

Corticospinal excitability is associated with hypocapnia but not changes in cerebral blood flow

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Key points

- Reductions in cerebral blood flow (CBF) may be implicated in the development of neuromuscular fatigue; however, the contribution from hypocapnic-induced reductions (i.e. P_{ETCO_2}) in CBF versus reductions in CBF *per se* has yet to be isolated.
- We assessed neuromuscular function while using indomethacin to selectively reduce CBF without changes in P_{ETCO_2} and controlled hyperventilation-induced hypocapnia to reduce both CBF and P_{ETCO_2} .
- Increased corticospinal excitability appears to be exclusive to reductions in P_{ETCO_2} but not reductions in CBF, whereas sub-optimal voluntary output from the motor cortex is moderately associated with decreased CBF independent of changes in P_{ETCO_2} .
- These findings suggest that changes in CBF and P_{ETCO_2} have distinct roles in modulating neuromuscular function.

Abstract Although reductions in cerebral blood flow (CBF) may be involved in central fatigue, the contribution from hypocapnia-induced reductions in CBF versus reductions in CBF *per se* has not been isolated. This study examined whether reduced arterial P_{CO_2} (P_{aCO_2}), independent of concomitant reductions in CBF, impairs neuromuscular function. Neuromuscular function, as indicated by motor-evoked potentials (MEPs), maximal M-wave (M_{max}) and cortical voluntary activation (cVA) of the flexor carpi radialis muscle during isometric wrist flexion, was assessed in ten males (29 ± 10 years) during three separate conditions: (1) cyclooxygenase inhibition using indomethacin (Indomethacin, 1.2 mg kg^{-1}) to selectively reduce CBF by $28.8 \pm 10.3\%$ (estimated using transcranial Doppler ultrasound) without changes in end-tidal P_{CO_2} (P_{ETCO_2}); (2) controlled iso-oxic hyperventilation-induced reductions in P_{aCO_2} (Hypocapnia), $P_{ETCO_2} = 30.1 \pm 4.5 \text{ mmHg}$ with related reductions in CBF ($21.7 \pm 6.3\%$); and (3) isocapnic hyperventilation (Isocapnia) to examine the potential direct influence of hyperventilation-mediated activation of respiratory control centres on CBF and changes in neuromuscular function. Change in MEP amplitude ($\%M_{max}$) from baseline was greater in Hypocapnia than in Isocapnia ($11.7 \pm 9.8\%$, 95% confidence interval (CI) [2.6, 20.7], $P = 0.01$) and Indomethacin ($13.3 \pm 11.3\%$, 95% CI [2.8, 23.7], $P = 0.01$) with a large Cohen’s effect size ($d \geq 1.17$). Although not statistically significant, cVA was reduced with a moderate effect size in Indomethacin ($d = 0.7$) and Hypocapnia ($d = 0.9$) compared to Isocapnia. In summary, increased corticospinal excitability – as reflected by larger MEP amplitude – appears to be exclusive to reduced P_{aCO_2} , but not reductions in CBF *per se*. Sub-optimal voluntary output from the motor cortex is moderately associated with decreased CBF, independent of reduced P_{aCO_2} .

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Abbreviations CBF, cerebral blood flow; cSP, cortical silent period; cVA, cortical voluntary activation; EMG, electromyography; MCAv, middle cerebral artery velocity; MEP, motor evoked potential; MVC, maximal voluntary contraction; M_{\max} , maximal M-wave; P_{aCO_2} , arterial partial pressure of carbon dioxide; P_{ETCO_2} , end-tidal partial pressure of carbon dioxide; P_{ETO_2} , end-tidal partial pressure of oxygen; pVA, peripheral voluntary activation; RR_{TMS} , maximal relaxation rate from transcranial magnetic stimulation; $\text{RR}_{\text{Tw,Pot}}$, maximal relaxation rate of the potentiated twitch; TCD, transcranial Doppler; TMS, transcranial magnetic stimulation; \dot{V}_i , inspired flow rate.

Introduction

Neuromuscular fatigue is defined as the inability of a muscle or group of muscles to maintain a given level of force (Bigland-Ritchie & Woods 1984; Gandevia 2001). It can be attributed to many factors, ranging from changes in cortical excitability to excitation–contraction coupling in the muscle tissue itself (Taylor & Gandevia 2001). Recent research suggests that central mechanisms of fatigue may predominate during dynamic exercise (Amann *et al.* 2006, 2007) and isolated isometric muscle contractions (Millet *et al.* 2009, 2012), and may be attributed in part to a reduction in cerebral blood flow (CBF) (Nybo & Nielsen 2001; Nybo & Rasmussen 2007; Secher *et al.* 2008).

