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THE ROLE OF NITRIC OXIDE IN PATIENTS WITH MOOD AND ANXIETY SYMPTOMS AND IN THEIR PHARMACOLOGICAL TREATMENT

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE ROLE OF NITRIC OXIDE IN MOOD AND ANXIETY SYMPTOMS AND THEIR PHARMACOLOGICAL TREATMENT, submitted by Nathalie Lara in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Panic disorder (PD) and major depressive disorder (MDD) have been associated with increased cardiopulmonary morbidity and mortality. However, the mechanisms explaining this association remain largely unknown. Nitric oxide (NO) is widely known as a modulator of bronchial and vascular tone. NO system dysregulations may contribute to the increased cardiopulmonary morbi-mortality displayed by these patients. This thesis evaluated the effects of cholecystokinin-induced panic attacks on NO measurements and respiratory variables, as well as the effect of paroxetine treatment on plasma NO metabolites (NOx) in healthy volunteers. NO concentration in exhaled breath was significantly decreased during cholecystokinin-5-induced panic attacks, mainly due to changes in minute ventilation. No changes in systemic or lung NO production were detected. Plasma NOx levels were increased at 8 weeks of paroxetine administration when compared to baseline. To further understand these findings it is necessary to perform the same investigation in PD and MDD patients.

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DEDICATION

To my parents Ana and Lucrecio for teaching me the importance of being persistent and brave. To my sister Alexandra and brother Enrique for all their support. To my friends for painting my life with nice colors.

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ABBREVIATIONS

BTG	β-thromboglobulin
Ca^{+2}	calcium
CV	cardiovascular
°C	Degree Celsius
CNS	central nervous system
CCK	cholecystokinin
CHD	coronary heart disease
df	degrees of freedom
DSM	Diagnostic and Statistical Manual of Mental Disorders
eNOS	endothelial nitric oxide synthase
GABA	gamma-aminobutyric acid
HV	healthy volunteers
Hz	hertz
HPLC	High-performance liquid chromatography
5-HT	5-hydroxytryptamine
HCl	hydrochloric acid
iNOS	inducible nitric oxide synthase
IV	intravenous
L	liter (s)
LDL	low density lipoprotein
MDD	major depressive disorder
μg	microgram (s)
μL	microliter (s)
μMol	micromole (s)
mg	milligram (s)
mL	milliliter (s)
min	minute (s)
ng	nanogram (s)
nL	nanoliter (s)

nNOS	neuronal nitric oxide synthase
[NO]exh	exhaled nitric oxide concentration
NO	nitric oxide
NOx	nitric oxide metabolites
VNO	nitric oxide production in exhaled breath
NOS	nitric oxide synthase
N	normal
PA	panic attack (s)
PD	panic disorder
PSS	panic symptom scale
ppb	parts per billion
p	probability
RR	respiratory rate
rpm	revolutions per minute
sec	second (s)
SSRIs	selective serotonin reuptake inhibitors
SEM	Standard error of the mean
SD	Standard deviation
VE	minute ventilation
VT	tidal volume

CHAPTER 1

General Introduction: The role of nitric oxide in patients with mood and anxiety symptoms and in their pharmacological treatment

1.1 INTRODUCTION

Panic disorder (PD) is defined as the recurrent experience of intense feelings of fear, accompanied by a series of symptoms that comprise a panic attack (DSM-IV, American Psychiatric Association, 1994). Among the most common psychiatric disorders present in primary care settings, PD has an estimated lifetime prevalence of 2 to 5 %. Women are more commonly affected than men (2-3:1). The average onset of the disorder occurs between the second and third decade of life[1].

The hallmark of PD is the occurrence of panic attacks, characterized by the sudden onset of psychological features (fear of dying, fear of losing control, fear of going crazy, derealization and depersonalization) and physiological symptoms (dyspnea, palpitations, chest pain, sweating, paresthesias, hot and cold flushes, tremor and faintness). PD is diagnosed when at least one of these recurrent panic attacks occurs with no obvious stimulus and has been present for at least one month. PD can be further complicated by the presence of agoraphobia, defined as the phobic avoidance resulting from panic attacks (DSM-IV).

Most of the diagnostic symptoms of a panic attack are also important clinical features of cardiovascular diseases; PD patients frequently consult with primary care physicians about the fear of dying from a coronary heart disease[2,3,4]. In addition, several studies have reported a significant association between PD and cardiovascular mortality and morbidity[5,6]. This increased cardiovascular mortality and morbidity may be explained by the increased cardiovascular (CV) risk factors that those patients with PD display. For instance, anxiety may promote deleterious behaviors such as cigarette smoking[7], or influence directly the pathological processes that precede an acute coronary event such as the presence of atherosclerosis[8]. In addition, it appears that the increased risk for sudden cardiac death found in PD patients persists even after correction for conventional CV risk factors[9]. Indeed, it has been shown that patients with high levels of phobic anxiety, a

common feature in PD patients, display an increased risk for sudden cardiac death even after correction for CV risk factors[9]. The acute physiological and neurochemical changes that occur during a panic attack may be responsible for this increased CV risk. However, at this time the exact pathophysiologic mechanism involved remains unsolved.

Currently, cholecystokinin (CCK) is recognized as the most abundant neuropeptide in the brain[10]. Two type of receptors, CCK-A and CCK-B, have been identified [11]. In humans, CCK-B receptors are more widely distributed in the central nervous system (CNS)[12]. The role of CCK in the neurobiology of PD comes from consistent findings in both animal and human studies. Animal studies suggest that stimulation of CCK receptors in the CNS induces anxiety responses[13,14,15]. CCK receptor antagonists were found to abolish this anxiogenic response[13,14]. In humans, CCK-B agonists such as CCK-4 and CCK-5 have been proven to be valid panicogenic agents, based on the fact that they elicit panic symptoms that resemble the usual panic attacks experienced by PD patients[16]. The potential involvement of CCK in PD is further suggested by the presence of genetic abnormalities of the CCK-B receptor found in PD patients[17]. Moreover, CCK is known to interact with many neurotransmitter systems such as the GABA-ergic, serotonergic and noradrenergic systems thought to be involved in PD[12].

Major depressive disorder (MDD) has been associated with increased morbidity and mortality after myocardial infarction[18]. MDD is an independent risk factor for development of CV disease[19]. The increased CV risk in MDD patients may be due to either an increase in deleterious behavior or to pathophysiologic processes such as autonomic abnormalities[20] or increased platelet reactivity. Indeed, it has been shown that MDD patients display increased platelet reactivity[21,22], and that this alteration is normalized by paroxetine, a first line antipanic and antidepressant treatment[23,24].

Nitric oxide (NO) is a gaseous neuromessenger synthesized from the amino acid L-arginine by a family of coenzymes known as nitric oxide synthases (NOS). Three

isoforms have been identified: isoform I in neuronal and epithelial cells (nNOS), isoform II in inflammatory cells (iNOS) and isoform III in endothelial cells (eNOS)[25,26]. Measurement of NO can be done directly by obtaining the NO concentration in exhaled breath or indirectly by sampling the metabolic end products of NO (NOx) in plasma[27].

