Psychiatry Research 209 (2013) 447–452

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Increase in nitric oxide levels and mitochondrial membrane potential in platelets of untreated patients with major depression

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ARTICLE INFO

Article history:
Received 4 April 2012
Received in revised form
26 September 2012
Accepted 22 December 2012

Keywords:
Major depressive disorder
Mitochondria
Platelets
Platelet mitochondrial membrane potential
Nitric oxide synthesis

ABSTRACT

Alterations in platelet activity have been associated with the onset of major depressive disorder (MDD) and with ischemic cardiovascular events through mechanisms that remain unknown. The present study evaluated nitric oxide (NO) levels, mitochondrial membrane potential (PMMP), and P-selectin expression in platelets from 30 untreated MDD patients and 30 matched controls by flow cytometry. In addition, tryptophan and serotonin concentrations were measured in the whole blood by high performance liquid chromatography. Patients were assessed with the Mini International Neuropsychiatric Interview and the Hamilton Depression Rating Scale. The patients had not had antidepressant treatment or any other pharmacological interventions for at least 1 year. MDD patients significantly differed from controls in levels of major fluorescent platelets for NO, PMMP, and P-selectin compared with those observed in control subjects. Serotonin concentrations in MDD patients did not differ from those in controls These results demonstrate that untreated MDD patients show increased platelet activation, suggesting an alteration in the platelet function.

1. Introduction

Major depression, like other mental disorders, is a significant health problem worldwide (Demyttenaere et al., 2004; Skala et al., 2006). Platelet activity and reduced serotonin (5-HT) concentrations have been linked to the onset of depression (Skala et al., 2006). However, Piletz et al. (2009) did not find changes in plasma nitrate concentrations in untreated depressed patients. Also, serotonin induces platelet aggregation and thrombogenesis in blood vessels from patients with major depressive disorder (MDD). It is a consequence of increased expression of the serotonin 5-HT_2 receptor which induces the increase in the concentration of intra-cellular calcium (Nair et al., 1999; Nemeroff and Musselman, 2000; Kitzlerová and Anders, 2007). Thus, a decrease in 5-HT concentration is considered an important event in the development of MDD.

Platelets are able to take up, store (more than 99% of the body's 5-HT is found in dense granules in platelets), and metabolize 5-HT (Carneiro and Blakely, 2006). Although 5-HT is regarded as a relatively weak platelet agonist, low concentrations of an agonist, such as epinephrine and adenosine diphosphate (which are frequently higher in patients with depression), can enhance platelet aggregation (Baumert et al., 2008; Doornbos et al., 2008).

Conversely, nitric oxide (NO) regulates platelet function and activity, and platelets can synthesize NO from L-arginine (Vignini et al., 2008). Further, endothelial and platelet-derived NO inhibits platelet aggregation, adhesion to the vascular wall, and platelet recruitment (Annie-Jeyachristy et al., 2008). Nevertheless, the function of NO in the onset of depressive disorders remains unknown. Recent evidence has shown that platelets from patients with ischemic disease, diabetes, or risk factors for cardiovascular disease have decreased sensitivity to the anti-aggregatory and vasodilator effects of NO (Chirkov and Horowitz, 2007), a phenomenon that has been termed *platelet NO resistance* (Rajendran and Chirkov, 2008).

There are conflicting data regarding the synthesis of NO in patients with depression. Low NO metabolite concentrations and reduced platelet endothelial NO synthase (eNOS) activity have been reported in subjects with MDD (Chrapko et al., 2004), and the negative effects on NO metabolites subside after administration of antidepressants (Chrapko et al., 2006). In contrast, Suzuki et al. (2001) and Yanik et al. (2004) observed significantly

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0165-1781/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.psychres.2012.12.024

higher plasma nitrate concentrations in depressed patients and subjects with psychiatric or anxiety disorders compared with healthy controls. Evidence suggests that NO inhibits the mechanisms that are activated by 5-HT, which has been linked to the onset of depressive disorders (Harkin et al., 2003, 2004). Also, NO synthase (NOS) inhibitors mitigate the negative effects of NO (Wang et al., 2008; Krass et al., 2011).

In this report, we examined intraplatelet NO synthesis, platelet mitochondrial membrane potential (PMMP), and the expression of P-selectin (a well-known indicator of platelet activation) in platelets from untreated patients with major depression.

