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EFFECTS OF COMBINED TREATMENT WITH IMIPRAMINE AND METYRAPONE ON THE IMMOBILITY TIME, THE ACTIVITY OF HYPOTHALAMO-PITUITARY-ADRENOCORTICAL AXIS AND IMMUNOLOGICAL PARAMETERS IN THE FORCED SWIMMING TEST IN THE RAT

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Major depression is frequently associated with the hyperactivity of the hypothalamic-pituitary-adrenocortical axis, and glucocorticoid synthesis inhibitors have been shown to exert antidepressant action. The aim of the present study was to examine the effect of joint administration of metyrapone (50 mg/kg) and imipramine (5 and/or 10 mg/kg) on immobility time, plasma corticosterone concentration, the weight of spleens and thymuses and the proliferative activity of splenocytes in rats subjected to the forced swimming test - an animal model of depression. Metyrapone alone (50 mg/kg) reduced the immobility time of rats in the forced swimming test and decreased plasma corticosterone level, but did not change immunological parameters. Joint administration of metyrapone and imipramine (5 and 10 mg/kg) produced a more pronounced antidepressant-like effect than either of the drugs given alone. The forced swimming procedure significantly increased the proliferative activity of splenocytes, that parameter being reduced only by co-administration of metyrapone and imipramine. Joint administration of metyrapone and imipramine inhibited to a similar extend the corticosterone level as did treatment with metyrapone alone (about twofold); however, the plasma corticosterone level in animals treated with metyrapone and the higher dose of imipramine did not differ from the concentration of this steroid in control, not-stressed rats. The obtained results indicate that metyrapone potentiates the antidepressant-like activity of imipramine and exerts a beneficial effect on the stress-induced increase in plasma corticosterone concentration and the proliferative activity of splenocytes. These finding suggest that a combination of metyrapone and an antidepressant drug may be useful for the treatment drug-resistant depression and/or depression associated with a high cortisol level.

Key words: *metyrapone, imipramine, forced swimming test, corticosterone, proliferative activity*

INTRODUCTION

Major depression is frequently associated with the hyperactivity of the hypothalamic-pituitary-adrenocortical (HPA) axis. Clinical studies have shown that depressed patients have an increased concentration of cortisol in the plasma and cerebrospinal fluid, an abnormal 24-hour pattern of cortisol and ACTH secretion and elevated levels of corticotropin-releasing hormone in the cerebrospinal fluid (1- 3). A high incidence of depression in Cushing`s syndrome, as well as the antidepressant action of cortisol synthesis inhibitors and antagonists of corticotropin-releasing hormone (CRH) receptors suggest that the hyperactivity of the HPA is involved in the pathogenesis of this disorder (4- 6). A large number of the obtained data indicate that the hyperactivity of the HPA axis in major depression can be induced by a weakened inhibitory feedback mechanism (7, 8). In fact, the synthetic glucocorticoid dexamethasone is less potent in lowering cortisol levels (basal and CRH-induced) in the blood in depressed patients than in healthy subjects (7- 9). Apart from the impairment of the feedback inhibition mechanism, the HPA axis hyperactivity in depression can be evoked by an elevated level of pro-inflammatory cytokines which are known to increase CRH synthesis (10, 11). In fact, major depression is often associated with not only neuroendocrine changes, but also dysregulation of the immune system, mainly with its activation. Chronic immune activation or an inflammatory process seems to be especially evident in treatment-resistant depression (12). A significant increase in the proliferative activity of peripheral blood mononuclear cells, isolated from the blood of treatment-resistant patients (prior to an antidepressant therapy), was observed in our recent study (unpublished data).

The dysfunction of the HPA axis is corrected during a clinically effective therapy with antidepressant drugs, while the persistence of dexamethasone non-suppression is often associated with the risk of relapse or lack of improvement (7, 13). The currently used antidepressant drugs show therapeutic efficacy in about 60-70% of depressive patients only (14). Therefore to the improve therapy, a combination of antidepressants from various pharmacological groups or a combination of an antidepressant drug and a substance that can enhance its effect is used in the clinic. Among agents which are expected to potentate antidepressant efficacy there are inhibitors of glucocorticoid synthesis. In fact, they have shown antidepressant-like properties in some animal models of depression (15, 16). Also clinical studies have demonstrated the antidepressant effects of metyrapone, aminoglutethimide and ketoconazole; however, these drugs are used mainly in drug-resistant depression (5, 17, 18). To data, in clinical studies, glucocorticoid inhibitors or antagonist of glucocorticoid receptors have been administered alone in relatively high doses, so their side-effects are occasionally observed (17). Combinations of a glucocorticoid inhibitor and an antidepressant drug should help decrease their doses and, in consequence, also side-effects. Among glucocorticoid inhibitors, metyrapone (which inhibits the enzyme 11-hydroxylase) has the weakest effect on gonadal hormone