Endothelial-derived NO plays an important role in the CV system through modulation of vascular tone, platelet reactivity and cardiac contractility[28], and has been implicated in the pathophysiologic processes that lead to CV disease[29]. Both endothelial and platelet-derived NO regulate platelet function, decreasing platelet aggregation and adhesion[30]. Considering that decreased NO production is associated with the pathophysiological mechanisms leading to CV disease[29], it is of concern that the study by Finkel et al. suggested that paroxetine induces a decrease in NO production[31]. However, this study involves several limitations such as comorbid ischemic heart disease in the subjects, lack of control for contaminants of NOx measurements and the method of analysis employed. In addition, they did not include a control group; therefore did not avoid the influence of psychopathology on NOx measurements.

The following series was designed to investigate the role of NO in the context of induced panic/anxiety and the effects of paroxetine administration on NO production in healthy volunteers (HVs).

In summary, this thesis investigated:

- The changes in NO concentration in exhaled breath and respiratory variables during CCK-5-induced panic attacks in HVs; it was hypothesized that CCK-5-induced panic attacks would reduce the NO concentration in exhaled breath and would alter respiratory variables.
- The changes in plasma NO metabolites after 8 weeks of paroxetine administration in HVs; based on previous data it was hypothesized that paroxetine would induce a decrease in plasma NO metabolites when compared to baseline.

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CHAPTER 2

Measurements of exhaled nitric oxide and plasma metabolites during CCK-5-induced panic attacks

*The contents of this chapter are represented in a manuscript submitted for publication (**Nathalie Lara**, Wendy E. Chrapko, Stephen L. Archer, François Bellavance, Irvin Mayers and Jean-Michel Le Mellédo). The author of this thesis played a major role in this study, including design and revision of protocol, research visits, data collection and analysis, and writing of the manuscript.

2.1. INTRODUCTION

Nitric Oxide (NO) is widely accepted as an important biomediator involved in regulation of vascular and bronchial tone.[1,2] NO has an important role in the pathophysiologic processes leading to cardiovascular and pulmonary diseases, such as reduction in NO endothelial release in atherosclerotic vessels[3] and increased NO production in respiratory diseases such as asthma.[4]

Panic attacks (PAs) are characterized by short periods of intense fear accompanied by marked physiological symptoms such as sweating, tremor, palpitations, dyspnea, dizziness, paresthesias, gastric sensations, hot flushes and/or cold chills and chest pain or discomfort. Patients suffering from panic disorder (PD) experience recurrent PAs.[5]

Recently, PD has been associated with increased cardiovascular mortality and morbidity.[6,7,8,9] Furthermore, there is a high association between pulmonary illnesses and PD, as evidenced by the increased prevalence of asthma in PD patients.[10] Respiratory alterations such as persistent respiratory irregularity and increased respiratory response to the respiratory stimulant doxapram have also been reported in PD patients.[11] Dysregulation of the NO system in PD patients may ultimately contribute to the increased cardiovascular risk and the respiratory dysfunction associated with PD. The exact pathophysiologic mechanism of respiratory alterations in panic remains unclear. For instance, it has been hypothesized that the dyspnea experienced by PD patients may be the result of the triggering of a "false

suffocation alarm", suggesting a central abnormality in the control of ventilation.[12] According to this theory, dyspnea would be the key symptom in the chain of events that lead to a PA. Endogenously produced NO plays an integral role in the physiological regulation of many airway functions such as bronchodilation and regulation of airway and pulmonary blood flow[13]; we therefore hypothesized that abnormal NO production could play a role in the occurrence of respiratory symptoms featured during PAs.

Cholecystokinin (CCK) type B receptor agonists such as tetragastrin (CCK-4) and pentagastrin (CCK-5) have been widely used to investigate the neurobiological changes taking place during PAs.[14] Intravenous bolus injections of CCK-4 and CCK-5 induce short-lived PAs in PD patients and to a lesser degree in healthy volunteers (HVs) (panic response rate 70%-100% and 17-70% respectively).[15] These PAs are reported by PD patients to be very similar to the naturally occurring PA.

The direct measurement of plasma NO concentration remains difficult *in vivo* because NO is rapidly inactivated by hemoglobin or oxidized to form several nitrogen dioxides (NOx). Plasma NOx concentration is used as a marker for endogenous NO production in humans.[1] Another valid method of measurement of endogenous NO production is to assess the NO excreted in the exhaled air, detected by a chemiluminescence assay.[16,17,18,19,20,21,22]

We tested the hypothesis that CCK-5-induced PAs in HV increase respiratory rate,

tidal volume and NO production in exhaled breath and plasma.

2.2. METHODS

2.2.1. Subjects

Twenty-four normotensive subjects, taking no medications and with no evidence of medical conditions at the time of the study, were administered the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) Axis I Disorders (SCID-I) and included in the study based on the lack of Axis I psychiatric disorders. The SCID is a clinical research tool that dramatically improves accuracy and standardization of psychiatric diagnoses.[23] As in previous studies, we used the Panic Symptom Scale (PSS) to assess the intensity of the panic response to CCK-5 and to differentiate non panickers from panickers (those meeting criteria for occurrence of a PA). [24] Seventeen out of twenty-four non-smoker subjects that received CCK-5 were defined as panickers (71%) and included in the study (13 males and 4 females mean age ± SEM 24.71±1.65 yr). Female subjects were tested during their early follicular phase. The research protocol was approved by the Health Research Ethics Board of the Faculty of Medicine and Dentistry of the University of Alberta and all subjects provided written consent.

2.2.2. Study Design:

A double-blind placebo-controlled cross-over study was used. Subjects were randomized to receive intravenously a 5 second-bolus of either placebo first and 3 to 7 days

later CCK-5 (50μg) (CLINALFA, Läufelfingen, Switzerland), or CCK-5 first and placebo 3 to 7 days later.

2.2.3. General Procedures

Subjects followed a nitrate/nitrite restricted diet[25,26] for 3 days before the test and fasted overnight (12 hours).[27] Forty-five minutes before injection, an intravenous catheter was inserted in the antecubital vein for drug infusion and blood collection. Shortly after, a face mask occluding the nose was placed on the subject. Measurements were taken breath by breath from baseline (30 seconds interval taken before injection) up to 3 minutes 40 seconds post-injection. Calculation of tidal volume, respiratory rate and minute ventilation were performed using Power-lab software (AD Instruments; Castle Hill, Australia).

2.2.4. NO measurement:

[NO]exh was measured as previously described[28], using chemiluminescence analysis (NO analyzer 280; Sievers Instruments, Boulder, CO). Ambient NO contamination was avoided by the use of NO-free air (<3 ppb of NO). To exclude nasal air, known to contain high levels of NO from nasal and paranasal origin[22], a non-rebreathing face mask was used (Hans Rudolph Inc; Kansas City, MO). NO production was calculated using the equation,

$$VNOstp = 0.826\ VE\ ([NO]exh) \qquad (1),$$

where VE is minute ventilation, 0.826 is the gases universal constant[29] and [NO]exh is the exhaled NO concentration. To obtain levels of NO released from the lower airways, we

calculated the mean and the integral function *(see online supplement 2.5.)* of the plateau seen in [NO]exh, as previously described in other studies.[20,30,31]

2.2.5. NOx measurement

NOx were measured by chemiluminescence in small volumes of plasma (10 μL) by converting it to NO using a strong reducing environment: vanadium (III) in hydrochloric acid 1N at 90°C.[28]

2.2.6. Statistical analysis

Following the experimental design, a linear model for cross-over design[32] was used to analyze the data. The following parameters were included in the model: the main effect of time, the main effect of treatment, the main effect of visit (first vs. second injection visit), the main effect of order of treatment (CCK-5 first vs. CCK-5 second), and the following interactions: treatment with time, visit with time, and order of treatment with time. A p value of less than 0.05 was considered significant. Bonferroni's multiple comparisons analyses were also done, where appropriate. All statistical analyses were conducted using SAS statistical software for Windows, Release 8.02 (SAS Institute Inc. Cary, NC)

2.3. RESULTS

The PSS score after CCK-5 injection was greater than after placebo injection (PSS Score means ± SEM of 29.65±2.72 after CCK-5 injection and 1.24±0.58 after placebo injection. $p<0.001$).