2. Methods

2.1. Participants

Thirty patients consecutively referred to the Clinical Services of the National Institute of Psychiatry in Mexico City were included in this study (age: 30.2 ± 10.6 years; range 18–43 years, female/male: 24/6). After the clinical evaluation of a board-certified psychiatrist, cases were included in this study based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DMS-IV TR; American Psychiatric Association, 2000). A different psychiatrist corroborated the diagnosis on the basis of the Spanish version of the Mini International Neuropsychiatric Interview (MIN; Heinze, 2000). Then, each subject was evaluated with the Hamilton Rating Scale for Depression (HRSD; mean score 24.2 ± 1.98; Hamilton, 1967).

To be included in this study, subjects had to be diagnosed with major depressive disorder; they had to have a score above 17 at the HRSD; they had to be free of any antidepressant and/or benzodiazepines for at least 1 year before the study; and they had to be without any other pharmacological intervention for at least 3 weeks before the study. Other inclusion criteria were low coffee intake (< 2 cups/day) and no alcohol consumption. Mental retardation, bipolar disorder, depression due to a medical condition, high suicidal risk, schizophrenia, psychotic symptoms that impeded psychiatric evaluation, obsessive-compulsive disorder, substance dependence, pregnancy, lactation, postmenopausal (or other causes of hypoestrogenism), hyperlipidemia, diabetes, use of anti-inflammatory or anti-aggregatory drugs (e.g., aspirin, clopidogrel), cardiovascular diseases, infectious diseases, allergy, or a refusal to participate represented exclusion criteria.

Thirty non-depressed controls, matched for gender and age with the MDD group (age: 34.5 ± 7.7; range 19–45 years, female/male: 24/6), were recruited from the general population. We ruled out major psychiatric conditions through the semi-structured interview MINI (e.g., high suicidal risk, bipolar disorder, substance dependence). In addition, other conditions that could have interfered with platelet activity (e.g., active smoking, diabetes, use of anti-inflammatory or anti-aggregatory drugs) were also excluded. All individuals underwent laboratory testing (i.e., complete blood count, biochemical testing, urine analysis, thyroid function test, and electroencephalogram). The protocol was conducted per the Helsinki declaration and with the approval of the Ethics Committee of the National Institute of Psychiatry. Both patients and control subjects resided in the Mexico City area and signed institutional written informed consent forms.

2.2. Sample collection

After the psychiatric and clinical evaluation, 10 mL of venous blood was collected from fasting individuals, between 08:00 and 09:00 h, into Vacutainer tubes (Becton & Dickinson, USA) that contained 10% K_3EDTA. Platelets were counted on an ADVIA 70 Hematology System (Ramsey, MN, USA). Previously, 1.0 mL of plasma had been collected and stored at −70 °C until needed.

2.3. Determination of serotonin (5-HT) and tryptophan (TRP)

2.3.1. Sample preparation

Samples were prepared according to Anderson et al. (1987), with the use of fluorescence detection for 5-HT and tryptophan (TRP), which is known to provide higher sensitivity in the determination of whole blood and platelet-rich plasma. Briefly, 250 μL of whole blood was mixed with a cold solution (4 °C), containing 25% ascorbic acid (50 μL) and 3.4 M $HClO_4$ (50 μL). The mixture was centrifuged at 7500*g* for 5 min at 4 °C, and the supernatant was filtered through a 0.45-μm membrane (Millipore, Inc, USA). Fifty microliters of the protein-free sample was used to analyze TRP and 5-HT by chromatography. The concentration of platelet serotonin was calculated by dividing the amount of 5-HT in whole blood by the estimated number of platelets in the blood.

2.4. High performance liquid chromatography (HPLC)

Elution and retention times of 5-HT and TRP from plasma samples were determined on an HPLC system (Waters, Inc) per Chin (1990), with modifications. Briefly, the column was a Novapak C-18 stainless-steel type, 150 mm × 3.9 mm I.D, packed with 4 μm, and the mobile phase consisted of an aqueous component—acetonitrile (95:5, v/v) with the aqueous component consisting of 12.16 mM citric acid, 11.6 mM $(NH_4)\bullet 2HPO_4$, 2.34 mM sodium octyl-sulphonate, 1.11 mM disodium EDTA, and 3.32 mM dibutylamine phosphate. The mobile phase pH was adjusted to 3.71 with 2 N NaOH after acetonitrile was added, filtered through a 0.45-μm membrane (Millipore, Inc.), and used at a flow rate of 1.0 mL/min.

