


The changes in, and relationship between, plasma nitric oxide and corticotropin-releasing hormone in patients with major depressive disorder

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Summary

There is strong evidence of roles of the hypothalamus-pituitary-adrenal axis and nitric oxide (NO) synthase-NO system in depression, but the relationship between them is unknown. The aim of this study, therefore, was to elucidate whether there is any correlation between NO and corticotropin-releasing hormone (CRH) in major depressive disorder (MDD) patients. In 16 outpatients with MDD and 18 healthy controls, the plasma amino acids citrulline (Cit) and arginine (Arg) were determined by high-performance liquid chromatography, and CRH levels were measured by radioimmunoassay. The Cit/Arg ratio was calculated as an index of NO synthesis. Correlations between NO and CRH were examined with the Spearman test. Before treatment, no significant correlation was observed between the plasma NO level and CRH levels in MDD patients. The plasma NO levels were significantly higher in MDD patients. A significant correlation was found between NO levels and Hamilton Depression Rating Scale (HAMD) scores in MDD patients. The plasma CRH levels were significantly higher in MDD patients than in controls. After monotherapy for 2 months, the NO levels had dramatically declined but were also higher than those in the controls. This study is the first report of the absence of a significant correlation between plasma NO and CRH levels, although both levels are elevated in MDD patients. Furthermore, the strong links between the plasma NO levels and the HAMD scores, as well as the increased NO reduction after remission, suggest that NO plays a key role in depression and may be an indicator of therapeutic success.

KEYWORDS

arginine, citrulline, corticotropin-releasing hormone, major depressive disorder, nitric oxide

1 | INTRODUCTION

Major depressive disorder (MDD) is a prevalent, often recurrent debilitating illness accompanied by severe functional impairment, high mortality, and a heavy health care burden.¹ Numerous hypotheses and

pathways have been suggested to be involved in MDD, but the underlying mechanism remains unclear.

Nitric oxide (NO) is a highly diffusible and reactive molecule synthesized and released with the assistance of nitric oxide synthases (NOSs), which convert arginine into citrulline, producing NO in the process.² NO has been shown to modulate the functions of different neurotransmitters, including norepinephrine, serotonin,

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glutamate and dopamine, and thus plays an important role in the neurobiology of major depression.³ Altered NO levels have been found in depression, not only in different brain regions^{4,5} and cerebrospinal fluid (CSF) but also in blood^{6,7} and exhaled gas.⁸ However, there are many inconsistent results in previous studies.⁹⁻¹² Our laboratory's previous data have also revealed increased plasma NO levels in patients with first-episode melancholic MDD.¹³ Much other work has been done to elucidate the contribution of NO to the pathophysiology of depression. However, there is disagreement regarding its specific function in depression, as has been reviewed by Dhir and Kulkarni.³

Previous animal experiments have indicated the co-localization of NOS1 with corticotropin-releasing hormone (CRH) in the hypothalamus paraventricular nucleus (PVN),¹⁴ and NO modulates the release of CRH.¹⁵ The hypothalamo-pituitary-adrenal (HPA) axis is the key regulating system for stress responses.¹⁶ Activity of the CRH neurons in the PVN forms the basis of HPA-axis activity.¹⁷ Several findings have suggested a central role of CRH in the neurobiology of depression. However, to date, there are inconsistent findings concerning the CRH levels (whether central or peripheral) in clinical depression patients. Decreased,^{18,19} increased,²⁰ or unchanged²¹ CRH levels have been reported in depression. A series of studies have reported a decreased number of nNOS neurons in the PVN in patients with depression²² but increased numbers of CRH neurons²³ and higher CRH-mRNA levels in the PVN in depressed patients.²⁴ Hence, the hypothesis that the NOS-NO system modulates HPA-axis function has been proposed in the past decade. Unfortunately, those data have come from various laboratories using different methods. Most conclusions have been derived from postmortem human brain slices or animal studies, and no studies of the relationship between NO and CRH have been conducted on special subtypes of depression in clinical patients. In fact, our previous data have yielded different results, showing no co-localization of NOS1 and CRH in the rat PVN;²⁵ moreover, no significant correlations were found between plasma NO and cortisol levels in either the control or the MDD group, and no significant correlations were found between plasma NO and corticosterone levels in the animal control group or in the CUS group.²⁵

In the present study, we aimed to further elucidate the relationship between plasma NO and plasma CRH in clinical MDD patients. To do so, we analyzed the changes in plasma CRH levels, NO levels and NO in pre- and post-treatment MDD patients.