levels (18). We found previously that combined treatment with imipramine and metyrapone produced a more potent antidepressant-like effect than either of the drugs given alone in the forced swimming test in rats (19). Additionally, preliminary studies (with 9 patients) indicated that joint administration of imipramine and metyrapone resulted in clinical improvement. The aim of the present study was to examine the effect of joint administration of metyrapone and imipramine on plasma corticosterone concentration, the weight of spleens and thymuses, the proliferation activity of splenocytes and immobility time in rats subjected to the forced swimming test (FST) - an animal model of depression.

MATERIALS AND METHODS

Animals

Male Wistar rats (200-250 g), purchased from a licensed dealer, were kept under standard animal house conditions (a room temperature of 23° C, a 12/12 h light/dark cycle, the light on at 08:00), with food and water *ad libitum*. The rats were randomly divided into 8 groups and were acclimatized for at least 1 week before the experiment. The experimental protocols were approved by and complied with the guidelines of the Local Ethics Committee at the Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Forced swimming test in rats

The animals were individually subjected to two trials during which they were forced to swim in a cylinder (40 cm high, 18 cm in diameter) filled with water (25° C) up to a hight of 15 cm. There was a 24-hour interval between the first and the second trial. The first trial lasted 15 min, while the second one was carried out for 5 min. The total duration of immobility was measured throughout the second trial (20). Imipramine (5 or 10 mg/kg) and metyrapone (50 mg/kg) were dissolved in distilled water; either at those drugs or distilled water (vehicle) was injected intraperitoneally (i.p.) three times: at 24, 5 and 1 h before the test. Imipramine was also co-injected with metyrapone in the doses and at the times stated above. Control rats were injected with the vehicle. Each group consisted of eight rats.

The assay of corticosterone in blood plasma

Blood for corticosterone level determinations was collected during decapitation 2 h after the last vehicle/drug injection and 1 h after the FST. Control groups were not subjected to the FST. The blood was collected on EDTA and centrifuged at 800 g for 15 min; the supernatant was removed and stored at -20° C until a consequtive analysis. Corticosterone was extracted from the plasma to ethanol and was measured by a radioimmunological method. Ethanol plasma extracts were dried under a nitrogen stream, dissolved in 0.1 ml of 0.05 mM phosphate buffer, pH=7.0, containing a 0.9 % NaCl and a 0.1% gelatin (Sigma Chemical Co), and were incubated with a 0.1 ml solution of 1,2,6,7-[^{3}H]-corticosterone (20000 dpm/sample; Amersham, s.a. 85 Ci/mmol) and with a 0.1 ml solution of a corticosterone antibody (Biogenesis) for 16 h at 4° C. Free and bound corticosterone was separated using dextran-coated charcol. The samples were incubated for 10 min at 4° C with 0.2 ml of a 0.05 % dextran (Dextran T 70, Pharmacia) and a 0.5 % charcol (activated, Sigma) suspension. After centrifugation at 1000 g for 20 min, 0.3 ml of the supernatants were placed in a

scintillator and their radioactivity was measured with a β-counter (Beckmann LS 335). Corticosterone content was calculated using a log-logit transformation. The assay sensitivity was 10 pg/tube. Intra- and interassay coefficients of the variation were lower than 5 and 8%, respectively.

Immunological experiments

The rats were decapitated two hours after the last vehicle/drug injection and 1 h after the FST. Their spleens and thymuses were aseptically dissected and weighed.

Preparation of cell suspensions

The spleens (of six animals from the selected groups) were gently crushed in a glass homogenizer. The spleen cells were resuspended in a RPMI-1640 medium (Sigma), and were centrifuged at 500 x g for 5 min. The cell pellets were resuspended in the same medium supplemented with antibiotics (50 μg/ml of penicillin, 50 μg/ml of streptomycin), a 10% fetal bovine serum and 2 mM of L-glutamine (all the reagents obtained from Sigma).

