Altered Diurnal Variation of Nitric Oxide Production in Patients with Panic Disorder

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The aim of this prospective study was to investigate the diurnal change in serum nitric oxide (NO) levels in active and remission phases of patients with panic disorder. This study included 15 patients fulfilling the criteria for panic disorder of Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition and 15 healthy controls matched for age and sex. All patients were receiving a selective serotonin reuptake inhibitor at therapeutic doses. The serum nitrite and nitrate levels of subjects were determined at 10:00 a.m. after overnight fasting and at 3:00 p.m. 2 hours after lunch. NO levels of all patients measured in the morning were significantly higher than those of controls. The patients were also divided into active and remission groups according to clinical status and Panic Agoraphobia Scale’s cut-off point. There were no statistically significant differences in serum nitrite and nitrate levels of the active group between the 10:00 a.m. and 3:00 p.m. measurements. In contrast, statistically significant differences were found in the serum levels of nitrite (p<0.05) and nitrate (p<0.05) in the remission group. Notably, the afternoon nitrite and nitrate levels of the remission group were higher than those of the morning levels as seen in control subjects. Thus, diurnal variation of NO production is altered in patients with panic disorder but is resumed in the remission phase. The present study suggests that serum NO levels are a good marker for evaluation of panic disorder.

oxidative stress; nitric oxide; diurnality; panic disorder; SSRI

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The biochemical mechanisms underlying the pathophysiology of panic disorder remain uncertain. Ability to recognize potential dangers in the environment is necessary for the survival of living beings. The mechanisms of fear and anxiety are related to various neurotransmitter systems including γ-aminobutyric acid (GABA) (Kalueff and Nutt 1996), serotonin (Olivier et al. 1992), noradrenaline (Charney and Redmond 1983), and cholecystokinin (Harro et al. 1993). The glutaminergic system (Sanger et al. 1991; Meldrum 2000) and nitric oxide (NO) are also considered to play an important role in mediating anxiety and stress response (Vincent 1994).

The neural structure of anxiety, i.e. the medial amygdaloid nucleus, dorsolateral periaqueductal gray matter, hypothalamic paraventricular nucleus (PVN), pituitary and hippocampus, are rich in NO synthesizing neurons (Vincent and Kimura 1992; Onstott et al. 1993). The PVN of the hypothalamus is an important locus mediating central NO actions and autonomic functions (Navarro-Oliveira et al. 2000; Stern et al. 2003). Okada et al. (2002) proposed that intracerebroventricular administered 3-morpholino-sydnonimine SIN-1 (a nitric oxide donor) activates central noradrenergic neurons innervating the PVN in rats. As a contradictory result, Givalois et al. (2002) showed that NO synthase (NOS) inhibitors produced an increase in plasma ACTH levels of adrenalectomized rats. They suggested that the central NO system had a negative influence on the activity of the hypothalamic-pituitary-adrenal (HPA) axis. According to Kishimoto et al. (1996), mRNA and protein for neuronal NO synthase (nNOS) in the PVN are increased in rats exposed to immobilization stress. It has been found that the effect of NO within the PVN mediates the release of GABA (Zhang and Patel 1998; Patel et al. 2001). There is an overall consensus about NO that it acts as a sympathoinhibitory substance within the central nervous system.

Serotonergic mechanism within the locus coeruleus (LC) is important in stress response. The release of 5 HT in the LC is facilitated by NO (Sinner et al. 2001). NO is also able to inhibit the function of monoamine transporters (Kiss and Vizi 2001). Suppression of panic attacks by a selective serotonin reuptake inhibitor (SSRI) suggests that serotonin is related to panic disorders. Acute administration of citalopram activates the HPA-axis at the hypothalamic level and long-term citalopram treatment desensitizes the HPA-axis at the pituitary level. Several studies have demonstrated that NO modulates the extracellular levels of serotonin in the central nervous system, while serotonin (5-HT) re-uptake may be influenced by the NO pathway (Wegener et al. 2003).

According to Marcourakis et al. (2002), low levels of platelet cyclic AMP, cyclic GMP and NOS activity in patients with panic disorder were improved by clomipramine. Suzuki et al. (2002) suggested that maprotiline, fluvoxamine, zonisamide, carbamazepine and diazepam could induce gene expression of type-II NOS in the brain.