Since Nybo and Nielsen (2001) first reported the association between reductions in CBF and impaired performance during prolonged exercise in the heat, research has examined the influence of decreased arterial P_{CO_2} (P_{aCO_2}), circulatory stress (Ide & Secher 2000) and inadequate cerebral oxygen delivery (Nybo & Rasmussen 2007; Rasmussen *et al.* 2007) as associated mechanisms contributing to fatigue. Rasmussen *et al.* (2010) suggested that impaired mitochondrial oxygen tension caused by inadequate CBF may compromise motor cortical output; however, the functional significance of reduced CBF and neuromuscular fatigue has yet to be investigated systematically in detail. Recently, Ross *et al.* (2012) indicated that the failure of voluntary motor drive during passive heat stress was related to reductions in CBF, which were mediated via hyperventilation-induced reductions in P_{aCO_2} . In their study, it is interesting to note that, at the point of thermal intolerance, the restoration of the partial pressure of end-tidal CO_2 (P_{ETCO_2}) towards baseline values restored decrements in voluntary drive despite CBF remaining below baseline levels. These findings are consistent with the view that reductions in P_{aCO_2} may independently impact motor drive and provide an important influence on neuromuscular fatigue.

Decreases in P_{aCO_2} have a profound effect on the neuromuscular system, ranging from the motor axons (Macefield & Burke 1991; Mogyoros *et al.* 1997) to the motor cortex (Kong *et al.* 1994; Kukumberg *et al.*

1996; Seyal *et al.* 1998; Dulla *et al.* 2005; Sparing *et al.* 2007). Hyperventilation-induced reductions in P_{aCO_2} are associated with the clinical presentation of tetany and paraesthesia, and are linked to spontaneous action potentials (Kong *et al.* 1994). At the peripheral level, an inverse relationship is observed between P_{aCO_2} and maximal M-wave (M_{\max}) amplitude, presumably due in part to a reduction in plasma calcium concentration (Macefield & Burke 1991). In the CNS, neuronal cells in the hippocampus increase in excitability during conditions of reduced P_{aCO_2} (Dulla *et al.* 2005). Similarly, reductions in P_{aCO_2} have been shown to increase corticospinal excitability, causing increased amplitude of the motor evoked potential (MEP) and a shortening of the MEP onset latency (Kong *et al.* 1994; Seyal *et al.* 1998); however, these findings are not universally accepted (Priori *et al.* 1995), probably due in part to methodological differences.

Hyperventilation may modulate both the cerebral vasculature and the neuromuscular system independent of changes in P_{aCO_2} . For example, Neubauer *et al.* (1983) reported a modest increase in total CBF (12%) following isocapnic hyperventilation, induced by carotid sinus nerve stimulation in anaesthetised cats. It was hypothesised that the increase in metabolic activity of the medulla during hyperventilation causes the release of metabolites that mediate local cerebral vasodilatation. Hayen *et al.* (2013) reported a 4% increase in CBF in response to negative intrathoracic pressures observed during large alterations in tidal volume. Similarly, hyperventilation may be associated with factors, such as an external focus of attention or the perception of effort (Neubauer *et al.* 1983; Sparing *et al.* 2007; Ruge *et al.* 2014), that have an excitatory effect on the motor cortex (Ruge *et al.* 2014).

Given the functional association between the cerebral vasculature and P_{aCO_2} , the question of primary interest is whether impaired neuromuscular function is specifically mediated by reductions in CBF or preceding reductions in P_{aCO_2} . Therefore, the purpose of this study was to examine the effects of reductions in CBF with and without reduced P_{aCO_2} on neuromuscular function. It was hypothesised that reductions in P_{aCO_2} , independent of changes in CBF, would have a significant role in mediating changes in neuromuscular function.

Methods

Ethical Approval

The experimental protocol and procedures were approved by the Bioscience Research Ethics Board at Brock University (BREB 12–167), and conformed to the latest revision of the *Declaration of Helsinki*. After an explanation of the experimental procedures and associated risks, all participants provided written informed consent and were screened by a physician.

Participants

Ten healthy male volunteers, who were free from cardiovascular, respiratory and neurological disorders, were recruited from the university and local community. The mean (\pm SD) age, height, mass and body mass index were 29 ± 10 years, 1.78 ± 0.06 m, 70.7 ± 7.6 kg and 22.2 ± 1.3 kg m⁻², respectively.

