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Decreased cerebrovascular response to CO_2 in post-menopausal females with COPD: role of oxidative stress

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ABSTRACT: Chronic obstructive pulmonary disease (COPD) is associated with cerebrovascular abnormalities and an overproduction of reactive oxygen species. We hypothesised that COPD patients have oxidant-related cerebrovascular dysfunction. Our main objective was to evaluate cerebrovascular reactivity and its relationship with oxidative stress in females with COPD.

We studied eight females with moderate COPD and 10 healthy female control subjects of similar age. Transcranial Doppler ultrasound assessed cerebral blood flow (CBF) velocity during hypercapnia. Plasma was assessed at rest for DNA oxidation, advanced oxidation protein products, lipid peroxidation, nitrotyrosine, antioxidant enzyme activity (glutathione peroxidase and catalase) and end-products of nitric oxide metabolism.

Moderate COPD patients showed decreased cerebrovascular sensitivity to carbon dioxide (CO_2) (COPD 1.17±0.54 *versus* control 2.15±0.73 cm·s^{-1}·mmHg^{-1}; $p<0.01$). COPD patients had higher levels of DNA and lipid oxidation, advanced oxidation protein products and higher glutathione peroxidase activity ($p<0.05$). Controlling for measures of oxidative stress (DNA and lipid oxidation, and advanced oxidation protein product) eliminates statistically significant differences between the COPD and control groups in the CBF sensitivity to CO_2.

Females with moderate COPD were found to have cerebrovascular dysfunction. Our results suggest that increased levels of systemic oxidative stress may have implications in the cerebrovascular dysfunction observed during hypercapnia in COPD.

KEYWORDS: Cerebrovascular circulation, chronic obstructive pulmonary disease, hypercapnia, reactive oxygen species, respiration, transcranial Doppler ultrasonography

Most recently, chronic obstructive pulmonary disease (COPD) is gaining attention for the consequential systemic manifestations and comorbidities resulting from the disease [1]. Although these patients are at a three- to four-fold increased risk of developing cardio- and cerebrovascular disease, limited information is available regarding the pathophysiology of vascular diseases in COPD. Extrapulmonary consequences, including altered arterial blood gas levels, pH imbalance, increased oxidative stress, vascular dysfunction and autonomic disturbances, all have the potential to alter the regulation of cerebral blood flow (CBF). In healthy individuals, CBF and ventilation increase linearly with arterial carbon dioxide tension (P_{a,CO_2}). It is well established that COPD patients have a decreased ventilatory output to carbon dioxide (CO_2) [2–4]. However, few studies have investigated the cerebrovascular response to CO_2 in these subjects, and how decreased ventilation can affect this response. Recent literature suggests that cerebrovascular sensitivity and ventilatory response are tightly linked in healthy individuals [5]. However, evidence suggests that COPD patients exhibit cerebrovascular disturbances [3, 6, 7], although the pathological onset and cause of these disturbances have not yet been comprehensively studied.

Furthermore, the role of oxidative stress pertaining to cerebrovascular dysfunction is of particular interest in COPD patients. COPD is associated with an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to an imbalance of oxidants–antioxidants, resulting in oxidative stress [8]. ROS have been implicated in the role of vascular

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dysfunction both directly (*via* dilatory effects of hydrogen peroxide), and indirectly through decreased bioavailability of nitric oxide (NO) *via* the promotion of superoxide anion ($O_2^{\cdot-}$) quenching to form peroxynitrite ($ONOO^-$) [9]. By the reduction of available NO, this reaction (*i.e.* $O_2^{\cdot-} + NO \rightarrow ONOO^-$) can subsequently affect cerebrovascular tone, resulting in vasoconstriction [10].

While a few studies have looked at cerebrovascular health in COPD patients [3, 6, 7, 11], no study has investigated how oxidative stress and antioxidant activity could be involved in the CBF regulation observed in COPD. Oestrogen is a sex hormone with beneficial vasoactive and antioxidant properties. After menopause, oestrogen levels decline sharply and oxidative stress is reported to be increased [12]. This may have an impact on females with COPD who are already at increased risk of exposure to oxidants.

Furthermore, the present collection of literature relating to cerebrovascular physiology and COPD represents exclusively male or mixed sex studies. Recent commentary states that the impact of COPD in females is significantly understudied, and evidence that does exist suggests important sex differences, raising debate as to the role of sex as a potential risk factor for developing COPD [13]. Evidence suggests that females are at increased susceptibility for the development of the disease (*e.g.* they incur COPD after a fewer number of cigarettes per lifetime compared with males), have increased prevalence rates and exhibit the poorest outcomes associated with COPD [14].

We therefore choose to study females exclusively to gain better understanding of certain physiological effects of COPD in this under-represented group. Thus, we sought to determine: 1) the extent to which cerebrovascular regulation is altered in females with moderate COPD, and whether there is a relationship to ventilation; and 2) whether the expected differences in CBF are explained by systemic oxidative stress markers or antioxidant activity. Some of these results have been previously presented in an abstract form [15].