A significant decrease in plateau [NO]exh levels (for both calculations of integral function and mean) was found after pentagastrin-induced PA (Fig.2-1), but not with placebo, as illustrated by time effect and treatment x time effect (Table 2-1). Bonferroni's *post-hoc* analysis showed a statistically significant decrease ($p<0.05$) in plateau mean [NO]exh at 40 seconds, 1 minute and 1 minute 20 seconds compared to baseline after CCK-5-induced PAs, and at 40 and 60 seconds in plateau integral [NO]exh. Statistically significant treatment by time interactions were found in tidal volume (Fig. 2-2), respiratory rate (Fig. 2-3) and minute ventilation (Fig. 2-4) (defined as the product of tidal volume and respiratory frequency), after CCK-5-induced PAs (Table 2-1). Bonferroni's *post-hoc* analysis showed significant increases ($p<0.05$) compared to baseline, in minute ventilation (Fig. 2-4) and respiratory frequency (Fig. 2-3) at 40 seconds, 1 minute and 1 minute 20 seconds after CCK-5-induced PAs, and at 40 seconds in tidal volume (Fig. 2-2). However, there were no statistically significant time, treatment and time x treatment effects in plateau NO production (VNO) (integral function and mean) after pentagastrin-induced PAs (Table 2-1). No significant changes were noted in plasma NOx after CCK-5-induced panic attacks (Table 2-1).

No order of visit effects or order of treatment effects were observed on any of the variables.

2.4. DISCUSSION

In the present study administration of CCK-5 induced intense panic symptoms compared to placebo. This is in accordance with previous CCK-5 panic challenge

investigations performed in healthy volunteers (HVs)[15]. Previous studies have shown that PD patients display an increased panic response to CCK-4 and CCK-5,[33,34] suggesting that CCK plays a role in the neurobiology of PD, either through an increase in central CCK neural activity or an increase in CCK receptor sensitivity.[35] However, the exact mechanism leading to the occurrence of PAs remains unclear.

In the present investigation significant increases in tidal volume (VT), respiratory rate (RR) and VE were found during CCK-5-induced PAs. These findings differ from a previous research report using CCK-4 in healthy volunteers. Bradwejn et al. showed that CCK-4-induced panic symptoms produced an increase in VE mediated by increases in VT but not in RR.[36] This discrepancy regarding the increase in RR between our study and Bradwejn et al. could be explained by the fact that we studied only panickers (seventeen) whereas Bradwejn et al.'s studied non panickers (six) and panickers (nine) together.[36] In their discussion Bradwejn et al. noted that the VE in panickers appear to be greater than in non-panickers, but the observed difference did not reach statistical significance; however, they did not provide information on RR in panickers vs. non panickers. Our findings of an increase in RR during CCK-5-induced PA are also supported by results of other studies which used different panic challenges and which showed an increase in VT and RR during induced PAs.[37,38] Another human study testing the effects of CCK-4 on airway resistance found no differences in spirometric variables after CCK-4-induced panic symptoms.[39] Findings from these studies and our results suggest that the CCK-induced changes in

respiratory function have a central rather than peripheral mechanism.

The present study is the first to investigate the changes in measurements of NO during induced anxiety/panic in humans. We found that CCK-5-induced PAs produced a significant decrease in exhaled NO levels, without affecting NO production (VNO). These findings can be explained by an increase in VE such that, with constant VNO, there is a dilutional decrease in $[NO]_{exh}$. Interestingly, these ventilatory changes and associated decreased $[NO]_{exh}$ were concomitant and occurred at the time of maximum intensity of panic symptoms.

During exercise, decreased $[NO]_{exh}$ and increase in VNO have been well documented for both tidal breathing and end-tidal plateau measurements of NO.[40] The decrease in exhaled NO concentration may be due to large increases in airflow rates, reducing the contact time between the airway wall and the exhaled air.[41] Despite the decreased [NO]exh , the large increases in ventilation during exercise can result in increased VNO. The decrease in [NO]exh observed during exercise can be also explained by the rapid uptake of NO by the blood, due to increased diffusion of NO. However, no changes in levels NO metabolites (NOx) in plasma have been found during heavy exercise.[41,42] In contrast in our study, no significant changes in NO production (VNO and NOx) were found during CCK-5-induced PAs. The phenomena observed during PA may be similar to those of exercise, except that there was no increase in NO production but a stable NO production resulting from balanced

changes in VE and [NO]exh.

Increased rates of CV/cerebrovascular morbidity [8] and mortality (mortality ratio for men suffering from PD was twice that of the predicted ratio)[43] have been described in PD patients. This increase in CV/cerebrovascular morbidity and mortality rates may be due to the existence of chronic CV risk in PD patients, illustrated either by elevated conventional risk factors such as smoking[44] or by chronic physiological states such as a sympathetic predominance, as suggested by the decreased heart rate variability consistently described in PD patients.[45] It appears that the increased risk for sudden cardiac death found in PD patients persists even after correction for conventional risk factors.[9] Indeed, Kawachi et al.[9] found that patients with high levels of phobic anxiety, a common feature in PD patients, display an increased relative risk (Relative risk: 3.01) of fatal coronary heart disease (CHD) and sudden cardiac death even after correction for conventional risk factors. The CV risk, and particularly the increased risk for sudden cardiac death associated with PD, is therefore also likely due to the acute physiological and neurochemical disturbances occurring during a PA. For example, specific panic symptoms such palpitations, sweating and shaking suggest an activation of the sympathetic system which could promote the occurrence of cardiac arrhythmia. We have, indeed, shown that pretreatment with intravenous propranolol blunts the panic response to CCK-4-induced PA.[46] The respiratory alterations that accompany a PA (such as hyperventilation) observed in our study could also contribute to the acute CV risk during PAs. Indeed, hyperventilation has been reported to potentially

precipitate coronary spasm in the presence or absence of atherosclerosis.[47] However, From our peripheral measurements of NO in HV it appears that lung NO does not play a major role in respiratory changes associated with acute PAs.

In summary, this is the first study to establish that the increased minute ventilation observed during CCK-B-agonist-induced PAs is due to increases in both VT and RR. We have also shown that during CCK-5-induced PAs there is a decrease in NO concentration in exhaled breath with no alteration of pulmonary or systemic NO production. This decrease in [NO]exh appears to be due to an increase in minute ventilation. However, these results need to be replicated in patients with PD, who are known to display a greater panic response to CCK-B receptor agonists than HVs. Since the present results only assessed the possible connection between peripheral actions of NO (such as cardiopulmonary regulation) and respiratory symptoms displayed during PAs, we cannot make any inference to the role of brain NO in the pathophysiology of PAs.

2.5. Online data supplement: Measurements of exhaled nitric oxide and plasma metabolites during CCK-5-induced panic attacks.