Proliferative response of splenocytes to mitogen stimulation in vitro

The proliferative response of spleen cells was earlier described by Kubera et al (21). 4 x 10^6 splenocytes per ml were stimulated with 2.5 μg/ml of concanavalin A (Con A). The cells were incubated in 96-well plates at 37 °C at a final volume of 0.2 ml for 72 h. Cell proliferation was determined by adding 10 μl (0.5 μCi) of ^{3}H thymidine per well (ICN Pharmaceuticals; s.a. 6.7 Ci/mmol) 16 h before the end of incubation. The cultures were harvested with an automatic cell harvester (Scatron), and ^{3}H thymidine was estimated using a liquid scintillation counter (Beckman LS 335).

Statistics

Eight animals per group were used for behavioral studies and for the determination of corticosterone level. Then, six animals were randomly chosen from each group for immunological examination. The obtained data were subjected to an analysis of variance (ANOVA), followed by Dunnett's test (behavioral study) or Duncan`s test (corticosterone level and immunological study).

RESULTS

Forced swimming test

Imipramine in a dose of 10 mg/kg, but not 5 mg/kg, and metyrapone (50 mg/kg) reduced the immobility time of rats in the forced swimming test, i.e. they showed antidepressant-like activity. Combined treatment with metyrapone and imipramine (at either dose) produced more potent inhibition of immobility than either drug given alone (*Table I*).

Plasma corticosterone concentration

The forced swimming test significantly (ca. 3-fold) enhanced plasma corticosterone level estimated 1 h after the stress procedure (*Fig. 1*). Administration of imipramine alone had no significant effect on that hormone

Table 1. The effect of metyrapone, given alone or in combination with imipramine, on the immobility time in the forced swimming test in rats

Drugs (mg/kg)	Immobility time (s)
Vehicle	257.0 ± 1.8
MET 50	205.6 ± 5.1 *
IMI 5	249.8 ± 3.8
IMI 10	199.6 ± 6.1 *
IMI 5 + MET 50	169.4 ± 10.3 *#
IMI 10 + MET 50	107.0 ± 3.2 *#

Metyrapone (MET) and imipramine (IMI) were given three times (24, 5 and 1h) before the test. The results represent mean ± SEM; n=8. The data were statistically evaluated by ANOVA, followed by individual comparisons using Dunnett`s test. * p<0.001 vs. vehicle-treated grup, # p<0.001 vs. IMI or MET-treated grup.