Involvement in oxidative mechanisms is another function of NO. NO and antioxidant defense system are contributing factors of pathology such as schizophrenia (Mahadik and Mukherjee 1996) and depression (Bilici et al. 2001; Suzuki et al. 2001). It has been suggested that panic disorder may cause oxidative stress. Kuloglu et al. (2002) showed that superoxide dismutase, glutathione peroxidase, and malondialdehyde levels of the panic group were significantly higher than those of controls.

NO production shows a daily fluctuation (Mitome et al. 2001). Endogenous rhythm of NO production may be affected by some variables, such as drugs and diseases. The aim of this prospective study was to investigate the diurnal change of serum NO level in active and remission phases of patients with panic disorder.

**Materials and Methods**

**Subjects**

This research was conducted with panic disorder cases, which applied to the Anxiety Unit of the Department of Psychiatry. The research pro-
Diurnal Variation of NO in Panic Disorder

Protocol was approved by the Local Ethics Committee of Inonu University Medical School. Written informed consents were obtained from all patients and controls.

This study included 15 patients (8 females and 7 males) fulfilling the criteria for panic disorder with or without agoraphobia according to Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition (DSM-IV) (APA 1994) and 15 healthy controls matched age and sex. The ages of patients varied between 18-44 years old (mean: 31.9±8.3). Each patient was evaluated by an experienced psychiatrist via semi-structured interview. Patients with any kind of clinical psychiatric disorder and any history of psychosis, primary major affective disorder, alcohol or drug abuse/dependence, any medical disorder, any other drug use, or antioxidant agent use (i.e., vitamins E and C) were excluded. In order to avoid the ingestion of exogenous nitrate, all subjects were deprived of food for 12 hours before serum sampling (Moshage 1997). For evaluating postprandial NO production, blood samples were taken 2 hours after lunch.

All patients received an SSRI at therapeutic doses (sertraline 62.5±25.0 mg/day in 4 patients, fluvoxamine 133.3±28.9 mg/day in 3 patients, fluoxetine 20 mg/day in 3 patients, paroxetine 26.7±11.8 mg/day in 3 patients, and citalopram 20 mg/day in 2 patients) for at least fifteen days.

To measure frequency, severity and avoidance level of panic disorder, Panic Agoraphobia Scale (PAS) was used. The patients were divided into active and remission groups according to clinical status and Panic Agoraphobia Scale’s (PAS) cut-off point. 10 patients were in an active period and 5 patients were in remission. Following the serum sampling anxiety levels were measured in both groups at 10:00 a.m. and 3:00 p.m. by using Beck Anxiety Inventory (BAI).

The control group included fifteen healthy volunteers. They had no panic symptoms and their PAS scores were “0”. This group was sex matched (8 females and 7 males) and age adjusted (17-38 years with a mean age of 32.2±5.3).

**Instruments**

*Panic Agoraphobia Scale (PAS)*. This scale was developed by Bandelow (1995). It measures panic attack frequency, evasion levels, anticipation anxiety, the level of restriction in social relationships, and belief of physical illness in panic cases. It includes 14 questions. The total scores of each item determine the intensity score. Tural et al. (2000) made an adaptation to Turkish, validity and reliability of this scale. The cut-off point of this scale is 12 according to Tural et al. (2000).

*Beck Anxiety Inventory (BAI)*. The Beck Anxiety Inventory is a 21-item self-report inventory. It is designed to measure the level of anxiety. The items consist of symptoms of anxiety. Information is obtained for the physical, cognitive, fear of losing control and emotional aspects. Each item is rated through 0 to 3. Higher total scores indicate higher levels of anxiety. This inventory was developed by Beck et al. (1988) and adapted to Turkish by Ulusoy et al. (1998).

**Biochemical measurements**

NO metabolism has a diurnal rhythm because fasting for 12 hours reduces levels of plasma nitrite and nitrate by 50%. In healthy volunteers having fasted around ~90% of plasma nitrite and nitrate is derived from NOS-derived NO. Taking this into account, serum sampling was done at 10:00 a.m. (after an overnight fasting). To be tested for postprandial serum nitrite and nitrate, a second blood sample was taken 2 hours after lunch.