2 | RESULTS

No significant differences were found in age, sex, race, years of education, body mass index (BMI) and Hamilton Depression Rating Scale (HAMD) scores between the MDD group and the control group (Table 1). Moreover, these demographic and clinical characteristics were similar between MDD and control patients in both male and female subgroups. After the first blood collection, all patients were treated with the selective serotonin reuptake inhibitor (SSRI) escitalopram at 10-20 mg/day. Thirteen patients had monotherapy, and another three patients underwent combination therapy with alprazolam (0.4-0.8 mg/day) because of insomnia and anxiety. Visits were made once per 2 weeks until 2 months after the first visit. The patients voluntarily joined the follow-up study. At the last visit, the patients' symptoms and HAMD scores were evaluated again, together with their blood samples being taken. Five patients dropped out for various reasons (1 for severe insomnia, 2 for nausea, and 2 for restlessness).

The total MDD group showed a significant increase in NO content compared with that in the healthy control group (median 1.45 vs 0.96, $Z = -4.28$, $P = .000$, Figure 1). Both male and female subgroup analyses revealed that MDD patients had a significantly higher plasma NO content than did healthy participants (males: median 1.36 vs 1.11, $Z = -3.00$, $P = .003$; females: median 1.52 vs 0.95, $Z = -2.94$, $P = .003$). A strong association was found between the plasma NO levels and HAMD scores in the MDD group ($\rho = 0.63$, $P = .008$; Figure 2). After treatment, the NO level markedly decreased compared with pretreatment levels (median 1.33 vs 1.39, $Z = -2.05$, $P = .041$; Figure 3).

The plasma CRH level also showed a significant increase in the total MDD group compared with the control group (median 19.48 pg/mL vs 10.55 pg/mL, $Z = -3.20$, $P = .001$, Figure 4). The change in CRH in female patients was more pronounced than that in male patients (male: median 18.08 pg/mL vs 11.64 pg/mL, $Z = -1.77$, $P = .077$; female: median 20.45 pg/mL vs 11.24 pg/mL, $Z = -2.37$, $P = .018$).

No significant correlation was observed between plasma NO levels and CRH levels ($r = .05$, $P = .91$, Figure 5).

3 | DISCUSSION

We found no significant correlation between plasma NO levels and CRH levels, although they both were significantly increased in the

TABLE 1 Sociodemographic and clinical profiles

Variables	MDD (n = 16)	CONT (n = 18)	P value ^a
Age/years	50.63 ± 2.975	46.17 ± 2.801	.2833
Gender (male/female)	7/9	8/10	.9118
Race (Han%)	100%	100%	1
Education/years	10.69 ± 0.9207	10.56 ± 0.7930	.9137
Body mass index	22.81 ± 0.5777	22.46 ± 0.5131	.6460
HAMD	30.50 ± 2.564	5.889 ± 0.6151	<.0001

^aSignificance at $P < .05$.

MDD, major depressive disorder; CONT, Control.