Experimental Design

Participants were given the opportunity to practise experimental protocols and acquaint themselves with the apparatus during a familiarisation session. Subsequently, participants reported to the laboratory on three separate occasions and were asked to abstain from strenuous exercise and the consumption of alcohol for 24 h and caffeine for 12 h prior to each session. To ensure sufficient recovery from the experimental protocol, sessions were separated by a minimum of 48 h. In a repeated-measures design, baseline and experimental measures were collected in the following conditions in this order: (1) cyclooxygenase inhibition via oral indomethacin (Indomethacin; 1.2 mg kg^{-1}) administration to selectively reduce CBF ($28.8 \pm 10.3\%$) without altering ventilation or arterial blood gases (Fan *et al.* 2011); (2) controlled hyperventilation (Hypocapnia) to reduce CBF by a comparable extent as condition 1 in the presence of reductions in P_{ETCO_2} ($P_{\text{ETCO}_2} = 30.1 \pm 4.5$ mmHg); and (3) isocapnic hyperventilation (Isocapnia) to selectively induce hyperventilation without changing P_{ETCO_2} or CBF. The Isocapnia condition was performed to examine possible confounds associated with hyperventilation and potential activation of the CNS respiratory control centres (Neubauer *et al.* 1983). Each condition was presented in a non-randomised order such that the breathing frequency (f_{br}) utilised in Hypocapnia induced a similar reduction in CBF observed during Indomethacin; the f_{br} utilised during Isocapnia was similar to Hypocapnia.

Indomethacin condition (Indomethacin). To reduce CBF independent of P_{ETCO_2} , participants ingested a 1.2 mg kg^{-1}

dose of indomethacin along with 150 mg of ranitidine to reduce the possibility of gastrointestinal irritation. Participants then rested quietly in the laboratory for 90 min to allow for drug concentrations to peak within the bloodstream (Xie *et al.* 2006). Indomethacin is a reversible and well-tolerated cyclooxygenase inhibitor that blunts the prostanoid-mediated dilatation of the cerebral vasculature, decreasing CBF (Parfenova *et al.* 1995) without concomitant changes in cerebral metabolic rate (Hohimer *et al.* 1985; Kraaier *et al.* 1992) or plasma catecholamines (Staessen *et al.* 1984; Wennmalm *et al.* 1984; Green *et al.* 1987). Additionally, evidence suggests that indomethacin reduces cerebrovascular reactivity to CO₂, causing enhanced central chemoreceptor activation (Xie *et al.* 2006; Fan *et al.* 2011) with little effect on the peripheral chemoreflex (Fan *et al.* 2011; Hoiland *et al.* 2015). Consequently, indomethacin has been used successfully to investigate changes in CBF independent of changes in P_{aCO_2} (Ainslie & Duffin 2009).

Controlled hyperventilation condition (Hypocapnia). To reduce P_{ETCO_2} and hence CBF, participants performed a controlled hyperventilation protocol in which they were instructed to breathe in synchrony with a metronome. The rate of the metronome was adjusted such that the hyperventilation-induced reduction in CBF was of similar magnitude to that observed during the Indomethacin trial. Dynamic end-tidal forcing (for more details, refer to the following section) was used to clamp end-tidal partial pressure of oxygen at iso-oxia (defined as the mean P_{ETO_2} during the 5 min baseline period).

Isocapnic hyperventilation condition (Isocapnia). To examine the potential direct influence of hyperventilation-mediated activation of the CNS respiratory control centres on CBF (Neubauer *et al.* 1983) and changes in corticospinal excitability (Sparing *et al.* 2007), participants breathed in synchrony with a metronome set at a rate identical to that of the Hypocapnia condition, while dynamic end-tidal forcing (for more details, refer to the following section) was used to clamp P_{ETCO_2} at eucapnia (defined as the mean P_{ETCO_2} during the 5 min baseline period).

Measurements

Respiratory measurements and dynamic end-tidal forcing. Participants breathed through a mouthpiece attached to a T-shaped two-way non-rebreathing valve (2700, Hans Rudolph, Inc., Shawnee Mission, KS). Inspired flow rate (\dot{V}_i) was measured using a pneumotach (3813, Hans Rudolph) while inspired and expired fractions of O₂ and CO₂ were sampled at a flow rate of 200 ml min^{-1} and analysed in real time using a gas analyser (ML206,

AD Instruments, Colorado Springs, CO, USA). The P_{ETO_2} and P_{ETCO_2} were clamped using a custom end-tidal forcing system designed by one of the co-authors (G.L.H.). Using solenoid valves to independently control gas flow from cylinders of compressed medical grade breathing air (21% O_2 , 0.03% CO_2 , balance N_2), 100% N_2 and 100% CO_2 , inspired air volumes were delivered to an air reservoir (~5 litres) via a humidification chamber (~500 ml). Inspired air volumes and gas fractions were determined using an algorithm (Koehle *et al.* 2009) incorporating tidal volume, P_{ETO_2} and P_{ETCO_2} error (i.e. the difference between desired and measured end-tidal partial pressures) to 'force' P_{ETO_2} and P_{ETCO_2} towards the desired values. Expired air was collected in a ~2 litres air reservoir, and was vented from the system using a low resistance one-way valve. In the event that the air volume in the inspired reservoir was lower than tidal volume, the negative pressure (created by the complete emptying of the inspired reservoir) would cause a high resistance one-way valve connecting the expired reservoir to open, allowing the participant to re-breathe expired air. Re-breathing prevents excessive fluctuations in P_{ETO_2} and P_{ETCO_2} values, while ensuring that the inspired air volume is sufficient during situations of irregular tidal volume.