Peaks that were eluted from the column under isocratic conditions were identified by a fluorescence detector (Waters, Inc.). The retention time for TRP was 5 min versus 7.84 min for 5-HT. The intra-assay (n=20, 8.55 μg/mL) and inter-assay (n=5, 8.98 μg/mL) variation coefficients for TRP were 1.77% and 3.08%, respectively; the intra-assay (n=20, 40.65 ng/mL) and interassay (n=4, 43.75 ng/mL) variation coefficients for 5-HT were 5.30% and 5.24%, respectively.

2.5. Platelet isolation

The remaining whole blood was centrifuged at 170*g* for 15 min, yielding a platelet-rich plasma (PRP) fraction, in which the total number of platelets was counted. The remaining PRP fraction was centrifuged at 1000*g* for 15 min, and the platelets were resuspended in HEPES–albumin buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L glucose, 1 mg/mL albumin bovine serum, 10 mmol/L HEPES, pH 7.3) and stored at room temperature (20–24 °C). It is important to mention that during the process of isolation and measurement of NO, PMMP and P-selectin underwent several centrifugations, which might induce mechanical activation of the platelets. Nevertheless, the differences observed between platelets from patients with MDD and the corresponding controls were consistent; this fact supports the reliability in the results described in the present work.

2.6. Measurements of NO levels, platelet mitochondrial membrane potential (PMMP), and P-selectin

We used flow cytometry to evaluate nitric oxide levels, platelet mitochondrial membrane potential and P-selectin expression. Platelets were recognized by laser scatter characteristic (FS/SS), and evaluated only in the zone R1 (zone previously reported by Leaver et al., 2006). The levels of NO in the platelets were evaluated using the fluorescent probe 4-amino-5-methylamino-2′-7′-difluorofluorescein diacetate (DAF-FM; Molecular Probes, Eugene, OR, USA; Alvarado et al., 2007b). DAF-FM is a reagent useful to detect and to quantify low concentrations of NO. It is essentially non-fluorescent until it reacts with NO to form a fluorescent derivate identified as benzotriazole. We isolated 2×10^7 platelets 2 h before, resuspended them in 1 mL HBSS–saline without albumin (140 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L glucose, 10 mmol/L HEPES; pH 7.3), and incubated them with 6 μmol DAF-FM for 30 min.

The NO and PMMP was determinate in platelets previously centrifugated at 1000*g* × 10 min, to eliminate the excess of probe. Nitric oxide levels in platelets were analyzed by flow cytometry (Becton Dickinson FacsCalibur, San Jose, CA, USA) using 1×10^5 platelets. Positive controls were platelets that had been incubated for 30 min with 1 mmol/L SNAP (S-nitrous-N-acetylpenicillamine, a nitric oxide donor, Molecular Probes) and DAF-FM diacetate. The number of platelets that were positive for NO was counted versus the number of platelets that were positive to NO in the controls (basal production). The concentration and time of incubation with DAF-FM diacetate were determined after assessing various concentrations and times of incubation with the probe.

PMMP was measured using the fluorescent probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$ (3)) (Molecular Probes; Alvarado et al., 2007a). Platelets (2×10^7) were incubated with 10 nmol/L $DiOC_6$ in 1 mL of HBSS for 30 min to analyze PMMP by flow cytometry in 1×10^5 platelets. Negative controls comprised platelets that were incubated with 50 nmol/L of the mitochondrial uncoupler CCCP (carbonyl cyanide m-chloro phenyl hydrazone, Molecular Probes). The identity of the population of platelets in the region R1, evaluated, was verified using one isotype control and anti-platelet monoclonal antibody: anti-h-P-selectin (RD Systems, Minneapolis, MN, USA). Previous reports are in agreement to the role of P-selectin as a marker of platelet activation (André, 2004; Mendoza-Sotelo et al., 2010). In this study, P-selectin expression was measured in 1×10^5 platelets which were incubated for 45 min with the antibody. In order to corroborate activation, platelets from both patients with MDD and controls were incubated with 10 μM ADP (an adequate activator to platelets) for 5 min. Afterwards, levels of NO, fluorescence to PMMP and P-selectin expression were evaluated.

2.7. Statistical analysis

Results are expressed as the percentage of positive cells to the presence of NO, fluorescence to PMMP, and P-selectin expression ± the standard deviation of the percentage. Data analysis from the total number of fluorescent platelets to NO,