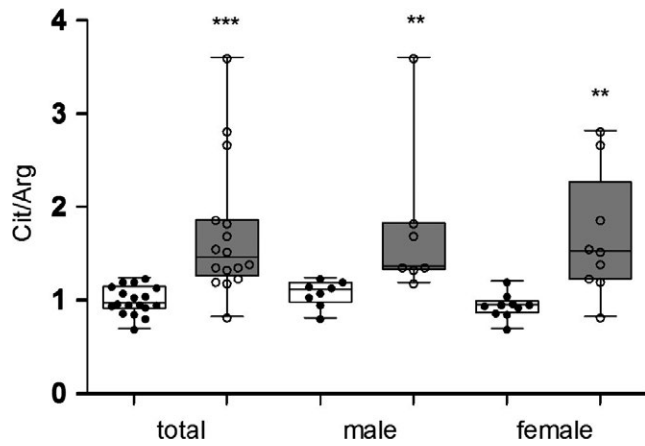


FIGURE 1 Plasma concentrations of nitric oxide (citruilline/arginine ratio) in (□) major depressive disorder (MDD) and (■) healthy controls. Both MDD patients and healthy participants were divided into male and female subgroups. In the total analysis, MDD patients showed a significant increase in the plasma NO (Cit/Arg ratio) content compared with that in the healthy control group. Both male and female subgroup analyses showed that MDD patients had significantly higher plasma NO content than that in healthy participants. The data are shown as the median, 25th-75th percentiles, and the range. Cit, citruilline; Arg, arginine; MDD, major depressive disorder. ** $P < .01$, *** $P < .001$

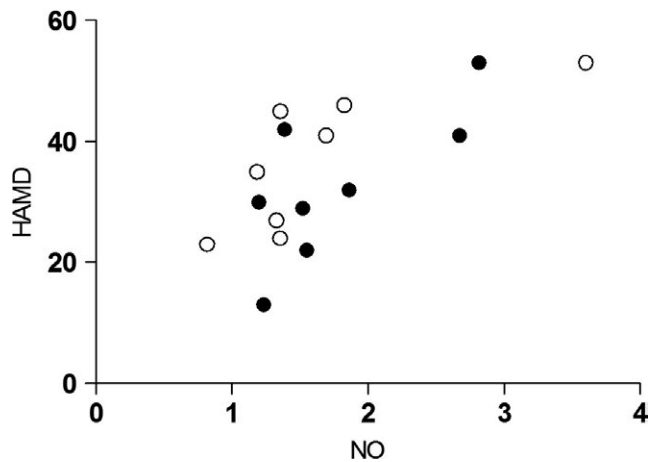


FIGURE 2 Correlation between plasma nitric oxide levels and Hamilton Depression Scale scores in major depressive disorder. A strong association between plasma NO levels and HAMD scores was observed in the MDD group. NO, nitric oxide; HAMD, Hamilton Depression Scale. (○) Male; (●) Female

MDD group compared with the control group. There was a strong association between plasma NO levels and HAMD scores before treatment.

Herein, we provide the first report of the lack of a significant correlation between plasma NO and CRH levels, in accordance with our previous data showing no co-localization for NOS1 and CRH in the CUS rat PVN; no significant correlations were found between plasma NO and corticosterone levels either in the CUS rat model or in the control group, and there was no significant correlation between plasma

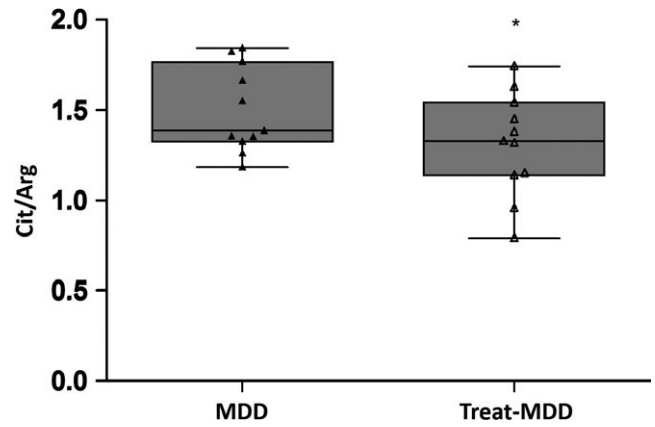


FIGURE 3 Change in the plasma concentrations of nitric oxide (citruilline/arginine ratio) between pre- and post- treatment in major depressive disorder. After treatment, the NO levels markedly decreased compared with pretreatment levels but were still higher than those in the control group. Cit, citruilline; Arg, arginine; MDD, major depressive disorder; treat-MDD, post-treatment in major depressive disorder. * $P < .05$, ** $P < .01$, *** $P < .001$