METHODS

Study participants

10 post-menopausal females with smoking-related COPD and 12 healthy, nonsmoking post-menopausal females (controls) were recruited for participation in this study. COPD subjects were recruited from participating outpatient medical clinics within the Calgary Health Region, Canada, and controls were recruited from the community. All study participants visited the Laboratory of Human Cerebrovascular Physiology at the University of Calgary, Calgary (1103 m elevation above sea level) for two testing sessions. Subjects were instructed to refrain from eating or drinking for 4 h prior to each testing session. The study was approved by the institutional Conjoint Health Research Ethics Board and conformed to the Declaration of Helsinki, and all participants provided written, informed consent.

Major inclusion criteria

Patients had physician-diagnosed COPD, with a smoking history of >10 pack-yrs and airflow obstruction (forced expiratory volume in 1 s (FEV_1)/forced vital capacity <70%; FEV_1 ⩽70% predicted). Patients were all ex-smokers (>1 yr), post-menopausal for ⩾12 months, able to walk independently outside or on stairs and had a body mass index < 35 $kg \cdot m^{-2}$. Participating controls were healthy volunteers, with no history of lung disease or regular cigarette smoking (<1 pack-yr). Complete exclusion criteria are listed in the online supplementary material.

Experimental protocol

Participants visited the laboratory on two occasions. The first day consisted of a medical screening questionnaire, pulmonary function testing and venous blood collection. After ~1 week, subjects returned for a CO_2 challenge test, which was conducted in most participants between 11:00 h and 14:00 h.

Pulmonary function test

Spirometry, measures of lung volumes and single-breath diffusing capacity were completed in all subjects according to the American Thoracic Society guidelines [16–18].

Protocol to measure the cerebrovascular and ventilatory response to euoxic hypercapnia

Subjects were comfortably seated in a semi-recumbent position at rest for 10 min for collection of baseline vascular and ventilatory variables. An arterialised capillary blood sample was taken from the middle finger after warming for 3 min. Blood samples were immediately analysed for oxygen tension, carbon dioxide tension (P_{CO_2}) from the mixed venous blood and acid–base balance (ABL 725 Radiometer; Brønshøj, Denmark). Briefly, using dedicated software (BreatheM v2.38; University Laboratory of Physiology, Oxford, UK), the technique of dynamic end-tidal forcing [19] was used to precisely target the desired end-tidal carbon dioxide tension (P_{ET,CO_2}) and end-tidal oxygen tension (P_{ET,O_2}). The ~10-min hypercapnic protocol progressed to hypercapnia at 9 mmHg above the resting P_{ET,CO_2}, while P_{ET,O_2} was held constant at baseline values. The protocol was designed as five 2-min stages of increasing P_{ET,CO_2}, in a stepwise fashion (*i.e.* 1, 3, 5, 7 and 9 mmHg above eucapnia).

Heart rate, oxygen saturation and continuous beat–beat blood pressure was measured *via* finger pulse photoplethysmography. Respiratory volumes were measured with a turbine and volume transducer (VMM-400; Interface Associates, Laguna Niguel, CA, USA), and respiratory flow direction and timing were obtained with a pneumotachograph (RSS100-HR; Hans Rudolf, Kansas City, MO, USA). Middle cerebral artery blood flow velocity was continuously measured using a 2-Hz pulsed Doppler Ultrasound system (TC22; SciMed, Bristol, UK). Peak blood flow velocity (v_{peak}) was used as a surrogate for global CBF. The power (P) signal acquired from the Doppler system is used as an indicator for changes in vessel diameter. Therefore, without a change in P, v_{peak} is considered to be a reliable index of flow.

Biochemical analyses

Venous blood was collected at rest into two EDTA and two serum separator tubes for biochemical analysis. Blood was centrifuged at 1,000 × *g* for 10 min at 4°C. Plasma and serum were separated into appropriate aliquots and frozen at -80°C until assays were performed. Assays to measure plasma levels of oxidative stress (8-hydroxy-2′-deoxyguanosine (8-OHdG), malondialdehyde (MDA), advanced oxidation proteins product (AOPP)) nitrosative stress (nitrotyrosine), antioxidant

enzyme activity (glutathione peroxidase (GPX) and catalase activities), and end-products of nitric oxide (nitrites and nitrates) were performed. Hormone and cholesterol analysis was performed by Calgary Laboratory Services. Further procedural details are provided in the online supplementary material.

Data and statistical analysis

The main outcome variable was the cerebrovascular sensitivity to CO_2. Secondary outcomes included the ventilatory and blood pressure responses to CO_2, and molecular markers related to vascular function (oxidative stress, antioxidants and end-products of nitric oxide (NOx)). An estimate of the cerebrovascular sensitivity (*i.e.* $v$peak sensitivity) to CO_2 was calculated for each individual as the slope of the regression relating $v$peak *versus* P_{ET,CO_2} during hypercapnia. Likewise, the minute ventilation (V'_E) sensitivity to CO_2 was calculated as the regression slope of V'_E against P_{ET,CO_2} during hypercapnia. The last 30 s of data of each hypercapnic stage was averaged and used in this calculation. Where percentage changes are reported, these data were normalised to the last 3 min of the isocapnia euoxia baseline period preceding the hypercapnic steps.