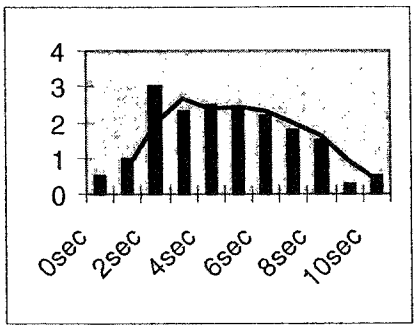
Classically, the mean function has been used to obtain the plateau (defined as the interval from 1/2 to 7/8 of the duration of the exhalation) of the exhaled concentration of NO,

$$\frac{\sum_{i=1}^{N} X(ti)}{N} \qquad (1),$$

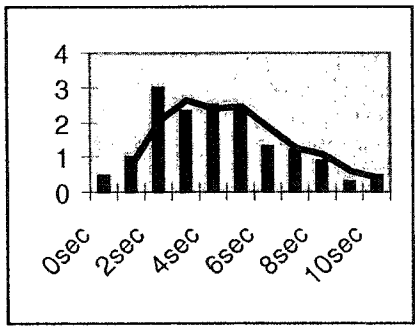
where $t_1 \leq t_i \leq t_N$ and [to, t_N] is the interval of time of the plateau, N is the number of observations and X(ti) is the value of the exhaled NO concentration at time t_i.
However, variations in the respiratory pattern will not be detected by this calculation and some important information can be missed. For this reason we used the integral function on the plateau on each breath,

$$\int_{to}^{t_f} f(t)\, dt \qquad (2),$$

where f(t) is the exhaled NO concentration (ppb) at time t and [to,t_f] is the interval of time of the plateau (sec). Therefore, more information about the behavior of the curve can be obtained, especially when the measurements of NO are done breath by breath with variable expiratory flows (Fig. 2-5).



Breath 1 [NO]exh plateau
Mean = 2.35
Integral=9.4



Breath 2 [NO]exh plateau
Mean= 2.4
Integral= 7.2

Figure 2-5. Differences in calculations of plateau NO exhaled.

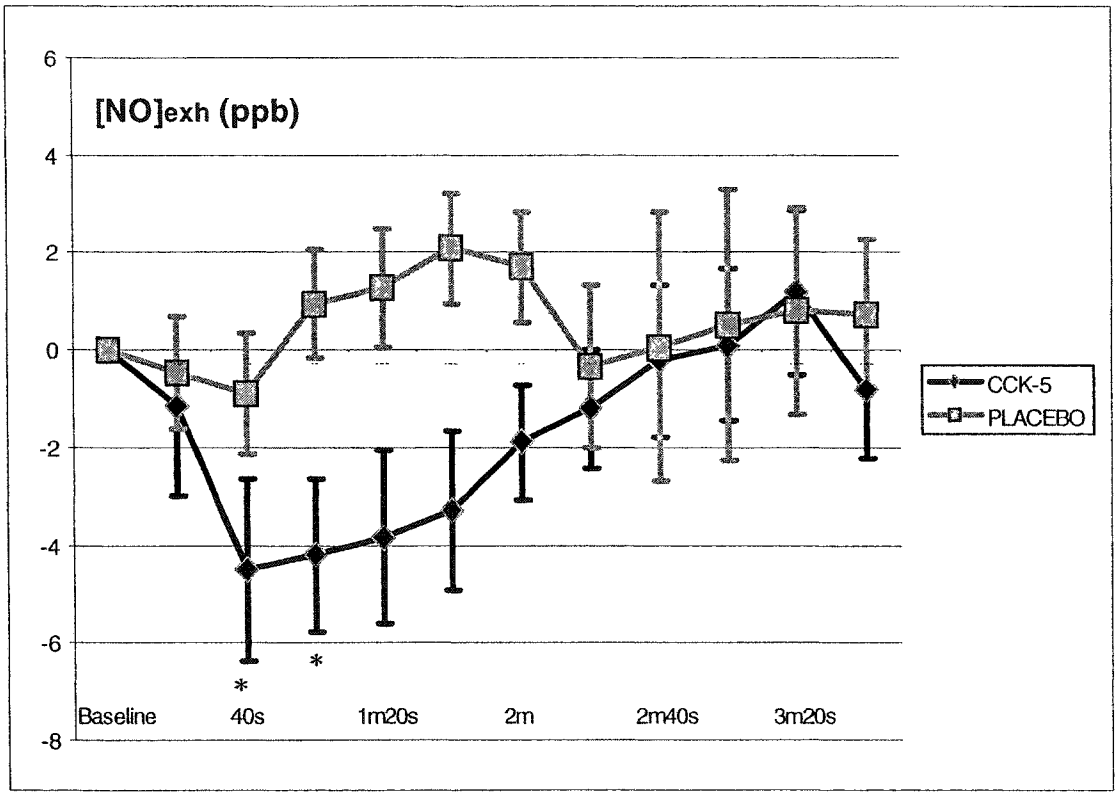


Figure 2-1. Changes (difference from baseline) in plateau [NO] exh (integral) post-CCK-5 induced panic attacks.

Bars indicate the means ±S.E.M. $p<0.05$. N=18. s = seconds. m = minutes.

* Significant difference from baseline after Bonferroni's *post-hoc* analysis ($p<0.05$)

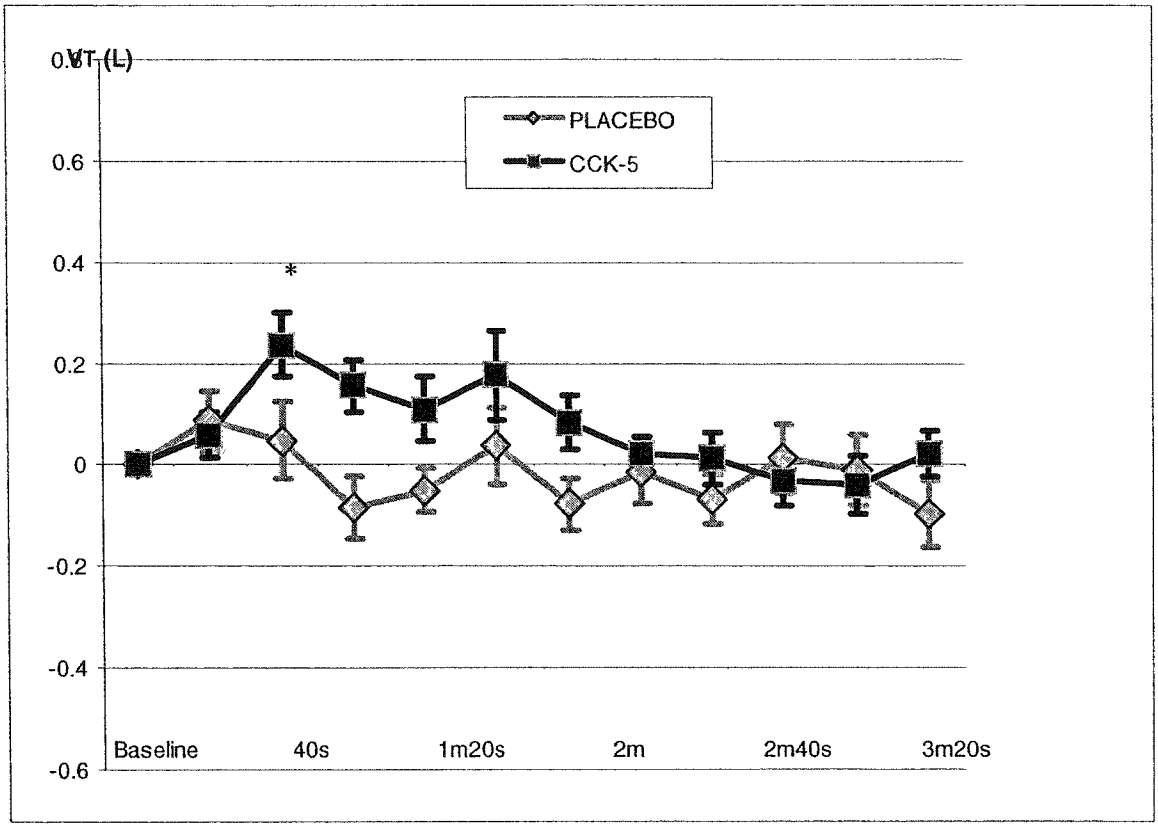


Figure 2-2. Changes (difference from baseline) in tidal volume (VT) post-CCK-5-induced panic attack