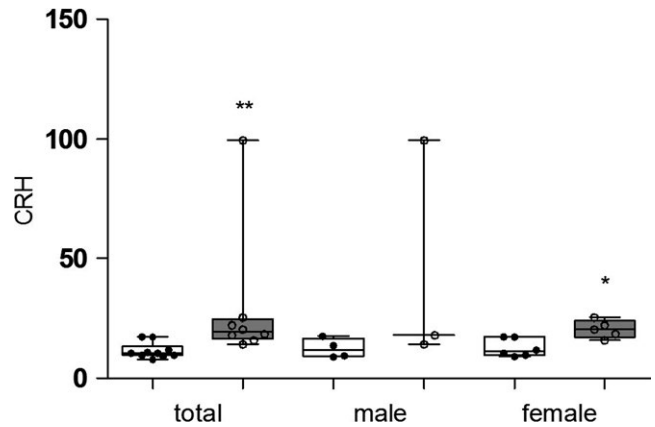


FIGURE 4 Plasma concentrations of corticotropin-releasing hormone in (□) major depressive subjects (MDD) and (■) healthy controls. The plasma CRH level also showed a significant increase in the MDD group compared with the control group. The changes in female patients were more pronounced than those in male patients. CRH, corticotropin-releasing hormone; MDD, major depressive disorder. * $P < .05$, ** $P < .01$

NO and cortisol levels in MDD patients.²⁵ Our results indicated that the NOS-NO system and HPA axis may function independently, contrary to some previous reports.^{14,15,22-24} However, most of the previous data have come from various laboratories using different methods. Furthermore, most of the conclusions came not from clinical patients but from postmortem human brain slices or animal studies, and most of those studies did not focus on special subtypes of depression.

In the present study, the levels of NO were also significantly higher in the MDD patients and declined after antidepressant treatment. In this study, the ratio of the amino acids citruilline and arginine (Cit-Arg ratio) was calculated as an index of NO synthesis. This method has been reported to be sufficiently accurate and reproducible,⁵ and it can be used as an effective index to reflect the NOS-NO system activity.²⁶⁻²⁸

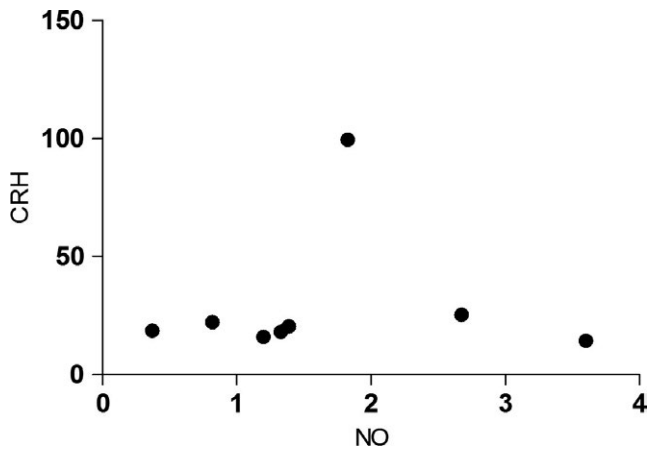


FIGURE 5 Correlation between plasma nitric oxide levels and corticotropin-releasing hormone levels in patients with major depressive disorder. No significant correlation was observed between the plasma NO levels and CRH level. CRH, corticotropin-releasing hormone; NO, nitric oxide

Furthermore, we selected a special subtype of depression (MDD) for study, using the same SSRI monotherapy. In addition, we collected blood samples before treatment. Our result was consistent with those from other laboratories.^{7,29} Our published data revealed increased plasma NO levels both in a male rat model of chronic unpredictable stress²⁵ and in first-episode MDD patients.¹³ Plasma levels of NO metabolites, i.e., nitrite and nitrate, which reflect plasma NO concentrations, have also been reported to increase depression.^{6,30} Of course, there are still some inconsistent findings in previous studies,⁹⁻¹² possibly because of different methods, different subtypes of depression or the different treatments the patients received, and multiple depression comorbidities. Therefore, in this study, we excluded the above factors.