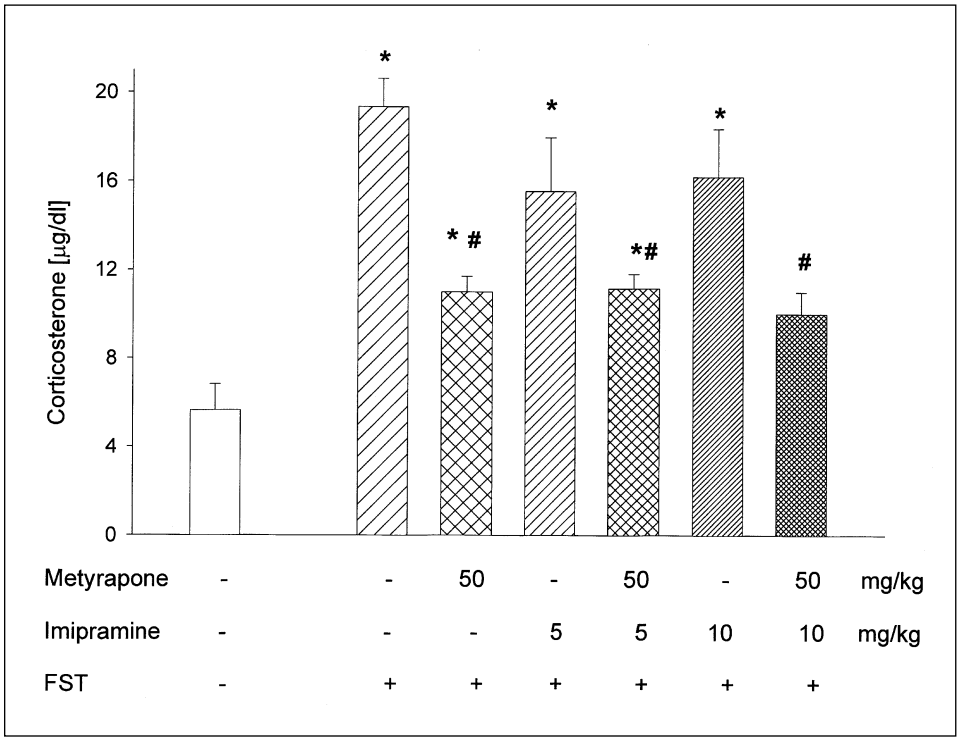

Fig. 1. The effect of exposure to the forced swimming test (FST) and metyrapone and imipramine administration on the plasma corticosterone concentration 1h after the FST. Metyrapone and imipramine were given three times (24, 5 and 1h) before the test. The control group was not subjected to the FST. The results represent mean ± SEM; n=8. The data were statistically evaluated by ANOVA, followed by individual comparisons using Duncan`s test. * p<0.01 vs. vehicle/no-FST group, # p<0.01 vs. vehicle or imipramine, respectively/FST group.

level; however, a tendency towards a decrease was observed. In animals treated with metyrapone alone, as well as in rats injected with metyrapone and imipramine at either dose corticosterone concentration was statistically significantly lower than in the respective groups not treated with metyrapone.

Thymus and spleen weight

There were no significant differences in the weight of thymuses in any of the groups studied (data not shown). The relative spleen weight (the weight of the spleen in mg divided by the body weight in g) was significantly reduced in rats treated with 10 mg/kg imipramine compared with the vehicle-treated/FST group (*Fig. 2*).

The proliferative activity of splenocytes in response to Con A

FST stress significantly increased the proliferative activity of splenocytes in response to Con A stimulation compared to the saline-treated controls. Pre-

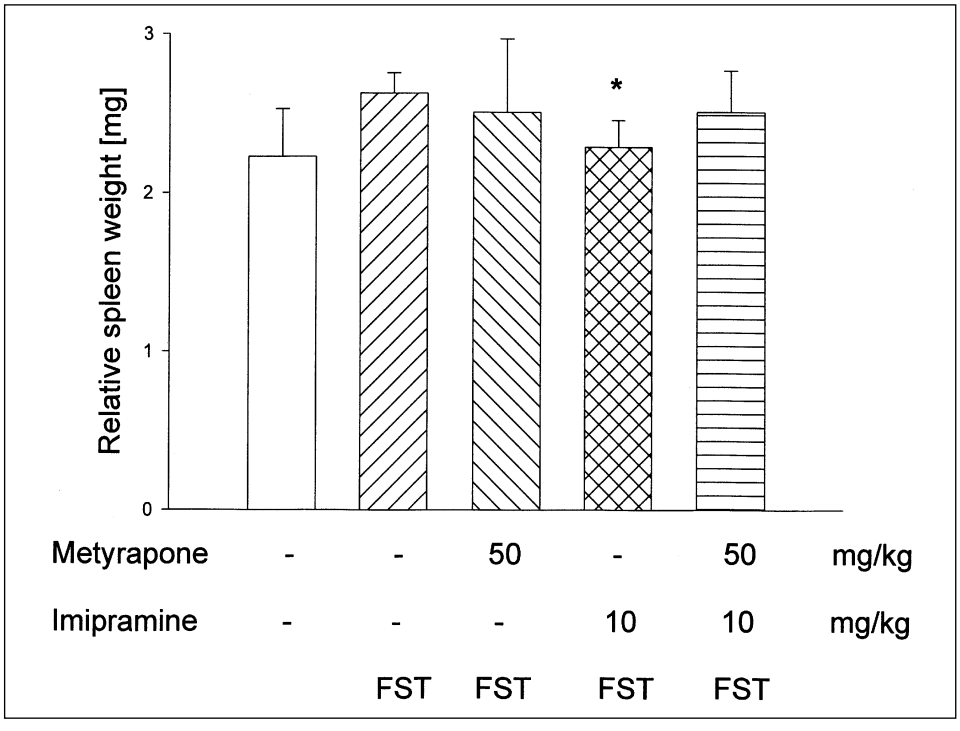

Fig.2. The effect of exposure to the forced swimming test (FST) and metyrapone and/or imipramine administration on the relative spleen weight. Metyrapone or imipramine were given three times (24, 5 and 1h) before the test. The control group was not subjected to the FST. The results are presented as mean ± SD; n=8. The data were statistically evaluated by ANOVA, followed by individual comparisons using Duncan`s test. * $p<0.01$ vs. vehicle/FST group.