Bars indicate the means ±S.E.M. $p<0.05$. N=18. s = seconds. m = minutes.

* Significant difference from baseline after Bonferroni's *post-hoc* analysis ($p<0.05$)

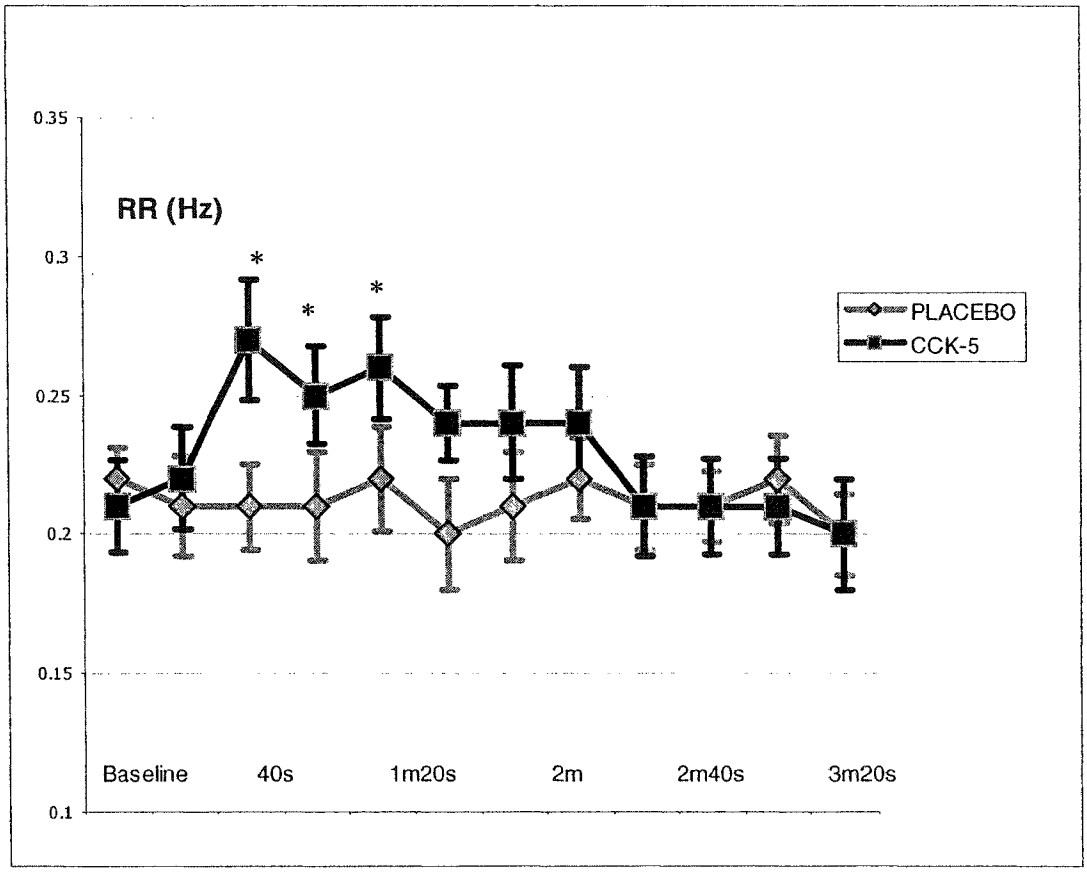


Figure 2-3. Changes in respiratory frequency during CCK-5-induced panic attacks. Bars indicate the means ±S.E.M. $p<0.05$. N=18. s = seconds. m = minutes.
* Significant difference from baseline after Bonferroni's *post-hoc* analysis ($p<0.05$)

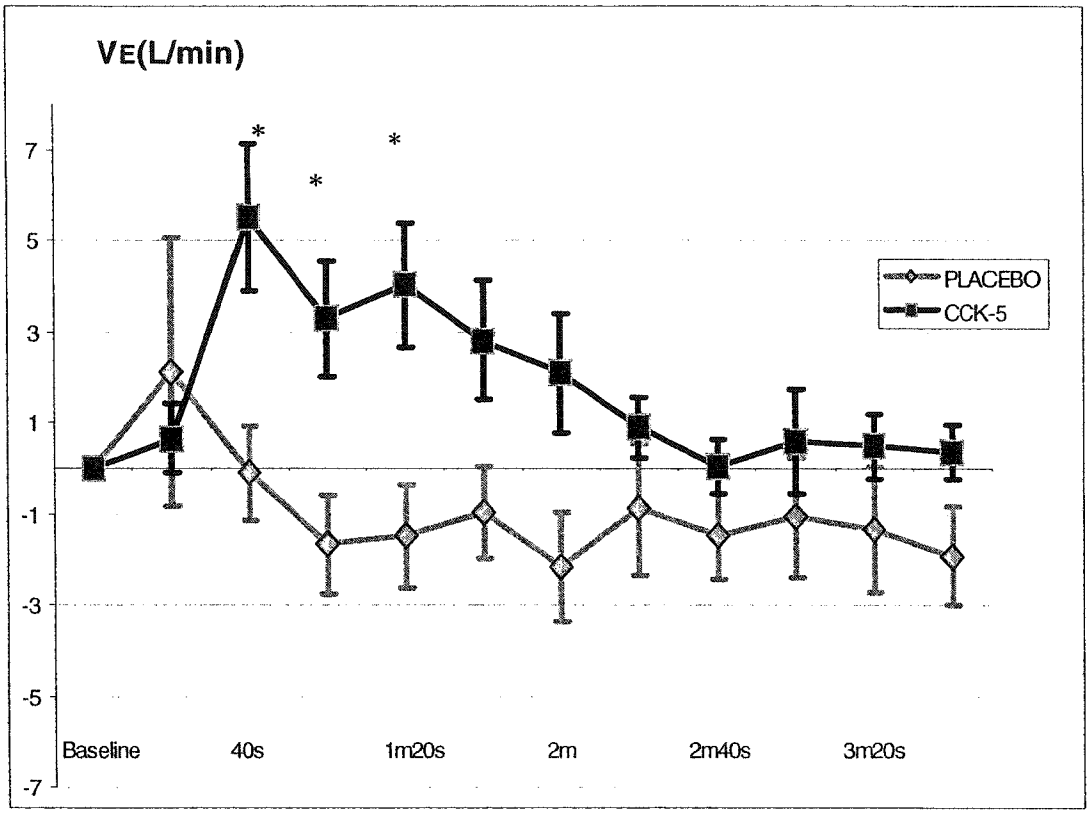


Figure 2-4. Changes (difference from baseline) in minute ventilation during CCK-5-induced panic attacks

Bars indicate the means ±S.E.M. $p<0.05$. N=18. s = seconds. m = minutes.