A strong association between plasma NO levels and HAMD scores was also revealed, thus indicating that the NO alterations were consistent with the severity of depressive symptoms. After antidepressant treatment, the concentrations of NO declined, as we have previously reported.¹³ Therefore, the plasma NO level may be a monitor of depressive systems or may forecast the outcome of anti-depression treatment. Interestingly, multiple antidepressants have been reported to change the NO levels in an animal's body,^{3,31,32} and clinical studies have also confirmed the NO modulatory activity of various antidepressants, particularly those belonging to the SSRI class.³ Hence, it is estimated that future antidepressants that may act partly on the NO signaling pathway might be helpful for the treatment of drug-resistant depression.

Our results indicated that the plasma CRH levels increased in MDD patients, in agreement with previous clinical results^{33,34} and postmortem human brain studies,^{23,24,35} thus revealing that hyperactivity of hypothalamic CRH neurons is involved in the progression of depression. It has also been shown that administration of CRH-R1 antagonists has clear antidepressant-like effects in depression.^{36,37} We observed a more significantly increased CRH level in female patients than in males, thus revealing a closer relationship between the HPA axis and female depression.

Several limitations should be noted in the present study. First, we collected only blood samples and not CSF samples of the patients, for

ethical reasons. Some researchers have proposed that the plasma levels of these neuroactive amino acids might, to a certain degree, reflect their brain levels.⁴ Moreover, some researchers have interpreted this finding as evidence that the plasma CRH measured had a hypothalamic origin.^{33,38} Second, our results came from a sample that was relatively small because we had to strictly control many factors. For example, the MDD patients had decreased appetites and decreased food intake, thus leading to a decreased metabolism and decreased synthesis of these amino acids. Consequently, we limited the BMI range in the present study.

In conclusion, this is the first report of a lack of a significant correlation between increased plasma NO and CRH levels, thus indicating that these two systems may function independently. Furthermore, the strong links between the plasma NO levels and the HAMD scores suggested that NO might be correlated with the severity of depression. The decreased NO levels after remission suggested that NO may be an indicator of therapeutic success and is useful for monitoring during therapy in MDD patients.

4 | METHODS

4.1 | Study design

This was a retrospective case-control study that included three parts: The first part was to analyze the plasma NO levels in MDD patients and the changes in NO levels in pre- and post-treatment patients. The second part was to evaluate the plasma CRH levels in MDD patients. The third part was to elucidate the relationship between NO and CRH in MDD patients. All subjects were evaluated through standard physical examinations, routine clinical laboratory tests and psychological assessments (including HAMD scores) when recruited. They had a follow-up once every 2 weeks; after 2 months of SSRI treatment, at the last visit, the patients' symptoms and HAMD scores were evaluated again, together with their blood samples being taken. The investigation was conducted in accordance with the latest version of the Declaration of Helsinki. All subjects signed informed consent forms, and the study was approved by the Medical Ethics Committee of the Fourth Affiliated Hospital of Zhejiang University School of Medicine.

4.2 | Subjects

Sixteen Chinese Han untreated outpatients with MDD (7 males and 9 females, mean age of 47 years) and 18 age- and sex-matched control subjects (8 males and 10 females, mean age of 45 years) were recruited to the study by the Department of Psychiatry of the Fourth Affiliated Hospital of Zhejiang University School of Medicine from Oct 2014 to Oct 2016. They were diagnosed with MDD according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) by qualified psychiatrists. The Mini International Neuropsychiatric Interview (Chinese modified version) was used to confirm the DSM-V diagnosis. The 24-item Hamilton Depression Scale (HAMD) was used to rate the severity of depression.