Using the variability (SD 0.57 $cm \cdot s^{-1} \cdot mmHg^{-1}$) and expected between-group difference of 0.75 $cm \cdot s^{-1} \cdot mmHg^{-1}$ from a previously published study [7], we determined that to detect a significant difference in the cerebrovacular sensitivity to hypercapnia, each group would have to consist of 11 participants (using an unpaired two-tailed t-test and setting α at 0.05 and β at 0.80).

Tests of normality and homogeneity of variance were performed and confirmed the appropriate use of parametric statistical procedures (SPSS Version 17.0; SPSS Inc., Chicago, IL, USA). The CO_2 sensitivity indices for CBF and V'_E were compared between groups using an independent t-test. The main effect of ''hypercapnia'' and the ''group × hypercapnia'' interaction was evaluated on physiological variables (respiratory (P_{ET,CO_2}, P_{ET,O_2}, V'_E, respiratory frequency (f_R), tidal volume and V'_E/maximum voluntary ventilation), cardiovascular (mean arterial blood pressure (MAP), cardiac frequency (f_C) and arterial oxygen saturation) and cerebrovascular ($v$peak and cerebrovascular conductance)) using a repeated measure ANOVA. The repeated effect of each CO_2 stage (*i.e.* 1, 3, 5, 7 and 9 mmHg) was evaluated on the aforementioned physiological variables. Individual cerebrovascular and ventilatory sensitivities were ranked, and Spearman's correlation coefficient was used to evaluate the strength of the relationship between V'_E and $v$peak sensitivity. Group differences in molecular markers (*i.e.* oxidants, antioxidants and NOx metabolism) were assessed using independent t-tests. *Post hoc* analysis assessed relationships between these molecular markers and main cardio- and cerebrovascular outcome variables using Pearson's correlations. Using exploratory analyses, we used several baseline factors (blood pressure, age, oxidative stress, antioxidant enzyme status and NO metabolism) as covariates in a one-way ANCOVA to find the best ''adjusted'' model to explain the variance in $v$peak sensitivity to CO_2 between groups. Data are presented as mean ± SD, and significance was set at $p \leqslant 0.05$. Confidence intervals are calculated at 95%.

RESULTS

Subject characteristics

Data were not obtained in two subjects (one control and one COPD subject) due to lack of a suitable middle cerebral artery signal. Furthermore, data from one COPD patient was excluded because the patient did not complete the CO_2 challenge to entirety, due to overwhelming breathlessness. A blood sample was not obtained in one control. Therefore, we analysed data from 10 controls and eight COPD patients.

Physical characteristics and pulmonary function tests of participants are summarised in table 1. Measurements of resting blood gases acquired by end-tidal and *via* capillary blood is summarised in table 2.

Physiological responses to hypercapnia

At rest, there were no significant differences between main vascular outcomes (*i.e.* $v$peak, MAP and f_C) between COPD patients and controls (table 3). In response to hypercapnia, the vascular response in COPD patients was blunted, as evidenced by only a 19% increase in $v$peak (*versus* 37% in controls; $p<0.01$) and an 8% increase in MAP (*versus* 12% in controls; $p>0.05$) from baseline (fig. 1a and b). The mean $v$peak sensitivity (unadjusted) for COPD patients was significantly decreased (COPD 1.17 ± 0.54 *versus* control 2.15 ± 0.73 $cm \cdot s^{-1} \cdot mmHg^{-1}$; $p<0.01$) (fig. 2a).

TABLE 1 Subject characteristics

	COPD		Controls	
	Mean ± SD	% pred	Mean ± SD	% pred
Subjects n	8		10	
Physical characteristics				
Age yrs	69.4 ± 4.3		64.3 ± 6.2	
BMI $kg \cdot m^{-2}$	26.5 ± 6.4		25.4 ± 2.3	
Total cholesterol $mmol \cdot L^{-1}$	5.16 ± 0.86		5.21 ± 0.87	
HDL cholesterol $mmol \cdot L^{-1}$	1.66 ± 0.41		1.46 ± 0.38	
Oestradiol $pmol \cdot L^{-1}$	69 ± 32		52 ± 16	
Progesterone $nmol \cdot L^{-1}$	2.2 ± 3.5		1.2 ± 0.5	
Smoking pack-yrs	42.9 ± 11.2#		0.16 ± 0.25	
Lung function				
FEV_1 L	1.28 ± 0.28#	63	2.52 ± 0.33	106
FVC L	2.55 ± 0.51#	95	3.24 ± 0.38	107
TLC L	5.55 ± 0.90	119	5.11 ± 0.48	100
RV L	3.04 ± 0.87#	159	1.78 ± 0.29	87
FRC L	3.57 ± 0.85#	134	2.47 ± 0.36	85
IC L	1.89 ± 0.35#		2.58 ± 0.40	
$D_{L,CO}$ $mL \cdot min^{-1} \cdot mmHg^{-1}$	12.1 ± 3.6#	64	21.8 ± 2.3	109

COPD: chronic obstructive pulmonary disease; % pred: % predicted; BMI: body mass index; HDL: high-density lipoprotein; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; FRC: functional residual capacity; IC: inspiratory capacity; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide. #: significantly different from controls at $p \leqslant 0.01$.