* Significant difference from baseline after Bonferroni's post-hoc analysis ($p<0.05$)

Variables	Time-effect	Treatment-effect	Treatment x time effect
[NO]exh *(i)*	F(11,165) =2.18, p=0.018	F(1,15)=0.04, p=0.84	F(11,160)=2.10, p=0.02
[NO]exh *(m)* (ppb)	F(11,165)=3.23, p=0.0005	F(1,15)=0.18, p=0.68	F(11,160)=1.93, p=0.039
VT (L)	F(11,165)=4.27, p<0.0001	F(1,15)=0.08, p=0.78	F(11,160)=2.27, p=0.01
RR (Hz)	F(11,165)=2.67, p=0.004	F(1,15)=2.44, p=0.14	F(11,160)=2.63, p=0.004
VE (L/min)	F(11,165)=2.91, p=0.002	F(1,15)=0.16, p=0.69	F(11,160)=3.69,p<0.001
VNO (i) (nL.sec/min)	F(11,165)=1.76, p=0.06	F(1,15)=1.52, p=0.24	F(11,160)=1.08, p=0.38
VNO(m) (nL.sec/min)	F(11,165)=1.60, p=0.10	F(1,15)=3.92, p=0.07	F(11,160)=1.32, p=0.22
NOx (μmol/L)	F(7,77)=1.21, p=0.31	F(1,11)=2.58, p=0.14	F(7,77)= 0.55, p=0.79

Table 2-1. Summary of Statistical results

Definition of abbreviations: [NO]exh *(i)*= NO exhaled concentration plateau integral; [NO]exh *(m)*= NO exhaled concentration plateau mean; VT= Tidal volume; VE= Minute ventilation; VNO (i)= NO production for plateau integral; VNO (m)= NO production for plateau mean; NOx= plasma NO metabolites.

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CHAPTER 3

Paroxetine-induced increase in metabolic end products of nitric oxide (NO)

*The contents of this chapter are represented in a manuscript submitted for publication (**Nathalie Lara MD**[*], Stephen L. Archer MD[Ø], Glen Baker PhD, DSc[*] and Jean-Michel Le Mellédo MD[*]). The author of this thesis played a major role in this study, including design and revision of protocol, research visits, data collection and analysis, and writing of the manuscript.

3.1. INTRODUCTION

The vascular endothelium synthesizes and releases several relaxing factors, including nitric oxide (NO). NO is synthesized from the amino acid L-arginine by a family of enzymes known as nitric oxide synthases (NOS). Three isoforms of nitric oxide synthases (NOS) have been identified and cloned: the neuronal (nNOS) and endothelial (eNOS) constitutive isoforms which are regulated by changes in intracellular free Ca^{2+} concentration, and the inducible (iNOS) isoform which is Ca^{2+}-independent. Platelets are an important site for NO production mediated by a Ca^{2+}-dependent endothelial type constitutive NOS isoform[1,2];however, this contribution of platelets to plasma NOx is not as important when compared to the contribution by the endothelium. Endothelium-derived NO not only participates in the modulation of the vascular tone, inducing vasorelaxation, but also inhibits several proatherogenic processes such as smooth muscle proliferation and migration, platelet aggregation and adhesion, and oxidation of low density lipoproteins (LDLs)[3]. Numerous CV disorders such as hypertension, heart failure and coronary heart disease (CHD) have been associated with alterations in the NO system[4]. In addition, conventional CV risk factors such as hypercholesterolemia, physical inactivity and smoking have been found to decrease endothelial NO production and more specifically NOx levels[5,6,7,8].

The direct measurement of plasma NO concentration remains difficult *in vivo* because NO is rapidly inactivated by hemoglobin or oxidized to form several nitrogen dioxides (NOx). Plasma NOx concentration is often used as a marker for endothelial NO production[9].

Major depressive disorder (MDD) has been associated with a 2.5 to 4 fold increase in occurrence of CV events in patients suffering from CV illness[10]. It has also been described as an independent risk factor for the future development of heart disease in healthy subjects[11].

Selective serotonin reuptake inhibitors (SSRIs) are effective therapeutic agents in the treatment of MDD and are associated with fewer CV side-effects when compared to tricyclic antidepressants[12]. *In vitro*, paroxetine is the most potent inhibitor of the reuptake of serotonin of all the currently available SSRI's[13]. Finkel *et al.* (1996) reported a paroxetine-induced decrease in plasma NOx of patients with ischemic heart disease treated for MDD[14]. However, that study was associated with multiple confounding factors, including the fact that it was performed in cardiac patients for whom cardiovascular medications (often affecting NO production) were not controlled for. Since decreased endothelium NO production is associated with increased cardiovascular risk[15], it is a concern that paroxetine-induced decrease in NO production may lead to a long term deleterious effect on the cardiovascular system.

It is therefore crucial, as a first and well controlled step, to test the effects of paroxetine on plasma NOx in healthy volunteers (HVs). The present study investigates the effects of 8 weeks of paroxetine treatment on NO production determined by plasma NOx in male HVs.

3.2. METHODS

3.2.1. Subjects

Eighteen male (mean age± SEM 24.89±1.26) non-smokers participated in the study. All subjects were normotensive, taking no medications, had no evidence of medical conditions at the time of the study and had no family history of coronary heart disease. The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) was used to exclude any Axis I psychiatric disorder. The research protocol was approved by the Health Research Ethics Board of the University of Alberta, Faculty of Medicine and Dentistry, and all subjects provided written consent. No dropouts occurred as a result of adverse events caused by paroxetine. Subjects were financially compensated for their participation in the study.

3.2.2. Experimental Design

Paroxetine was initiated gradually at a dose of 10 mg/day for 3 days and then increased to 20mg/day for 8 weeks. After completion of the eight week of treatment, paroxetine dose was decreased from 20mg/day to 10 mg/day for 5 days, and then discontinued. Venous plasma NOx concentrations were measured at baseline, after 8 weeks of paroxetine administration and 6 to 10 days post-discontinuation.

3.2.3. General procedures

To prevent contamination of NOx measurements by nitrites/nitrates contained in

certain types of food, subjects followed a nitrate/nitrite-restricted diet for 3 days before the test and fasted overnight (12 hours)[16,17,18]. In addition, subjects were instructed to avoid taking any medication that could interfere with the NO measurements for the same period of time previous to the test[19,20].

Venous blood samples were obtained from the antecubital vein at baseline, after 8 weeks of paroxetine administration and 6 to 10 days post-discontinuation

Assessment of changes in physical activity was performed by the 7-day recall interview[21] at the same time intervals.

3.2.4. NOx measurements

Each plasma sample was centrifuged at 3000 rpm for 10 min at 4°C. To decrease sample viscosity an equal volume of NO-free double distilled deionized water was added to plasma samples. NO does not exist *per se* in the blood but is rapidly inactivated by the interaction with hemoglobin or oxidized to form several nitrogen oxides (NOx), in particular, nitrates. NOx can be measured in small volumes of plasma (0.1 mL) by converting them to NO which in turn is measured by chemiluminescence, using a strong reducing environment: vanadium (III), 1N HCl at 90°C[9]. An antifoaming agent is added to avoid the foam caused by plasma proteins (Dow Corning, Midland, MI).