Cerebro- and cardiovascular measurements. Bi-lateral middle cerebral artery velocity (MCAv) was assessed using a 2 MHz pulsed transcranial Doppler ultrasound (TCD) system (Doppler-Box, Compumedics GmbH, Singen, Germany). The probes were positioned over the temporal window and were held in place using a secure and comfortable head frame (M600 Headframe, Spencer Technologies, Seattle, WA, USA). TCD signals were identified and optimised using techniques described by Willie *et al.* (2011). Heart rate (HR) was measured using a three lead electrocardiogram (MLA2340, AD Instruments) and non-invasive measurement of oxygen saturation was measured using a pulse oximetry (ML320/F, AD Instruments) device placed on the left ring finger. Beat-to-beat blood pressure was measured by finger photoplethysmography (Nexfin, Bmeye, Amsterdam, Netherlands) and was normalised during the data analysis to two repeated manual sphygmomanometer measurements of the brachial artery. All respiratory, cerebrovascular and cardiovascular data were acquired continuously at 1 kHz (PowerLab 16/30, AD Instruments).

Electromyography (EMG) and torque measurements. Electromyography activity of the flexor carpi radialis muscle was measured using surface electrodes (HushTM Disk Electrodes, Natus Medical Inc., San Carlos, CA, USA). To ensure low impedance values (<5 M Ω), the skin surface was shaved and abraded with electrolyte gel (Nuprep, Weaver and Company, Aurora, CO, USA) then cleansed

with isopropyl alcohol. The motor point of the flexor carpi radialis was located by applying a low-intensity electrical stimulus to the muscle. The point that elicited the largest EMG response at the lowest stimulation intensity was identified as the motor point. The recording electrode was placed over this motor point and the reference electrode was placed 10 mm distal to the recording electrode in a bipolar configuration. A 100 mm circular self-adhesive ground electrode (Dermatode, Delsys, Boston, MA, USA) was placed over the olecranon process. EMG signals were band-pass filtered between 10 and 2000 Hz acquired at a sampling rate of 3 kHz using an analog to digital converter (KeyPoint G4, Dantec, Denmark) and analysed offline (MATLAB 2012b, MathWorks, Natick, MA, USA). Participants were positioned with their forearm in a custom made apparatus (hip and elbow joint angle ~ 135 deg) designed to isolate isometric forearm flexion of the wrist. A non-compliant cuff was placed around the participant's forearm, approximately 10 cm proximal to the styloid processes, and the carpal to distal phalanges of the right hand were placed between two aluminium plates. These plates were affixed tangentially to a calibrated load cell and the forearm was positioned such that the styloid process was aligned with the axis of rotation. Torque measurements were acquired using an analog to digital converter at a sampling rate of 3 kHz (LabView 2010, National Instruments, Austin, TX, USA) and analysed offline (MATLAB 2012b).

Motor cortex stimulation. Stimulation of the motor cortex was achieved using transcranial magnetic stimulation (TMS) with a 70 mm figure-of-eight coil (Magstim Rapid², Whitland, Wales, UK). Initially, the cortical site for optimal activation of the flexor carpi radialis was systematically identified using repeated stimulations (separated by ~ 10 s) at different locations of the scalp until the largest amplitude MEP was recorded. In all cases, the optimal site of activation was ~3–5 cm contralateral to the vertex, 45 deg relative to the sagittal plane and marked on the silicone swim cap used to cover the participant's scalp, allowing for easy identification and slight adhesion to the coil. The resting motor threshold was estimated by constructing a stimulus response curve, consisting of four TMS stimuli every 10 s at each 5% increment, ranging from 35 to 90% of stimulator output. The resultant data were fitted with a sigmoidal curve of best fit, and solved to determine the stimulator output intensity (%) required to produce an MEP with peak-to-peak amplitude of 50 μV (Carroll *et al.* 2001). The resultant motor threshold was $70 \pm 3\%$ and the intensity for subsequent stimulation was delivered at $1.3 \times$ motor threshold ($91 \pm 8\%$). The maximum within-trial error was calculated at ~10% for MEP responses.

Peripheral motor nerve stimulation. A compound M-wave was evoked using a handheld probe with anode and cathode (inter-electrode distance of 2 cm) in series, placed in the bicipital groove and proximal to the cubital space (cathode distal to the anode). The anode and cathode were attached to a constant current stimulator (KeyPoint G4, Dantec) that delivered a square wave pulse of 2 ms duration. The median nerve was stimulated at 0.5 mA increments once every 10 s, ranging from 5 mA until reaching a plateau in the peak-to-peak M-wave amplitude (i.e. M_{\max}). To ensure a supramaximal response, subsequent motor nerve stimulation was delivered at $1.3 \times M_{\max}$. The maximum within-trial error was calculated as $\sim 2\%$ for M_{\max} responses. M_{\max} is representative of maximal EMG in response to activation of the entire motor neuron pool.