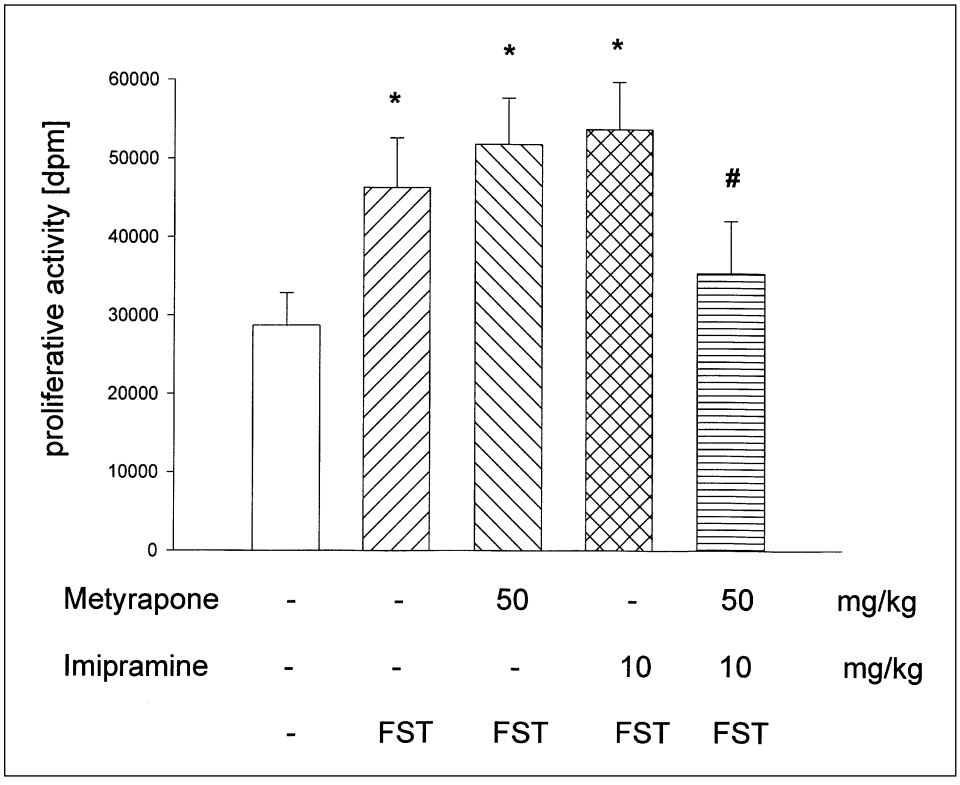

Fig.3. The effect of exposure to the forced swimming test (FST) and metyrapone and/or imipramine administration on the proliferative activity of splenocytes. Metyrapone and imipramine were given three times (24, 5 and 1h) before the test. The control group was not subjected to the FST. The results are presented as mean ± SD; n=6. The data were statistically evaluated by ANOVA, followed by individual comparisons using Duncan`s test. * $p<0.01$ vs. vehicle/no-FST group; # $p<0.01$ vs. imipramine/FST group.

treatment of the stressed rats with metyrapone, imipramine or a combination of metyrapone and imipramine had no effect on splenocyte proliferation compared to saline-treated, stressed animals; nevertheless, a significant decrease in splenocyte proliferation was observed in imipramine and metyrapone groups compared to imipramine-treated, stressed animals (*Fig. 3*).