**Chemicals**

All solutions were prepared with distilled-deionized water. Heparin, Histopaque-1077, ZnSO₄, NaOH, glycine, CuSO₄, sulfanilamide, NaNO₂, KNO₃, Na₂B₄O₇.10H₂O, were purchased from Sigma Chemical Co. (Germany); KH₂PO₄, Na₂HPO₄, were from Merck Co. (Germany), Cadmium (Cd) granules were from Fluka (Chemische Fabrik AG, Bushs, Switzerland).
Nitrite and Nitrate Determinations

Direct measurement of NO in vivo is difficult due to its short half-life. NO rapidly decomposes into stable end products nitrite and nitrate (Vincent 1994). Because of the oxidation of nitric oxide to nitrite and nitrate to terminate its effect, nitrite and nitrate levels were accepted as an index of NO production. The long half-life in plasma of about ~1, 5 hours, increased concentrations of nitrate in plasma at least indicate recent NO production. Nitrate was assayed by a modification of the cadmium-reduction method (Cortas and Wakid 1990). Nitrite product was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine. After samples were deproteinized with somogy reagent, Cu-coated Cd reduced the nitrate to nitrite in glycine buffer at pH 9.7. The reduction was followed by pseudo-first order reaction kinetics, at 90 minutes intervals. Greiss reagent colored the reduced samples. Then, the absorbances were read against the blank at 545 nm. Concentrations of samples were determined by comparison to a standard solution of sodium nitrite (0-100 $\mu$mol/liter).

Statistical analysis

Chi-square, Mann-Whitney’s U-test, Spearman correlation and Wilcoxon signed ranks tests were used for statistical analysis. The data were expressed as the means±s.d..

RESULTS

The morning nitrite levels of the panic group were higher than those of the controls (Table 1). This difference was statistically significant between the two groups ($p=0.044$). There was no statistically significant difference in the morning nitrate levels between patients and controls ($p=0.059$). In the afternoon measurements, there was no statistically significant difference in nitrite and nitrate levels between the two groups. The afternoon nitrite and nitrate levels of all subjects tend to be higher than those of morning levels.

There was a statistically significant difference between the morning and afternoon BAI scores in the panic group ($p=0.025$). Anxiety levels were found to be lower in the afternoon (Table 2). There was a statistically significant difference between the morning and afternoon BAI scores ($p=0.033$) in the active group, whereas no difference was found in the remission group (Table 3). Anxiety levels at 3:00 p.m. decreased markedly in the active group. Perhaps, the patients in the active group were more anxious at the initial blood sampling, and more relaxed at 3:00 p.m. BAI levels did not differ at the 10:00 a.m. and 3:00 p.m.

<table>
<thead>
<tr>
<th>Table 1. Nitrite and nitrate levels of patients and controls</th>
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<tr>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
</tr>
<tr>
<td>10:00 a.m.$^1$</td>
</tr>
<tr>
<td>3:00 p.m.$^1$</td>
</tr>
<tr>
<td>$p^3$</td>
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<tr>
<td>Nitrate</td>
</tr>
<tr>
<td>10:00 a.m.$^1$</td>
</tr>
<tr>
<td>3:00 p.m.$^1$</td>
</tr>
<tr>
<td>$p^3$</td>
</tr>
</tbody>
</table>

$^1$ $\mu$mol/liter.
$^2$ Mann-Whitney’s U-test (between PD and Controls).
$^3$ Wilcoxon signed ranks test.
$^4$ No Significant.
3:00 p.m. in the remission group. There was a significant difference in the PAS total scores between the active and remission groups.

Consequently, we compared the nitrite and nitrate levels between the active and remission groups (Table 1). There was no statistically significant difference between 10:00 a.m. and 3:00 p.m. levels of nitrite ($p>0.05$) and nitrate ($p>0.05$) in the active group. In contrast, the afternoon nitrite and nitrate levels were higher than the morning levels in the remission group and controls. Taken together, these results indicate that the diurnal variation of NO production is altered in patients with panic disorder but is resumed in the remission phase.

**Table 2.** PAS and BAI scores of the panic patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>Controls</th>
<th>$p^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS</td>
<td>16.7±10.1</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>BAI a.m.</td>
<td>19.7±17.6</td>
<td>1.9±1.3</td>
<td>0.000</td>
</tr>
<tr>
<td>BAI p.m.</td>
<td>11.1±13.1</td>
<td>2.1±1.4</td>
<td>0.004</td>
</tr>
<tr>
<td>$p^2$</td>
<td>0.025</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

1 Mann-Whitney’s U-test.
2 No Significant.

**Table 3.** PAS and BAI scores of the active and remission groups

<table>
<thead>
<tr>
<th></th>
<th>Active Group</th>
<th>Remission Group</th>
<th>$p^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS</td>
<td>21.4±8.9</td>
<td>7.2±3.9</td>
<td>0.002</td>
</tr>
<tr>
<td>BAI a.m.</td>
<td>21.3±15.5</td>
<td>16.4±22.7</td>
<td>NS³</td>
</tr>
<tr>
<td>BAI p.m.</td>
<td>8.9±7.4</td>
<td>15.4±21.1</td>
<td>NS</td>
</tr>
<tr>
<td>$p^2$</td>
<td>0.033</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