Indices of neuromuscular function

A schematic outlining the neuromuscular measurements and corresponding analyses is displayed in Fig. 1. Maximal voluntary contraction (MVC) torque was quantified as the mean torque over a 100 ms epoch immediately prior to TMS during the cortical voluntary activation (cVA) protocol (Fig. 2); the maximum torque value was selected for analysis.

Peripheral neuromuscular function indices. M_{\max} was calculated as the mean peak-to-peak amplitude of

the EMG response to six consecutive supra-maximal stimulations of the peripheral motor nerve (see *Peripheral motor nerve stimulation* above). The maximum relaxation rate of the potentiated twitch ($RR_{\text{Tw,Pot}}$) elicited using peripheral nerve stimulation and superimposed twitch response following TMS (RR_{TMS}) were calculated as the steepest rate of decline in force production following stimulation during the peripheral voluntary activation (pVA) and cVA protocols, respectively (Fig. 2). RR_{TMS} was normalised to total torque during the contraction (i.e. product of the voluntary contraction and interpolated twitch). Both $RR_{\text{Tw,Pot}}$ and RR_{TMS} are indicative of changes in peripheral neuromuscular function (Todd *et al.* 2005).

Cortical-spinal indices. Peak-to-peak MEP amplitude was normalised to M_{\max} to reflect the changes in corticospinal excitability independent of potential changes in muscle fibre action potentials (Gandevia *et al.* 1999). The cortical silent period (cSP) is representative of cortical inhibition and was analysed using validated procedures (Julkunen *et al.* 2013) following TMS during the cVA protocol.

Voluntary activation indices. cVA was quantified using the twitch interpolation technique in which the torque response to TMS was examined during a series of sub-maximal and maximal voluntary contractions. cVA was quantified using the equation (Todd *et al.* 2003):

$$\text{cVA} = 1 - (\text{SIT}/\text{ERT}) \cdot 100\%$$

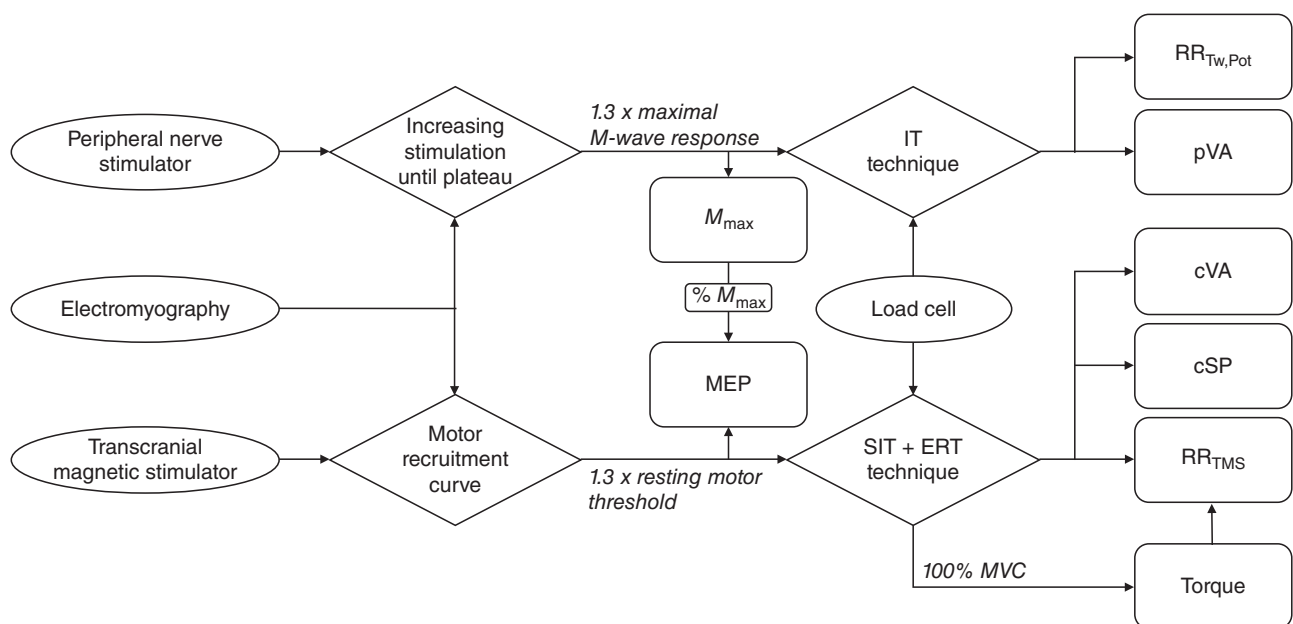


Figure 1. Schematic outlining the neuromuscular measurements and corresponding analyses