3.2.5. Measurements of plasma levels of paroxetine

Paroxetine plasma levels were obtained on the last day of week eight of treatment,

and subjects were instructed to take that day's dose 3 hours prior to blood sampling (absorption phase). The purpose of this measurement was to assess compliance of the subjects to paroxetine administration. As a result, two subjects were excluded from the analysis because of lack of detection of paroxetine levels, suggesting that they were not taking paroxetine. Paroxetine plasma levels were analyzed by gas chromatography with electron-capture detection, using a method designed in the laboratory of one of the authors (GBB)[22]. Briefly, this involved basification of the plasma samples, extraction with an organic solvent and derivatization under anhydrous conditions with heptafluorobutyric anhydride followed by analysis on a gas chromatograph equipped with a capillary column, an electron-capture detector and a printer/integrator (Hewlett-Packard Canada, Mississauga, Canada).

3.2.6. Statistical analysis

Repeated measurements ANOVA with time as the within subjects variable, was used and applied to measurements of plasma NOx. *Post hoc* two-tailed paired t-test analyses were also done where appropriate. Values are expressed as mean ± S.E.M. A p value less than 0.05 was considered significant. All statistical analyses were conducted using SPSS statistical software for Windows (SPSS Inc. Chicago, IL).

3.3. RESULTS

Based on the analysis of data obtained for the 16 subjects for whom plasma levels of paroxetine indicated medication compliance, a main time effect was detected for NOx

measurements [F(2,14)=6.78, p=0.009]. After 8 weeks of paroxetine treatment, NOx plasma levels were significantly higher than baseline and post-discontinuation (Fig. 1). No statistically significant differences were observed between NOx levels at baseline and post-discontinuation [t=0.35, df=15, p=0.74]. No significant changes in physical activity were detected after 8 weeks of paroxetine treatment compared to baseline and post-discontinuation [F(2,14)=1.59, p=0.22].

Paroxetine plasma levels were 277.8 ± 63.6 ng/mL (mean±SEM). Because of the presence of a nitrogen atom in the chemical structure of paroxetine, we assessed the possible direct contribution of paroxetine itself to NOx levels measured by chemiluminescence in opposition to a pharmacodynamic effect. The maximum paroxetine plasma level observed was 860.6 ng/mL. Human plasma samples obtained from a healthy volunteer were incubated with known concentrations of paroxetine HCl for 1 hour at 37 °C. Then, NOx were measured by chemiluminescence as described previously. We found, at this plasma concentration, that paroxetine could contribute up to 1.05 μMol/L of the total NOx. Conservative correction by systematic subtraction of 1.05 to from the NOx levels of each subject obtained after 8 weeks of paroxetine administration did not alter the statistical significance of the paroxetine-induced increase in NOx plasma levels [Time effect: F(2,14)=5.27, p=0.02] (Fig.3-1). The statistical significance of the results of all the analyses performed remained when the two subjects in whom compliance to paroxetine administration was doubtful were included in the analysis.

3.4. DISCUSSION

Our results suggest that 8 weeks of paroxetine administration induced a substantial increase in NOx plasma levels. A causal effect of paroxetine is supported by the normalization of NOx following discontinuation of paroxetine. A strength of our investigation is the tight control of factors known to influence NOx levels such as physical activity or intake of nitrites/nitrates with regular diet were controlled for. Unfortunately, the timing of our plasma sampling for paroxetine levels (only three hours post paroxetine intake, i.e. during the absorption phase), prevented us from obtaining meaningful correlations between NOx and paroxetine levels.

Our results are in contradiction to the findings by Finkel *et al*[14]. These authors observed that paroxetine, but not nortriptyline, induced a decrease in plasma NOx levels in depressed patients suffering from ischemic heart disease[14]. However, the concomitant use of cardiac drugs such as NO donors, physical activity and the effect of dietary intake of nitrates/nitrites on NOx measurements were not taken into account in that study. The clinical evolution and severity of the ischemic heart disease which has been associated with alterations in NO production[23,24] were other factors that were not controlled for. Noteworthy, Finkel *et al.* used the Greiss method for NOx measurements, which is considered less proficient than chemiluminescence[25]. Our results imply an increased activity of eNOS in the endothelium, whereas the findings from Finkel's study imply a decreased eNOS activity. Contrary to both studies' assumptions, Angulo *et al.* observed that paroxetine decreased the expression of nNOS but did not affect the expression of

eNOS in rats; however, the eNOS expression was measured in rat penises[26].

Our findings of a paroxetine-induced increase in NOx are supported by reports of the effects of paroxetine on functions in which NO plays a role and more particularly on the dysregulation of platelet activation. Rupture of atheromatous plaques and subsequent occlusive thrombus formation is believed to be responsible for most acute coronary syndromes. Injury of the endothelium leads to platelet adherence and activation, followed by thrombus formation which, when occurring in a coronary vessel, is the acute precipitating factor in most acute coronary syndromes[27]. Both endothelium and platelet-derived NO are available for platelet regulation, decreasing platelet aggregation and adhesion[28]. Musselman et al. have shown that a 6 week treatment with paroxetine normalized the increased platelet activation found at baseline of depressed patients[29]. Pollock et al. have shown that paroxetine induced a decrease in platelet factor 4 (PF4) and β-thromboglobulin (BTG), two proteins that are secreted from the α-granules of platelet upon activation[30] and that have been reported to be increased in depressed patients with comorbid coronary heart disease[31]. Although in an indirect manner, these results are at odds with the hypothesis of paroxetine-induced reduction in peripheral NO production suggested by Finkel *et al.*'s findings, which should be associated with a paroxetine-induced increase in platelet activation[14]. However, the possibility that the effect of paroxetine on platelet reactivity is related to another platelet agent such as serotonin[32,33] cannot be excluded. Although serotonin derived from activated platelets can trigger the release of NO by the endothelial cells through the activation of $5HT_1$-serotonergic receptors[34], Markovitz *et al.* have shown that sertraline (another SSRI)-induced alterations

of serotonin content and receptor density were not responsible for the treatment-induced normalization of the increased platelet activation found in depressed patients[35].

Since impairment of the synthesis of endothelium-derived NO has been extensively characterized in patients with atherosclerosis, risk factors for coronary heart disease and acute coronary syndromes[4], paroxetine-induced decrease in NO production would be expected to be detrimental to patients with coronary heart disease. However, investigations of paroxetine administration have provided evidence that it is safe from a cardiovascular point of view[36]. Our findings demonstrating a paroxetine-induced increase in NO production are compatible with these results as well as with the results of a study suggesting that SSRIs may confer a protective effect against myocardial infarction (MI)[37], an effect that remains to be confirmed in prospective studies. Paroxetine[38] and other SSRIs have been associated with an increase risk of abnormal bleeding[39,40] illustrated by echymoses, epistaxis, internal bleeding and menorrhagia suggestive of decreased platelet function. This again is more consistent with a paroxetine-induced increase in NO rather than a decrease in NO, since both endothelial and platelet-derived NO decrease platelet aggregation and adhesion[28]. The bleeding alterations induced by SSRIs are relatively rare, perhaps reflecting the inter-individual variability that we found in the paroxetine-induced increase in NOx concentrations. Due to the complex mechanisms involved in platelet activation and occurrence of cardiac events, it cannot be excluded, however, that the effects of paroxetine on NO can be offset, compensated for or potentiated by its actions on other systems involved in platelet activation, cardiovascular risk and cardiac regulation.