DISSCUSION

Our results show that metyrapone, a glucocorticoid synthesis inhibitor, reduces immobility time in rats subjected to the forced swimming procedure. These data are in line with our (19) and other (15, 16, 22) earlier findings, which indicated antidepressant-like properties of metyrapone in various animal models

of depression (the forced swimming test, olfactory bulbectomy and a restraint stress paradigm). Metyrapone reduces immobility time with efficacy comparable to that of tricyclic antidepressants, e.g. imipramine (our previous and present data; 19) or desipramine (16). Joint administration of metyrapone and imipramine shortens immobility time more potently than does either of the drugs given alone. The reduction of immobility time evoked by metyrapone may be a consequence of inhibition of the stress-induced corticosterone secretion. Like other inescapable stress paradigms, the forced swimming test increased corticosterone level in the blood. In the model used in the present study (in which the animals were subjected to a pre-test 1 day before the experiment) blood corticosterone concentration 1 hour after the forced swimming test was about threefold higher than in control, non-stressed animals. The increases in the corticosterone level depended on the kind and duration of stress and were usually the highest shortly after the application of single stress. It was found that immediately after 20 min of acute forced swimming, corticosterone plasma level increased about fortyfold (23). In line with our results, Baez and Volosin (15) showed that the forced swimming test procedure (the same as that used in our study) evoked a ca.1.5-2-fold increase in corticosterone concentration 1 h after the test. We found that systemic administration of metyrapone (three times, at 24, 5 and 1 h before the test) not only reduced the immobility time in rats subjected to the forced swimming procedure, but also suppressed the stress-induced rises in blood corticosterone level by ca. 50 %. The effect of metyrapone on immobility time was the subject of several studies, but its action on plasma corticosterone level in rats subjected to the forced swimming was previously determined by Baez and Volosin (15) only, who administered metyrapone once in the higher dose (150 mg/kg). Despite the differences in metyrapone administration, in the present study and in the paper cited above that inhibitor decreased ca. twofold plasma corticosterone level 1 hour after Porsolt`s test. Moreover, Baez and Volosin (15) demonstrated that the effect of metyrapone on immobility time and corticosterone level was reversed by corticosterone supplementation in forced swimming test, which proved that the antidepressant-like action of metyrapone stemmed from its effect on corticosterone level. Metyrapone, an 11-β-hydroxylase inhibitor, blocks the synthesis and the subsequent release of corticosterone (in rats) or cortisol (in humans) into the bloodstream. It was found that metyrapone suppressed plasma corticosterone concentration in stressed animals, but did not change the basal level of that steroid (24). This fact seems to be of great importance, since in the light of the corticosteroid receptor hypothesis of depression, attenuation of elevated, but not basal, glucocorticoid levels is beneficial to the treatment of depression. Glucocorticoids act via two distinct receptors: the high-affinity mineralocorticoid receptor (MR), and the low-affinity glucocorticoid receptor (GR). MRs are primarily involved in the regulation of basal glucocorticoids level and function, while GRs which are activated by high concentrations of steroids are more important to restoring homeostasis after stress (25). The blockade of GR

produced an antidepressant effect in experimental and clinical studies; however, due to the lack of a specific GR antagonist and because of the adverse effects of mifepristone, this treatment strategy has not been studied in detail so far (5, 9, 17). On the other hand, the MR antagonist spironolacton impairs the response of antidepressant drugs (9). Therefore the inhibitory effect of metyrapone on the stress-induced corticosterone concentration only seems to be sufficient for the treatment of depresion. In comparison with other glucocorticoid synthesis inhibitors such as, aminoglutethimide and ketoconazole, metyrapone acts more selectively on glucocorticoid synthesis and weakly affects gonadal steroid secretion (5, 18). However, by inhibiting 11β-hydroxylase metyrapone causes an increase in the concentration of corticosteroids 11-deoxy precursors including the positive modulator of $GABA_A$ receptor - tetrahydrodeoxycorticosterone (18). Hence, the antidepressant effect of metyrapone may be connected not only with the reduction of plasma corticosterone concentration, but also with the action of bioactive corticosterone precursors on GABA-ergic transmission (26).