TABLE 2 Comparison of end-tidal and capillary blood gases, haematocrit and acid–base balance between chronic obstructive pulmonary disease (COPD) and control groups

	COPD	Controls
Subjects n	7	8
pH	7.44±0.02	7.46±0.09
P_{c,CO_2} mmHg	37.1±4.8	34.9±4.3
P_{c,O_2} mmHg	59.5±7.8	59.8±6.8
P_{ET,CO_2} mmHg	32.5±2.7	33.6±2.8
P_{ET,O_2} mmHg	90.3±4.0	89.4±6.2
[HCO_3^-] mmol·L^{-1}	25.7±2.9	24.7±4.9
[tHb] g·dL^{-1}	16.1±0.7	13.5±2.7

Data are presented as mean±SD unless otherwise stated. P_{c,CO_2}: capillary carbon dioxide tension; P_{c,O_2}: capillary oxygen tension; P_{ET,CO_2}: end-tidal carbon dioxide tension; P_{ET,O_2}: end-tidal oxygen tension; [HCO_3^-]: bicarbonate ion concentration; [tHb]: total haemoglobin concentration.

Ventilatory output was decreased in COPD patients at the end of the CO_2 challenge ($p \leqslant 0.05$) (fig. 1c); however, there was only a trend towards a statistical difference when considering the V'_E sensitivity to CO_2 (0.90±0.61 *versus* 1.83±1.26 L·min^{-1}·$mmHg^{-1}$; $p=0.07$) (fig. 2b). Increases in ventilation were achieved primarily by an increase in expired tidal volume (V_{TE}), rather than f_R (table 3). Using inspiratory tidal flow (volume of inspired tidal breath (V_{TI})/ inspiratory time (t_I)) as an indication of respiratory drive, COPD patients showed a similar drive to breathe at rest between compared with controls, whereas V_{TI}/t_I was reduced during hypercapnia in COPD patients (table 3).

Each individual v_{peak} and V'_E sensitivity score was ranked amongst all participants. There was a significant positive correlation between the ranking of ventilatory and cerebrovascular sensitivity to hypercapnia. Subjects with low cerebrovascular sensitivity to CO_2 had a corresponding low ventilatory sensitivity and *vice versa* ($r=0.61$, $p<0.01$).

Biochemical analysis: oxidative stress, antioxidant enzyme activity and NO level

Oxidative stress and antioxidant activity

Results of biochemical assays are summarised in table 4. COPD patients showed significantly higher levels of oxidative stress as indicated by increased plasma 8-OHdG, MDA and AOPP ($p \leqslant 0.05$). These patients also had significantly higher antioxidant enzyme activity in the form of GPX ($p \leqslant 0.01$). The ratio between oxidative stress and antioxidant activity (*i.e.* 8-OHdG and GPX) was significantly higher in COPD patients than controls (1.03±0.50 *versus* 0.62±0.23, respectively; $p \leqslant 0.05$).

Vascular parameters and oxidative stress

We performed (within group) correlation analysis between plasma oxidative stress/antioxidant markers and vascular outcomes to measure the strength of the relationship between these variables. We did not find any significant relationships between oxidative stress (8-OHdG, AOPP, MDA and

TABLE 3 Physiological variables at rest and during hypercapnia

	Baseline isocapnia		Hypercapnia		
	COPD	Controls	COPD	Controls	p-value
Subjects n	8	10	8	10	
Respiratory					
P_{ET,CO_2} mmHg	34.0±2.6	35.2±3.1	41.6±2.7	43.0±3.3	$\leqslant 0.05^{\#}$
P_{ET,O_2} mmHg	90.1±3.9	88.8±6.6	90.3±4.3	89.0±7.5	
V'_E L·min^{-1}	7.6±1.3	8.2±1.7	14.5±5.5	23.3±9.5	$\leqslant 0.05^{\#,+}$
f_R breaths·min^{-1}	14±4	12±4	16±3	16±4	$\leqslant 0.05^{\#}$
V_{TE} L·$breath^{-1}$	0.59±0.18	0.72±0.22	0.95±0.38	1.50±0.61	$\leqslant 0.05^{\#,+}$
V_{TI}/t_I L·s^{-1}	0.34±0.11	0.34±0.07	0.59±0.21	0.82±0.33	$\leqslant 0.05^{\#,+}$
t_I/t_{tot}	0.38±0.05	0.41±0.05	0.39±0.05	0.43±0.04	
V'_E/MVV %	18±5*	10±3	32±8	26±10	$\leqslant 0.05^{\#,+}$
Cardiovascular					
MAP mmHg	86.6±9.8	87.6±12.3	92.8±9.2	97.7±12.2	$\leqslant 0.05^{\#}$
f_C beats·min^{-1}	67±11	67±9	70±11	71±10	
S_{a,O_2} %	94±2*	96±1	95±2	96±1	
Cerebrovascular					
v_{peak} cm·s^{-1}	46.9±6.9	46.6±15.0	55.8±9.6	63.3±19.6	$\leqslant 0.05^{\#}$
CVC cm·s^{-1}·$mmHg^{-1}$	0.55±.08	0.54±0.16	0.60±0.08	0.65±0.17	$\leqslant 0.05^{\#,+}$