Ovals represent measurement apparatus, diamonds represent experimental protocols and squares represent outcome variables. M_{\max} , maximal M-wave; MEP, motor-evoked potential; IT, interpolated twitch; SIT, superimposed twitch; ERT, estimated resting twitch; MVC, maximal voluntary contraction; $RR_{\text{Tw,Pot}}$, maximal relaxation rate of the potentiated twitch; pVA, peripheral voluntary activation; cVA, cortical voluntary activation; cSP, cortical silent period; RR_{TMS} , maximal relaxation rate of the superimposed TMS.

where SIT represents the superimposed twitch elicited by TMS ($MEP = 82 \pm 15\% M_{max}$) during an MVC and ERT represents the estimated resting twitch response. Due to increased cortical and spinal excitability induced by muscular activity, ERT was estimated by determining the y -intercept of the linear regression between the superimposed twitch amplitude and voluntary torque during MVC, 50% MVC and 75% MVC (Fig. 2). Similarly, pVA was quantified using the interpolated twitch technique in which the torque increment elicited by peripheral motor nerve stimulation was examined. pVA was quantified using the following equation (Merton 1954):

$$pVA = 1 - (SIT/Q_{Tw,pot}) \times 100\%$$

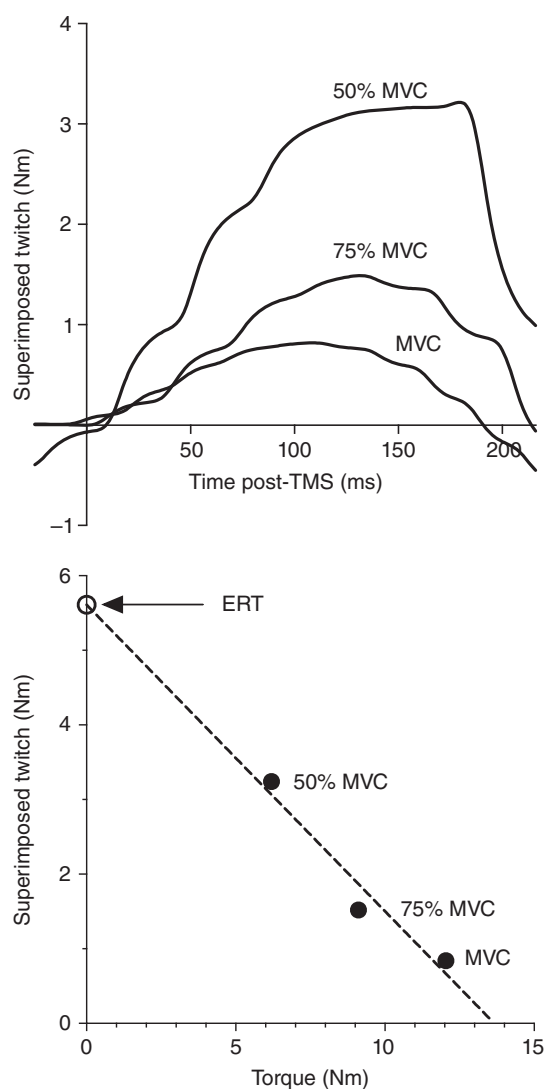


Figure 2. Torque increments and estimated resting twitch
Representative data illustrating (A) torque increments caused by superimposed TMS during MVC, 50% MVC and 75% MVC, and (B) interpolation of estimated resting twitch. MVC, maximal voluntary contraction; ERT, estimated resting twitch.

where SIT represents the superimposed twitch elicited by peripheral motor nerve stimulation and $Q_{Tw,pot}$ represents the potentiated twitch elicited ~ 1 s following MVC. Both cVA and pVA represent the percentage of motor units voluntarily activated by the CNS during maximal contractions; however, cVA assesses the mechanisms distal to the motor cortex, whereas pVA is restricted to mechanisms distal to the site of electrical stimulation (i.e. the peripheral motor nerve).

Experimental Protocol

Upon arrival to the laboratory, participants' height and mass were measured using standard laboratory equipment. Participants were positioned in a semi-recumbent position on an examination table with their back through neck supported by a backrest and their right forearm supported on a side table (hip and elbow angle ~ 135 deg). Following instrumentation (~ 20 min), participants rested quietly prior to data collection.