The substantial amplitude of the observed paroxetine-induced increase in NOx

(40%) associated with the normalization after paroxetine discontinuation suggests a true clinically relevant effect of paroxetine on endothelium-derived NO production. In comparison, 8 weeks of exercise training induced an increase of 58% in NOx plasma levels[41].

By assessing the effect of paroxetine on NOx in HVs, we avoided the potential confounding effects of the depressive illness and its recovery on NOx levels and were able to assess the pharmacological effect of paroxetine in isolation from its therapeutic effect. Paroxetine is primarily used to treat patients with MDD or anxiety disorders; therefore, we cannot extrapolate with certainty our results in HVs to these clinical populations. It will be important to compare our results to those of patients with anxiety disorders and MDD, especially since alterations in platelet reactivity have been described in patients with MDD. We have also initiated investigations assessing the effect of paroxetine on platelet eNOS activity in depressed patients.

Whether the effect of paroxetine on endothelial NO production is specific or shared with other classes of antidepressants or with antidepressants from the same class remains undetermined. Suzuki et al. found that the plasma NOx levels in depressed patients treated with imipramine, amitriptyline or mianserin were decreased, but measurement of NOx were performed on samples collected after depression recovery, 2 weeks after the last dose of antidepressants and potential confounders were not considered[42]. Several other factors such as a small sample size, associated benzodiazepine treatment and measurement of NOx by high-performance liquid chromatography (HPLC), which carries more disadvantages in the determination of nitrites/nitrates, limit the results

of this study. In addition, Finkel *et al.* (1996), found that nortriptyline had no effects on NOx levels in depressed patients[14]. In an attempt to answer the important question of a potential class effect, we are currently studying the effects of fluoxetine on NOx levels and platelet eNOS.

One of the limitations of our study is the short period of administration of paroxetine (8 weeks). This was related to the fact that the study was performed on HVs. Further studies in depressed patients should include assessments of NO production after longer treatment duration to assess whether the paroxetine-induced increase in NO production is long lasting or transient.

In conclusion, due to the association between depression and increased CV risk, it will be essential to assess the effects of paroxetine-induced increase in NO production on the CV outcome of depressed patients and more importantly in depressed patients with comorbid CHD, controlling for relevant confounding factors.

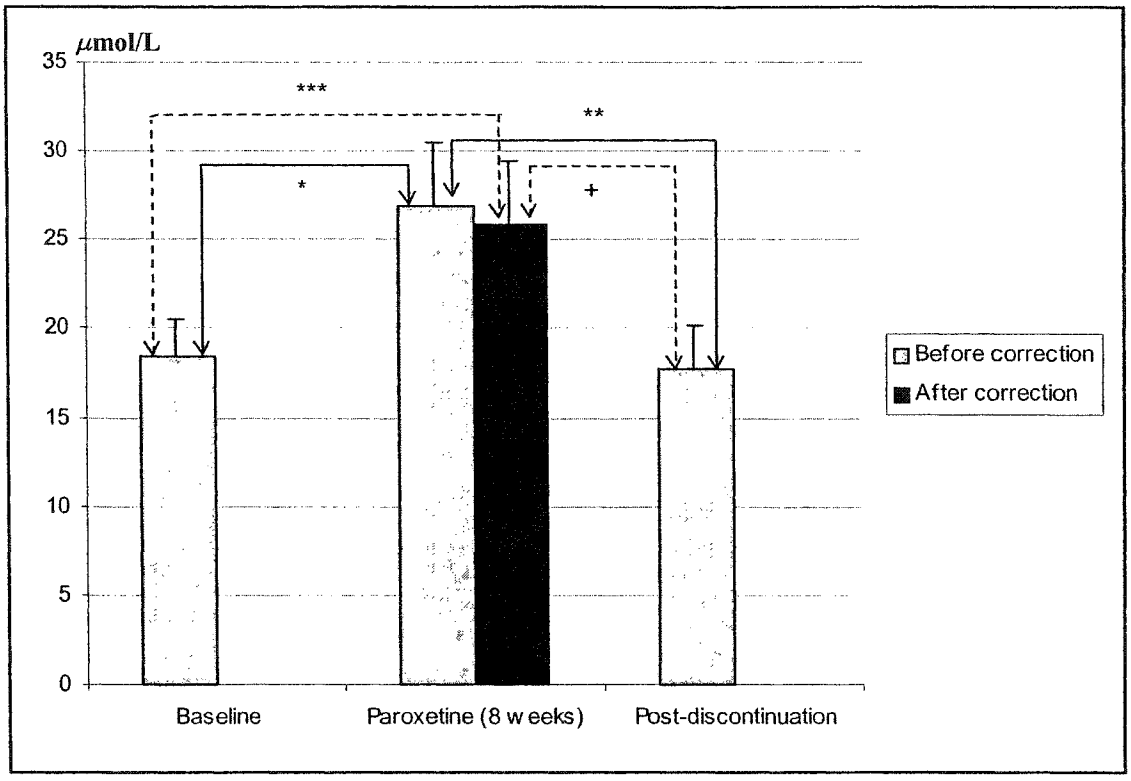

Figure 3-1. NOx plasma levels at baseline, 8 weeks of paroxetine administration and post-paroxetine discontinuation, before and after correction for paroxetine-NO contribution. Bars indicate means± S.E.M. N=16. *Two-tailed paired t-test *post-hoc* analysis [t=2.48, df=15, p=0.03] **Two-tailed paired t-test post-hoc analysis [t=3.30, df=15, p=0.005] ***Two-tailed paired t-test *post-hoc* analysis [t=2.177, df=15, p=0.046] +Two-tailed paired t-test *post-hoc* analysis [t=2.92,df=15, p=0.01]

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CHAPTER 4

General discussion and conclusions

4.1. GENERAL DISCUSSION AND CONCLUSIONS

This thesis is the first investigation of the changes in NO measurements during induced-anxiety/panic in humans, as well as of the effect of paroxetine administration on plasma end metabolic products of NO in healthy volunteers.

In chapter 2, it was shown that the concentration of exhaled NO was decreased during CCK-5-induced panic attacks, an effect probably mediated by increases in minute ventilation since lung or systemic NO production remained unaltered. Increases in both respiratory rate and tidal volume observed during CCK-5-induced panic attacks provoked a significant increase in minute ventilation. Since these results only assessed the possible connection between peripheral actions of NO and respiratory symptoms displayed during panic attacks, we cannot make any inference as to the role of brain NO in the pathophysiology of panic attacks. To further elucidate the possible link between NO and increased cardiopulmonary morbidity and mortality in PD, this study needs to be performed in PD patients.

In chapter 3, the data presented showed that administration of paroxetine produced an increase in end metabolic products of NO (NOx), contrary to the previous study by Finkel et al. that reported decreased NOx plasma levels after paroxetine administration[1]. However, that study was limited by several confounding factors. Paroxetine-induced increased levels of NOx may be responsible for the improvement of some variables, such as reduction of platelet reactivity[2], involved in the progression of cardiovascular pathology which is closely associated with mood disorders[3]. Due to the association between depression and increased cardiovascular risk, it will be important to assess the effects of the paroxetine-induced increase in NO production on the cardiovascular outcome of depressed patients.

4.2. REFERENCES

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