Data are presented as mean±SD unless otherwise stated. COPD: chronic obstructive pulmonary disease; P_{ET,CO_2}: end-tidal carbon dioxide tension; P_{ET,O_2}: end-tidal oxygen tension; V'_E: expired minute ventilation; f_R: respiratory frequency; V_{TE}: volume of expired tidal breath; V_{TI}: volume of inspired tidal breath; t_I: inspiratory time; t_{tot}: total time of respiratory duty cycle; MVV: maximum voluntary ventilation; MAP: mean arterial blood pressure; f_C: cardiac frequency; S_{a,O_2}: arterial oxygen saturation; v_{peak}: peak cerebral blood flow velocity; CVC: cerebrovascular conductance (CVC=v_{peak}/MAP). *: $p \leqslant 0.05$, significantly different from controls at baseline; $^{\#}$: significant main effect of hypercapnia; $^{+}$: significant group interaction with hypercapnia.

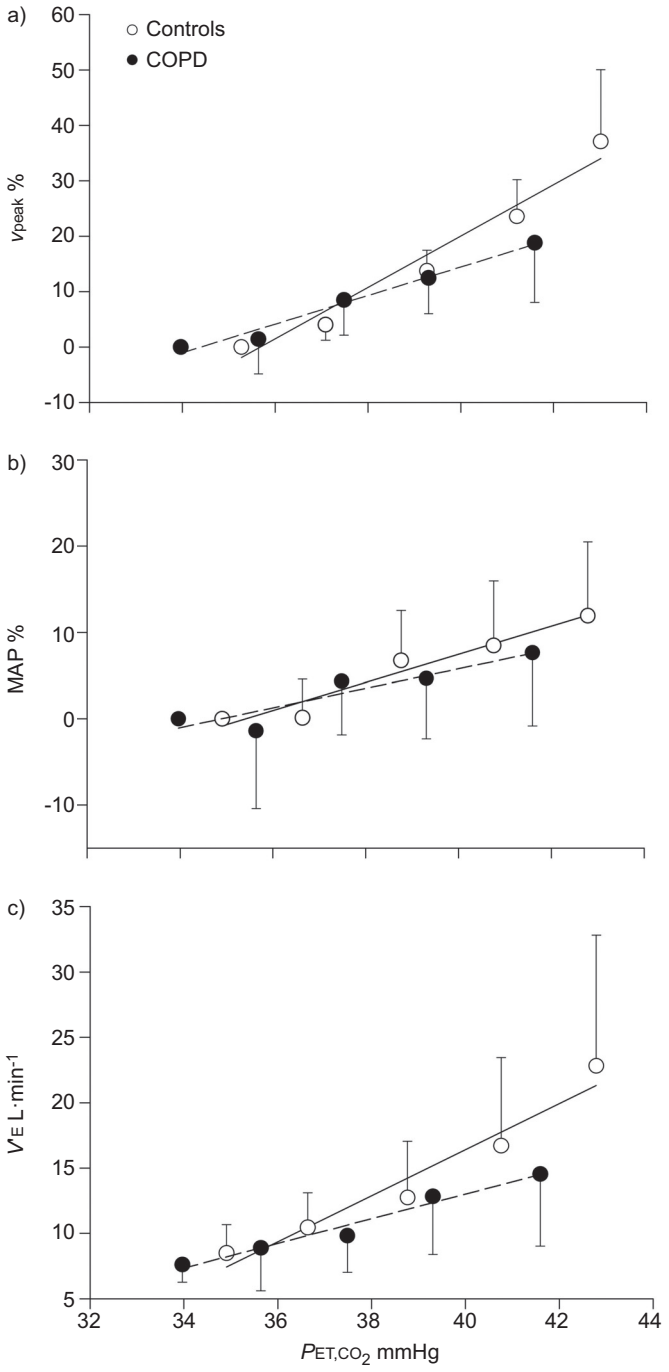

FIGURE 1. Change in a) peak cerebral blood flow velocity (v_{peak}), b) mean arterial pressure (MAP) and c) minute ventilation (V'_E) during incremental steps of euoxic hypercapnia in chronic obstructive pulmonary disease patients demonstrate a decreased cerebral blood flow response to hypercapnia ($p<0.05$). P_{ET,CO_2}: end-tidal carbon dioxide tension. Error bars represent the standard deviation.