1 Mann-Whitney’s U-test.
2 Wilcoxon signed ranks test.
3 No Significant.

**Discussion**

Several lines of evidence have suggested that some abnormalities of NO level and NOS gene expression may underlie a wide range of psychiatric disorders (Herken et al. 2001; Johnson 2001; Suzuki et al. 2003). NO-derived products such as NO$_3^-$ and NO$_2^-$ may interact with neuromodulators to modify monoamines and thereby change their regulatory action on synaptic transmission (Fossier et al. 1999). NO generators have been found to inhibit the uptake of dopamine, noradrenaline and 5-hydroxy tryptamine into striatal and hippocampal synaptosomes (Pogun et al. 1994; Lonart and Johnson 1995). NO within the PVN regulates sympathetic outflow via an inhibitory mechanism probably involving a GABA system (Patel et al. 2001).

Frisch et al. (2000) demonstrated in the mice that the functional inactivation of the endothelial NOS gene induced profound behavioral changes such as an increase in anxiety-related behavior that was related to changes in brain monoaminergic systems. Some other animal studies showed that pharmacological inhibition of NO synthesis had anxiogenic effects (Lino de Oliveira et al. 1997), and diminished the effects of anxiolytic agents (Quock and Nguyen 1992; Caton et al. 1994). These studies show that NO levels below the cytotoxic doses have an anxiolytic effect and/or they potentialize the effects of the anxiolytic
agents. Our presented study confirms this data.

In the control group, we observed that nitrite and nitrate levels had a diurnal rhythm. Afternoon nitrite and nitrate levels were higher than those of morning levels. However, nitrite and nitrate levels of the patients after taking a drug in the morning were statistically significantly higher than those of the controls. This result has supported the idea that SSRI increases level of NO (Marcourakis et al. 2002; Wegener et al. 2003). On the other hand, increased anxiety in the morning may contribute to an increase in NO levels via oxidative stress. That increase in NO levels could have an inhibitory effect on the autonomic nervous system, which might be responsible for decreased BAI levels in the afternoon. Increased nitrite and nitrate levels in the afternoon are already very well documented. The increased NO production reduces the oxidant stress. Some feed-forward mechanism prevents degradation of NO, which is produced by neighboring cells (Ennezat et al. 2001). Elevation of NO level induces synthesis of the extracellular superoxide dismutase. Elevated intracellular GSH increases the ability of cells to scavenge strong oxidants (Cepinskas et al. 2002). NO production could also alter 5-HT transmission by increasing re-uptake of 5-HT (Grahn et al. 2000). This relationship can activate some feedback mechanisms for homeostasis and contribute to some additive curative effect.

Brain aversive system, the neural structure of anxiety, commands defensive behavior and elaborates aversive emotional and motivational states (Graeff et al. 1993). Increased levels of NO could be related to the recognition of avoidance behavior. The study by Telegdy and Kokavszky (1997) on the effect of NO in passive avoidance learning on rats disclosed that NO was able to facilitate the learning and consolidation of memory in a passive avoidance paradigm. Holscher and Rose (1993) showed that chicks trained under the effect of NOS inhibitors presented significant deficits in learning the avoidance behavior. Our presented data disclosing the increase in the NO levels also implies a positive effect on learning this attitude. The level of anxiety was higher in the mornings in the active panic group while the level decreased in the afternoon with the influence of learning.

It seems that both the anxiety levels and the differences between morning and afternoon levels have decreased. Since anxiety did not increase in the morning, there wasn’t any additional increase in NO levels. Nitric oxide levels increased in the afternoon as expected. The actualization of diurnality in the NO metabolism in remitted patients has been considered as the biological rhythm of NO production in recovery process.

There are some limitations in our study. The design of this study is not to compare NO levels of panic patients with or without SSRI treatment. It is difficult to be certain whether the disease or SSRI, causes the difference in nitrite and nitrate levels. The other limitation is the small sample size.

In conclusion, an understanding of the intracellular oxidative mechanisms involved in the pathophysiology of panic disorder can provide some benefits for pharmacologic treatment. Minimizing oxidative stress through NO donors or some antioxidants may be a secondary strategy for the future treatment of panic disorder.

References
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