Exclusion criteria were pregnancy; a serious suicide attempt; current use of another medication that could interact with an SSRI;

current substance or alcohol abuse disorders; previous attendance at or completion of one of the study treatments; a primary diagnosis of schizophrenia, bipolar disorder, eating disorders or other psychiatric diagnoses, involving Axis I and II disorders; other physical illnesses (diabetes, endocrine disorders, hepatitis, cancer, or chronic infections); or an abnormal body mass index ($BMI \leq 18$ or $BMI \geq 25$).

4.3 | Blood sample collection and management

Fasting venous blood samples were collected into anticoagulated tubes (containing EDTA) in the morning (08.00 hours). After being centrifuged (4°C , 12 000 g) for 15 minutes, the plasma samples were divided into aliquots and immediately stored at -80°C in the laboratory for further measurement of NO and CRH levels.

4.4 | High-performance liquid chromatography (HPLC) with fluorescence detection (FLD) for plasma amino acid analysis

The protocol was the same as that previously published.¹³ The plasma samples were deproteinized with acetonitrile (1:1 v/v), lyophilized and diluted with 60% (v/v) methanol before analysis. The plasma amino acid levels were determined by HPLC, which included pre-column derivatization with o-phthalaldehyde (Cat: O120; Pickering Laboratories, Inc., Mountain View, CA, USA) and 3-mercaptopropionic acid (MPA, CAS: 107-96-0, Acros Organics, Somerville, NJ, USA), reverse phase separation with an Agilent 1100 series HPLC system and FLD (Agilent Technologies, Santa Clara, CA, USA). Two mobile phases were used in the detection. The linearity of the detector response to standards was in the range of 0.9–158.2 $\mu\text{mol/L}$ (Cit) and 1.8–178.0 $\mu\text{mol/L}$ (Arg). The intraday relative standard deviations for the peak area were as follows: Cit, 0.97% and Arg, 0.77%, respectively. The interday relative standard deviations for the peak area were as follows: Cit, 4.29% and Arg, 3.18%, respectively.

4.5 | Radioimmunoassay for CRH

Plasma samples were extracted and concentrated to 1 ml and analyzed using a competitive RIA kit (Phoenix Pharmaceutical Company, Harbor Boulevard, Belmont, CA, USA). Anti-CRH serum can elicit a 100% cross reaction with CRH (1–41), but no cross response with prepro-CRH (125–151), cortisol, adrenocorticotrophic hormone, vasopressin and brain natriuretic peptide Acros Organics 45. The limit of detection (LOD) was 10 pg/mL. The 50% binding intercept was 145 pg/mL. The CRH recovery from plasma was 62%, and the intra- and inter- batch variation coefficient were 6.7% and 10.8%, respectively.

4.6 | Statistical analysis

The results of the measurements are presented as the median (quartile). The comparisons between the groups were conducted on the basis of nonparametric statistics, because the small sample size did not provide enough data to discriminate between Gaussian and non-Gaussian distributions, thereby increasing the risk of an incorrect P

value if running parametric tests. A Mann-Whitney U test was used to measure the differences between the two groups. The comparison between two related samples was analyzed using the Wilcoxon test. Correlations between binary variables were examined with the Spearman test. Pearson correlation analysis was used to assess the correlation between continuous variables. SPSS version 11.5 software was used for the statistical analyses. Differences were considered significant if the two-sided P value was .05 or less.

DISCLOSURE

All authors declare no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

Author Yunrong Lu designed the study and wrote the protocol. Authors Yan Zhang, Yingbo Rao and Yu Zhang performed the sample collection, HPLC and RIA analysis. Authors Xi Chen and Han-fen Lou conducted the psychological tests. Authors Haiyan Xie and Ping Fang were responsible for the patient recruitment and follow-up. Author Li-wei Hu undertook the statistical analysis, and author Yunrong Lu wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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