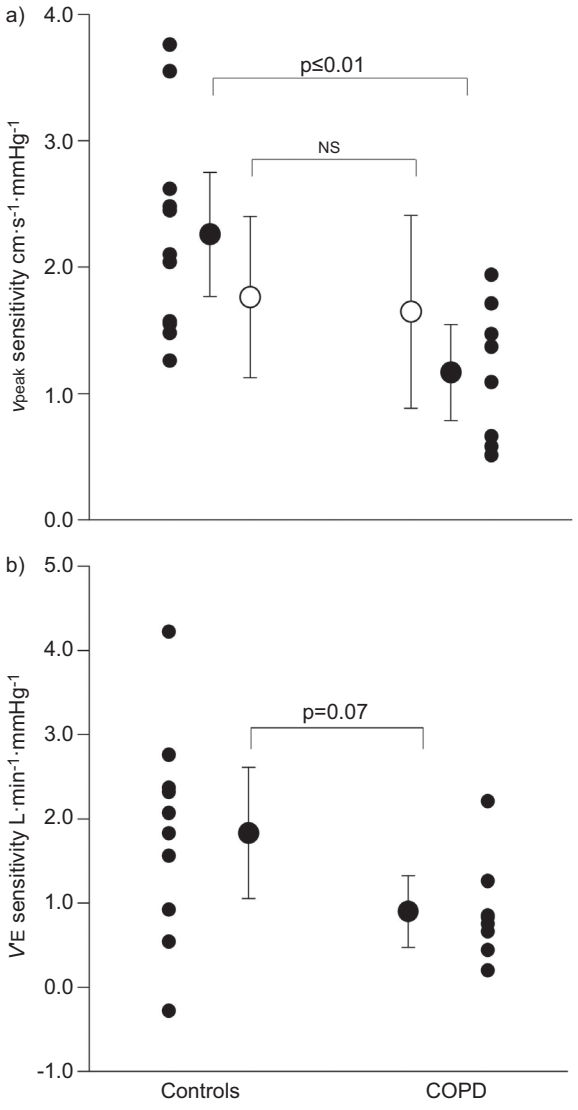

FIGURE 2. Physiological responses to carbon dioxide in chronic obstructive pulmonary disease (COPD) patients and controls. Cerebrovascular sensitivity indices were calculated as the slope of the line relating peak cerebral blood flow velocity (v_{peak}) or minute ventilation (V'_E), respectively, to the increases in end-tidal carbon dioxide (+9 mmHg above rest). Mean values are presented with 95% confidence intervals. a) Cerebrovascular sensitivity to carbon dioxide. Individual (small closed circles) and unadjusted mean (large close circles) responses are plotted for both COPD and controls. The cerebrovascular sensitivity is decreased in the COPD group when comparing the unadjusted means (COPD y=1.22x+5.11; controls y=2.16x+31.03). Once means are adjusted for oxidative stress (8-hydroxy-2'-deoxyguanosine, malondialdehyde and advanced oxidation proteins product) (open circles), no significant difference (NS) between groups exists. b) Ventilatory sensitivity to carbon dioxide. Individual (small closed circles) and mean (large closed circles) responses are plotted for both COPD and controls. There is a trend to decreased ventilatory response during hypercapnia in COPD patients compared with contols (COPD $y=0.89x+6.28$; controls $y=1.75x+5.50$).

nitrotyrosine) and v_{peak} sensitivity, MAP or cerebrovascular conductance. However, COPD patients with higher catalase activity were found to be associated with higher v_{peak} sensitivity ($r^2=0.59$, $p<0.05$).

A one-way ANCOVA was conducted using ''group'' (COPD or control) as the fixed factor, and cerebrovascular sensitivity as the dependent variable. A preliminary analysis evaluating the homogeneity of regression (slopes) assumption indicated the relationship between the covariates and dependent variable did not differ significantly (8-OHdG: F ratio (F)(1,14)= 1.45, p=0.248; AOPP: F(1,14)=0.466, p=0.506; MDA: F(1,14)=

TABLE 4 Plasma oxidative stress markers, antioxidant enzyme activity, and end-products of nitric oxide metabolism in chronic obstructive pulmonary disease (COPD) patients and controls

	COPD	Controls
Subjects n	8	10
Oxidative stress markers		
8-OHdG $\mu g \cdot L^{-1}$	$9.6 \pm 0.7^{\#}$	8.5 ± 1.3
MDA $\mu mol \cdot L^{-1}$	$23.4 \pm 4.7^{\P}$	13.1 ± 4.7
AOPP $\mu mol \cdot L^{-1}$	$292.1 \pm 125.1^{\P}$	134.8 ± 70.0
Nitrotyrosine $nmol \cdot L^{-1}$	93.1 ± 102.0	38.7 ± 34.2
Antioxidant enzyme activity		
Catalase $\mu mol \cdot L^{-1} \cdot min^{-1}$	10.5 ± 6.6	9.0 ± 7.1
GPX $\mu mol \cdot L^{-1} \cdot min^{-1}$	$16.9 \pm 4.9^{\P}$	9.5 ± 3.2
NO end-products		
NOx $\mu mol \cdot L^{-1}$	$4.9 \pm 2.2^{\#}$	10.9 ± 6.4

Data are presented as mean ± SD unless otherwise stated. 8-OHdG: 8-hydroxy-2′-deoxyguanosine; MDA: malondialdehyde; AOPP: advanced oxidation protein products; GPX: glutathione peroxidase; NOx: nitrogen oxides (end-products) of nitric oxide metabolism). #: $p \leqslant 0.05$ *versus* controls; ¶: $p \leqslant 0.01$ *versus* controls.

