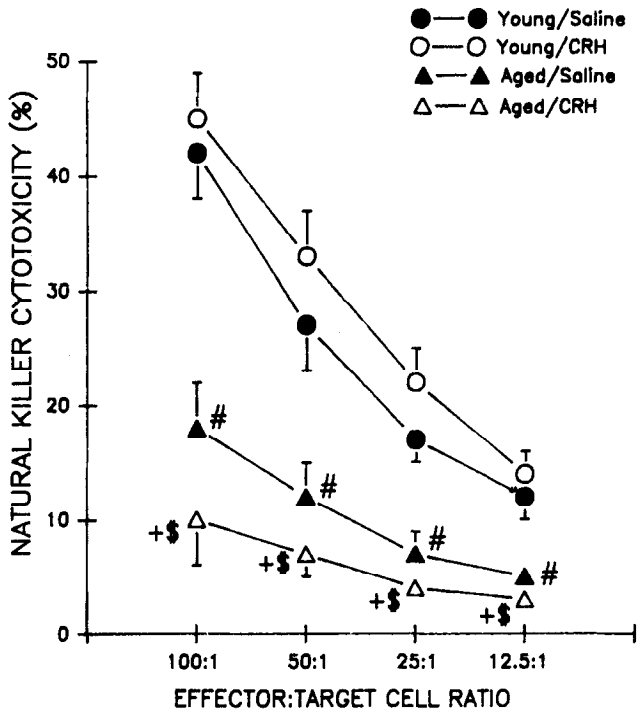

FIG. 5. Effects of age on CRH (200 pmol, icv)-induced suppression of splenic NK cell activity. Results were obtained in 6–10 animals/group and are depicted as the mean ± SEM. A repeated measures ANOVA across the four effector to target cell ratios demonstrated a significant group effect ($F = 12.7$; df = 3,26; $P < 0.001$), a significant ratio effect ($F = 169.9$; df = 3,78; $P < 0.001$), and a significant group by ratio interaction ($F = 14.3$; df = 9,78; $P < 0.001$). Planned comparisons demonstrated significant ($P < 0.05$) between aged/saline and aged/CRH (+), young/saline and aged/saline (#), and young/CRH and aged/CRH ($). Lytic values were similar in the young/saline and young/CRH groups.

(43). Likewise, *in vivo* studies involving young animals have demonstrated that autonomic blockade, chemical sympathectomy, or β-receptor antagonsism abolished the suppression of cellular immunity after the administration of central CRH (17, 44), interleukin-1 (19), or footshock stress (45). In humans, we have also demonstrated that acute release of catecholamines during physical exercise mediates suppression of lymphocyte proliferation via β_2-adrenergic mechanisms (46) and that chronic sustained elevation of sympathetic tone and release of NPY is negatively correlated with NK activity in aged individuals as well as in depressed patients and persons undergoing severe life stress (18).

CRH induced a further decrement of NK activity in aged, but not adult, rats, which might be due to the greater activation of the sympathetic adrenal medullary system, exaggerated release of epinephrine, norepinephrine, and NPY, and dose-dependent suppression of cytotoxicity by these neurotransmitters (47). Alternatively, the lymphocytes of aged rats may have an increased sensitivity to the inhibitory effects of catecholamines, particularly epinephrine. Recently, Ackerman and colleagues (11) and Bellinger *et al.* (48) found an age-related denervation of splenic lymphoid tissue with an associated up-regulation of splenocyte β_2-adrenergic receptors (49). Since epinephrine perferentially binds at β_2-adrenergic receptors and produces a suppression of NK activity at physiological concentrations (10^{-9} M) (47), CRH-induced elevations in the circulating concentration of epinephrine are likely to produce a greater inhibitory effect on the lytic activity of lymphocytes from aged *vs.* young animals. These data are consistent with those of Zalcman *et al.* (49), who found that immune responses of aged mice are more susceptible to increased sympathetic activity after stress. Finally, regulation of β-receptors may be impaired in the aged rat, exacerbating the immunosuppressive effects that follow acute elevations of circulating plasma epinephrine. For example, DeBlasi *et al.* (50) reported that down-regulation of β-receptors after restraint stress is slowed and diminished in aged rats compared to young animals, suggesting that aged rats lose the ability to regulate β-receptor number in response to agonist availability.

These findings of increased sympathetic activity, as measured by circulating concentrations of the sympathetic neurotransmitters norepinephrine and NPY, contrast with anatomical and biochemical data that show sympathetic denervation of the splenic tissue during senescence (11) and a decrease in norepinephrine content in the spleens of aged rats (11, 48). Since inhibition of NK cells *in vitro* requires micromolar concentrations of norepinephrine (43, 47), further studies using such techniques as *in vivo* microdialysis are necessary to quantitate the amount of norepinephrine released within the spleen by CRH and to define further the role of increased sympathetic tone within splenic tissue in the suppression of immune function in aged rats.

The pathophysiological consequences of the abnormal regulation of autonomic outflow and immune function in aged rats has not been investigated in the present study. However, some evidence exists that stress-induced tumor growth after inoculation with virally transformed cells is accelerated in aged rats (51). In addition, NK cells have a demonstrated ability to lyse target cells that have undergone malignant transformation (10, 52). Finally, in experiments in animals, NK cells have been shown to play an important part in immune surveillance against the establishment of primary tumors as well as in controlling the spread of distant metastases (10, 52, 53).

Controversy exists as to whether circulating basal titers of glucocorticoids are elevated in aged rats. The present study found similar basal concentrations and peak corticosterone responses after CRH administration in the aged and young animals and a dissociation between adrenocortical activation and acute suppression of NK activity, consistent with the findings of Chiueh *et al.* (37) and Lorens and colleagues (5). However, an increased release of glucocorticoid after acute stress has been reported, in which responses diverge between the aged and young animals at about 4 h from the onset of the stress (28). An impaired sensitivity to feedback inhibition of the pituitary-adrenal axis (54) may not have been revealed in the present study, which had a time course limited to 3 h.

Previous studies using young Wistar animals, rather than Fischer rats, have found that central CRH (200 pmol) induces at least a 2-fold elevation in circulating concentrations of norepinephrine (21) and about a 50% reduction of splenic NK activity 1 h after infusion (22, 44). In contrast, the present

study using Fischer rats found only a transient elevation in norepinephrine and no change in natural cytotoxicity after CRH treatment. Additional experiments are necessary to determine the CRH dose-response effects on sympathetic measures and immune function in aged and young Fischer animals, thereby evaluating possible strain differences in autonomic sensitivity after CRH treatment.

The central mechanisms that mediate the differential effects of age on CRH-induced elevations in catecholamines and NK activity remain to be elucidated, including an age-associated increase in CRH release *in vivo* (28) and/or augmentation of an ultrashort positive feedback loop of CRH on its own release (55). In addition, the persistent elevation of plasma catecholamines after central CRH administration in aged rats suggests a decreased sensitivity with age to the inhibitory feedback signal of increased sympathetic activity.

In summary, CRH acts within the brain to stimulate the activity of the sympathetic nervous system and reduce splenic NK activity. These findings show an age-related increase in autonomic outflow after CRH treatment. Furthermore, these data are consistent with the hypothesis that age-related changes in central nervous system regulation of sympathetic activity may influence *in vivo* modulation and suppression of natural cytotoxicity in aging.

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