The experimental protocol is illustrated in Fig. 3. Initial baseline respiratory, cerebrovascular and cardiovascular measurements were collected over a period of 5 min, followed by baseline neuromuscular measurements (~ 15 min). The neuromuscular testing consisted of eliciting six M_{max} and six MEP responses, each separated by 10–12 s (the timing between stimulation was randomised to minimise any confounds of a participant anticipating the stimulus). Subsequently, participants performed three sets of voluntary isometric contractions; each contraction was 3 s in duration and consisted of an MVC, followed by a 50% MVC and a 75% MVC. Participants viewed a monitor that displayed the target torque for sub-maximal contractions (i.e. 50% MVC and 75% MVC), with the target calculated as a percentage of the previous MVC. Throughout each contraction, single-pulse TMS was delivered to the motor cortex during a period of stable torque production (approximately 1 s following contraction onset) and each contraction was separated by 10 s. Participants then performed three MVCs, each separated by 10 s. For each MVC, peripheral nerve stimulation was used to elicit a superimposed twitch during a period of stable torque production (approximately 1 s following contraction onset) and a potentiated twitch approximately 1 s following each contraction. Following baseline, the experimental intervention was initiated (either Indomethacin, Hypocapnia or Isocapnia). Once participants stabilised (90 min wash-in for Indomethacin; approximately 5 min for Hypocapnia and Isocapnia), 5 min of respiratory, cerebrovascular and cardiovascular measurements were collected followed by the same neuromuscular testing protocol.

Statistical Analysis

Normal distribution was confirmed upon visual inspection of the data by frequency distribution. Linear mixed model analyses were conducted to assess the change in respiratory, cerebrovascular, cardiovascular and neuromuscular variables in response to the experimental intervention (dependent variable), with fixed effects for conditions (three levels: Indomethacin, Hypocapnia and Isocapnia), and a random intercept. To adjust for potential differences in baseline measurements across conditions, baseline values were entered into the model as a covariate. Pairwise comparisons, using a Bonferroni correction, were used to identify main effects. Cohen's d effect sizes were calculated using the mean and standard deviation of the difference between conditions and interpreted using the following classification: $d = 0.20$ – 0.49 = small effect, $d = 0.50$ – 0.79 = moderate effect and $d > 0.8$ = large effect (Cohen 1988). All statistical analyses were conducted using SPSS 20 (SPSS Inc., Chicago, IL, USA). Data are given in the text as the difference from baseline between conditions (mean \pm SD) and 95% confidence interval (CI), whereas data are presented in the tables as absolute values for baseline, experimental and change from baseline measurements (mean \pm SD). Data are presented in the figures as individual baseline, experimental measurements with underlying bars representing mean values.

Results

Respiratory, cerebrovascular and cardiovascular responses

Respiratory, cerebrovascular and cardiovascular responses to the experimental conditions (expressed as absolute measurements) are presented in Table 1.

Respiratory variables. Compared to Indomethacin, significant differences in change from baseline for f_{br} (7.0 ± 4.7 breath min^{-1} , 95% CI [3.9, 10.2], $P < 0.01$, $d = 2.1$; 7.5 ± 4.3 breath min^{-1} , 95% CI [4.3, 10.7], $P < 0.01$, $d = 2.5$) and consequently, \dot{V}_i (7.5 ± 3.6 l min^{-1} , 95% CI [3.0, 9.6], $P < 0.01$, $d = 1.8$; 6.1 ± 2.4 l min^{-1} , 95% CI [4.0, 8.2], $P < 0.01$, $d = 2.5$) were observed during Hypocapnia and Isocapnia conditions, respectively. By design, the change from baseline in P_{ETCO_2} was greater during Hypocapnia than during Indomethacin (-11.0 ± 3.6 mmHg, 95% CI [-14.4, -7.7], $P < 0.01$, $d = 3.1$) and Isocapnia (-11.3 ± 4.2 mmHg, 95% CI [-14.7, -8.0], $P < 0.01$, $d = 2.7$). There were no significant differences in P_{ETO_2} (all $P \geq 0.56$) in response across all conditions.

Cerebro- and cardiovascular variables. The change from baseline in MCA_v during both Indomethacin