0.081, p=0.780). Using these covariates, the ANCOVA was nonsignificant (F(1,13)=0.033, p=0.858), thus eliminating the difference in cerebrovascular sensitivity previously observed between groups (COPD 1.65 ± 1.08 $cm \cdot s^{-1} \cdot mmHg^{-1}$ *versus* controls 1.76 ± 1.01 $cm \cdot s^{-1} \cdot mmHg^{-1}$) (fig. 2a).

Based on our findings that suggest a potential role between oxidative stress and cerebrovascular dysfunction in females with COPD, we incorporated additional statistical analyses to offer a clinical perspective. Using the adjusted model, participants were assigned to either ''COPD'' or ''control'' group according to the plasma levels of oxidative stress. Positive predictive and negative predictive values (PPV and NPV, respectively) were calculated, where the prevalence rate of COPD was 44% (eight out of 18). Individuals were identified as having 'COPD' if the level of plasma oxidative stress was above the expected mean concentration (of 8-OHdG, MDA or AOPP). Individuals were identified as 'healthy/control' if all three levels of oxidative stress markers were below the adjusted mean oxidative stress concentration. Results indicated the PPV to be 93.5% and the NPV to be 100%.

DISCUSSION

We present novel data indicating a link between cerebrovascular reactivity and measures of systemic oxidative stress in COPD patients. The major finding in this study is an impaired cerebrovascular response to hypercapnia in females with COPD. We report a blunted cerebrovascular dilatory response in most, but not all, COPD patients, even at modest levels of CO_2 administration. As predicted, higher levels of systemic oxidative stress markers were found in the COPD patient cohort. As suggested in other populations [10, 20], in this context increased oxidative stress could explain the differences observed between the cerebrovascular sensitivity to hypercapnia observed between COPD patients and healthy control subjects.

Physiological response to hypercapnia

It is well known that in a healthy population, increased P_{a,CO_2} induces cerebrovascular dilation, leading to an increase in CBF [21]. This response depends on several cooperative pathways: 1) the chemical stimulus (pH/H^+) at the central chemoreceptors; 2) the ventilatory response (*e.g.* respiratory muscles); and 3) the vasodilatory response in the small vessels of the cerebral circulation. Insufficiencies at any one of these levels can lead to an abnormal hypercapnic response.

Our findings show COPD patients to have a lower CBF response to CO_2 compared with healthy controls (19% *versus* 41%, respectively). We did not find evidence of chronic cerebrovascular dilation in COPD patients. Our results are supported in both an animal model [22], and hypercapnic patients with severe COPD [6]. Cigarette smoking is known to induce both acute and chronic cerebrovascular dilation in healthy individuals, but cerebrovascular reactivity appears to be maintained. In young adults, cerebrovascular deficiencies are only observed following acute smoking (after smoking for 1 min) [23]. Similarly, in a healthy older population, smoking status was not a significant factor in determining the sensitivity to hypercapnia [7]. Interestingly, in this same study, BERNARDI *et al*. [7] found that individuals who were current smokers had mild COPD and significantly lower cerebrovascular sensitivity to hypercapnia compared with individuals who only had mild airflow obstruction, without a smoking history. Overall, however, the cerebrovascular reactivity in mild COPD patients did not differ from matched healthy controls. We now show that normocapnic patients with moderate COPD show cerebrovascular abnormalities. We believe that frequent stimuli specific to individuals with COPD (*e.g.* cigarette smoking and occurrence of frequent arterial oxygen desaturations) may exhaust the normal vascular response *via* constant vasodilation/constricting cycles. Acute effects of smoking cause an immediate constriction of the pial arteries followed by vasodilation, which is likely to be mediated by nicotine that stimulates NO release [24]. Extensive reviews on the topic suggest that increased oxidative stress may lead to either a decreased generation or bioavailability of NO leading to vasomotor dysfunction specific to the vascular endothelium [25, 26].

We found that patients with COPD showed a trend towards a decreased ventilatory response to hypercapnia. Both mechanical (respiratory) limitations and desensitisation of the central chemoreceptor have been implicated in the explanation of this pathology. A decreased ventilatory response provides an avenue for increased cerebral dilation in COPD participants. This is contrary to what we observed in patients, as the cerebrovascular response was blunted. In healthy individuals, XIE *et al.* [27] showed that decreased cerebrovascular responsiveness to CO_2 stimulates the ventilatory response, suggesting that cerebrovascular sensitivity to CO_2 has great influence on the $\dot{V}'E$ responsiveness of the central chemoreceptors. It is possible that the mechanisms involved in the control of breathing and cerebrovascular regulation in COPD patients are independently altered (*i.e.* two separate mechanisms affect these outcomes).