In the present study imipramine used in either dose tended to decrease corticosterone level, but its effect was not statistically significant. In contrast to acute effects, long-term administration of antidepressants has been found to decrease ACTH and corticosterone levels in the blood, and CRH concentration in the hypothalamus (13, 27). All the same, the effect of antidepressant drugs on HPA axis activity depends on the time of their administration, the kind, time and duration of stress, as well as on the time of blood collection. Regarding the forced swimming test, subchronic administration of antidepressant drugs decreased the stress-induced corticosterone concentration in some experiments, but in others no effect or only a tendency towards it was observed (28-31). The above data indicate that threefold administration of imipramine is not enough to obtain a significant decrease in corticosterone level. Joint administration of metyrapone and imipramine inhibited corticosterone level to a similar extend as did metyrapone alone (ca. twofold); however, plasma corticosterone level in animals treated with metyrapone and the higher dose of imipramine did not differ from the concentration of this steroid in control, non-stressed rats. To our knowledge, the effect of any antidepressant drug, administered jointly with an inhibitor of glucocorticoid synthesis, on corticosterone level has not been studied so far. The lack of a stronger inhibitory effect of metyrapone administered jointly with imipramine (compared to that of metyrapone alone) on corticosterone concentration is opposed to the action of these drugs on immobility time. As was shown in our previous study, dopamine $D_{2/3}$ and 5-HT_{1A} receptors were involved in the synergistic action of imipramine and metyrapone on immobility time (19). The lack of a synergistic action of metyrapone and imipramine on corticosterone level may be connected with the fact that antidepressant drugs are believed to inhibit the HPA axis activity be enhancing the glucocorticoid feedback mechanism, a mechanism which is inhibited by metyrapone (8, 32).

Antidepressant drugs are known to affect not only the levels of neurotransmitters and hormones, but also some immunological parameters which are disturbed in depression (11, 33, 34). The main finding of immunological studies is that the forced swimming test causes a significant increase in the proliferative activity of T cells, and that joint application of the suboptimal doses of metyrapone and imipramine significantly reduces this activity in comparison with imipramine alone. These results suggest that forced swimming stress may initiate accumulation and/or activation of spleen T lymphocytes with great proliferative ability in response to T cell mitogens. This is in line with some other data which have shown an immunostimulatory effect of acute stress on the proliferative activity of splenocytes in response to T cell mitogens (33, 35, 36). In contrast to splenocytes, acute stress reduces the proliferative activity of peripheral blood lymphocytes in humans and animals (33, 37, 38). It has been found that the spleen of acutely stressed animals contains relatively more T cells and a more rapidly proliferating subset of T cells: naive $CD4^+$ cells (33). On the other hand, a decrease in the number of T cells is observed in the blood of stressed animals. Changes in the redistribution of T lymphocytes are mediated by stress-induced increases in plasma corticosterone and by alterations in either the expression or the affinity of surface adhesion molecules on leukocytes and/or endothelial cells (39, 40).

Although glucocorticoids are involved in the regulation of proliferative activity, the obtained results do not confirm their participation in metyrapone and imipramine action on the proliferation of splenocytes. Metyrapone decreases corticosterone level, but has no effect on proliferation. Moreover, combined treatment with metyrapone and imipramine inhibits the proliferation of splenocytes, but decreases corticosterone level to a similar extend as does metyrapone alone. The lack of correlation between corticosterone level and the proliferative activity of splenocytes suggestes that the synergistic action of metyrapone and imipramine is probably connected with changes in the neurotransmitter level and/or their receptors. Of possible mediators, $GABA_A$ receptors may be involved, since both tricyclic antidepressant drugs and metyrapone are known to increase the levels of tetrahydroallopregnanolone and tetrahydroallodeoxycorticosterone, respectively, i.e., neuroactive steroids, $GABA_A$ agonists and on the other hand $GABA_A$ agonists potently decrease T cell proliferation (18, 41, 42).

In the present study we observed a reduction in the relative spleen weight in imipramine-treated, stressed animals. A similar effect was demonstrated by Connor et al. (28) after administration of desipramine (an imipramine metabolite) to rats subjected to the forced swimming test, this change being correlated with the increased number of erythrocytes in the blood. It is concluded that the reduction in spleen weight is probably due to the mobilization of erythrocytes within the spleen and their redistribution from the spleen to the peripheral blood (28).

In conclusion, subchronic pretreatment with metyrapone and imipramine induces more potent antidepressive activity in the forced swimming model of depression than does either of the drugs given alone. Co-administration of these drugs also evokes beneficial change in the immunological system, i.e. inhibition of the stress-induced proliferative activity of splenocytes. The latter treatment reduces the stress-induced corticosterone concentration, however to a similar extend as does metyrapone alone. These findings may help to choose a proper drug combination for the treatment of drug-resistant and/or high cortisol level-related depression.

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