Molecular markers

Oxidative stress and antioxidant status

Oxidative stress represents an unfavourable imbalance between ROS and antioxidants, either from the overproduction

of oxidants, or the depletion of antioxidants. In addition to endogenous sources of ROS (normal cellular metabolism), COPD patients are exposed to exogenous forms of free radicals from environmental pollutants and/or cigarette smoke. Our findings indicate a significant increase in both systemic oxidative stress markers (8-OHdG, MDA and AOPP) and increased antioxidant enzyme activity (GPX) in COPD patients compared with healthy control subjects, and are in similar agreement with other published data [28–31]. In contrast to our results, increased glutathione has previously been shown to be negatively correlated with lung function in patients with chronic airflow limitation [30]. We suspect that there may be an adaptive response involved in the oxidative stress–antioxidant enzyme response, and that the high level of oxidants stimulates the antioxidant enzymatic system in an effort to counteract the high burden of oxidants. Recent reports suggest a decrease in antioxidant capacity in both healthy smokers and patients with COPD, when compared with nonsmoking controls [29, 32]. Furthermore, antioxidant status was not different between current or ex-smokers in either the healthy subjects or those with COPD in these groups, implying that the disease state itself is a determinant of systemic oxidative stress rather than current smoking habit [32].

Nitric oxide and vascular parameters

Smoking is known to alter NO bioavailability [33] and cause endothelial dysfunction [34]; however, less is known how this affects COPD patients. NO is produced by the conversion of L-arginine to L-citrulline in the presence of NO synthases. One possible explanation for the decrease in NOx that we observed is that increased ROS ($O_2^{\cdot-}$) reacts with NO forming $ONOO^-$, consequently leading to the formation of 3-nitrotyrosine, thereby reducing the availability of NO. Although nitrotyrosine tended to be higher in COPD subjects ($p=0.11$), we did not find a significant negative correlation between NOx and nitrotyrosine, as expected, suggesting that other mechanisms regulate NO metabolism, such as NO synthase [35]. We have previously shown in healthy older females that higher levels of NOx are associated with a decrease in resting arterial blood pressure [20]; however, resting MAP did not differ between COPD patients and control subjects, and thus no relationship was found between NOx and MAP. This same study [20] suggests that in a healthy ageing population, increased ROS and $ONOO^-$ may in part be a detrimental contributor in the determination of cerebrovascular tone. We anticipated NOx to have a greater involvement in the cerebrovascular indices measured, since it is known that tone of cerebral blood vessels is influenced by NO under resting conditions, and the loss of NO bioavailability produces vasoconstriction [10]. Furthermore, increases in CBF during hypercapnia also appear to be dependent on production of NO [36].

Limitations

A limitation of our study is the use of end-tidal measurements as an indication of arterial gas concentrations. Caution should be taken in making this comparison, particularly in elderly populations and individuals with chronic lung disease, due to steeper alveolar–arterial gradients. To account for this limitation, we obtained capillary blood samples to measure blood gases at rest. When considering P_{CO_2}, protons and bicarbonate, good agreement has been shown between arterial and capillary samples in patients with chronic lung disease [37]. In COPD patients, we found that P_{ET,CO_2} was significantly lower than capillary P_{CO_2} (P_{c,CO_2}), consistent with increased alveolar dead space and a widened alveolar–arterial gradient. However, based on the P_{c,CO_2}, we can conclude that, on average, the COPD group is not chronically hypercapnic. Direct arterial blood samples are invasive and at the risk of compromising patient recruitment, were not included in our study.

Our sample size was calculated to detect differences in our main outcome variable (*i.e.* v_{peak} sensitivity to CO_2). We do, however, acknowledge that our sample size may be insufficient to detect correlational differences between oxidative stress markers and measures of cerebrovascular function, and the possibility of a type II error does exist. As our sample size addresses our main outcome variable, it is our view that larger scale studies need to be undertaken to further investigate these relationships, which would furthermore include comparison between males and females, offering important information in regards to sex-related differences.

We considered the confounding effects of current smoking status and past smoking history between our two groups when deciding on selection criteria for the study. We ruled out the known immediate autonomic and cardiovascular effects of nicotine on vascular tone by requiring all COPD subjects to have quit smoking for >1 yr prior to entering the study. Because little is known about the cerebrovascular effects of smoking in COPD patients, we first wanted to identify outstanding differences between a COPD patient and a matched control. Indeed, a third ex-smoker control group would provide a valuable comparison.

Conclusion

This study is the first to show altered cerebrovascular responses to hypercapnia in females with moderate, smoking-related COPD. We show increased oxidative stress in COPD patients, and we believe that this may contribute to the cerebrovascular impairments observed in these patients. Future research is needed to address interventional strategies aimed at minimising systemic oxidative stress, thus providing direct evidence of the relationship between oxidative stress and cerebrovascular function.

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STATEMENT OF INTEREST

A statement of interest for R. Leigh can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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