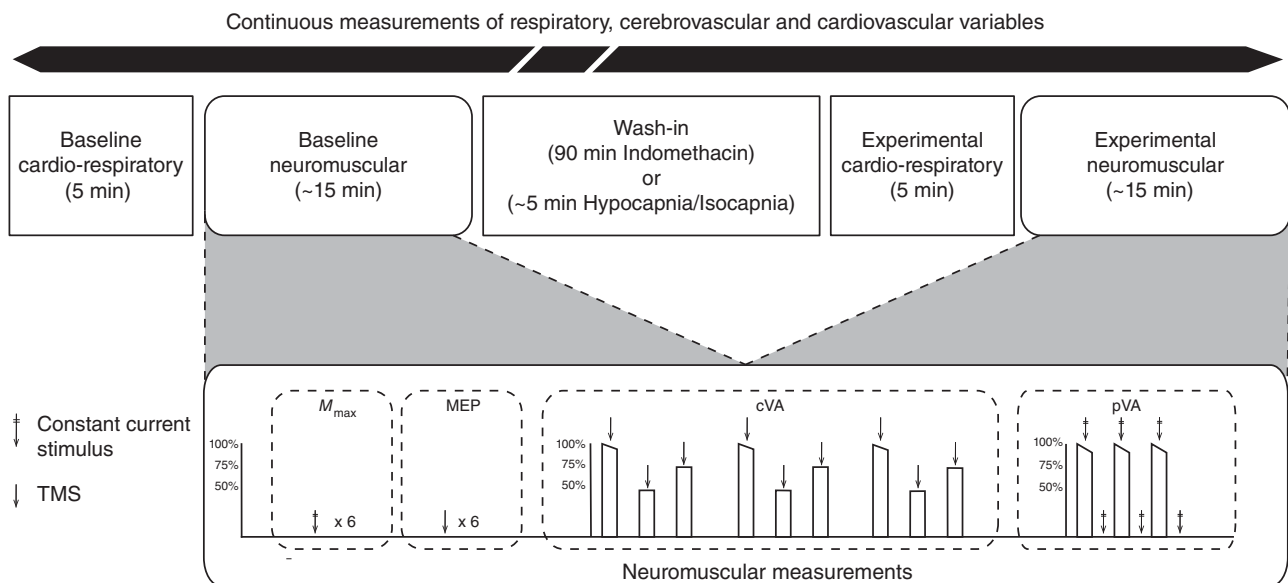


Figure 3. Schematic outlining the experimental protocol

Initial baseline respiratory, cerebrovascular and cardiovascular measurements were collected over a period of 5 min, followed by baseline neuromuscular measurements (~15 min). The neuromuscular testing (detailed in the dashed rectangle) consisted of eliciting six maximal M-wave (M_{max}) and six motor evoked potential (MEP) responses followed by the determination of cortical voluntary activation (cVA) and peripheral voluntary activation (pVA). Following baseline, the experimental intervention was initiated (either Indomethacin, Hypocapnia or Isocapnia). Once participants stabilised (90 min wash-in for Indomethacin; ~5 min for Hypocapnia and Isocapnia), 5 min of respiratory, cerebrovascular and cardiovascular measurements were collected followed by the same neuromuscular testing protocol. Respiratory, cerebrovascular and cardiovascular variables were measured continuously throughout the experiment. Arrow, TMS; arrow with cross, constant current stimulus.

Table 1. Baseline, experimental and change from baseline to experimental (Delta) cardio-respiratory data during Indomethacin, Hypocapnia, and Isocapnia conditions

	Indomethacin			Hypocapnia			Isocapnia		
	Baseline	Experimental	Delta	Baseline	Experimental	Delta	Baseline	Experimental	Delta
P_{ETO_2} (mmHg)	109.2 ± 6.9	107.3 ± 5.8	0.2 ± 0.3	110.5 ± 7.3	110.0 ± 9.8	0.8 ± 1.2	109.9 ± 6.7	106.0 ± 8.0	-0.3 ± 1.0
P_{ETCO_2} (mmHg)	41.1 ± 4.2	40.6 ± 3.0	-0.5 ± 3.0	41.6 ± 2.9	30.1 ± 4.5	-11.5 ± 2.8	42.1 ± 3.2	46.3 ± 2.7	-0.2 ± 1.3
MCA_v (cm s^{-1})	61.2 ± 7.8	43.4 ± 8.1	-17.7 ± 7.1	65.3 ± 9.6	50.9 ± 8.1	-14.3 ± 4.8	62.1 ± 6.6	62.1 ± 5.8	0.0 ± 2.1
f_{br} (breaths min^{-1})	14.2 ± 2.4	13.9 ± 2.2	-0.3 ± 2.5	14.5 ± 2.8	20.5 ± 4.6	6.0 ± 4.7	13.9 ± 2.9	21.1 ± 5.2	7.2 ± 3.5
\dot{V}_i (l min^{-1})	8.8 ± 2.1	9.0 ± 1.4	0.2 ± 1.4	9.5 ± 1.4	17.1 ± 6.1	7.5 ± 5.2	9.5 ± 1.6	15.9 ± 1.4	6.4 ± 1.7
HR (beats min^{-1})	59 ± 6	51 ± 6	-7.7 ± 3.8	58 ± 6	67 ± 10	9.1 ± 7.0	61 ± 8	64 ± 8	3.3 ± 4.1
S_{pO_2} (%)	97.5 ± 0.9	98.3 ± 0.7	0.7 ± 0.6	97.2 ± 2.1	98.7 ± 0.8	1.4 ± 2.2	97.1 ± 1.1	97.2 ± 1.0	0.1 ± 0.7
MAP (mmHg)	91 ± 8	96 ± 7	4.8 ± 9.4	93 ± 14	102 ± 13	9.1 ± 5.6	90 ± 10	97 ± 12	6.2 ± 3.8

P_{ETO_2} , end-tidal partial pressure of oxygen; P_{ETCO_2} , end-tidal partial pressure of carbon dioxide; MCA_v , MCA velocity; \dot{V}_i , inspired flow rate; HR, heart rate; S_{pO_2} , oxygen saturation; MAP, mean arterial pressure. Values are mean ± SD; $n = 10$ recordings.

($-17.7 \pm 7.1 \text{ cm s}^{-1}$ or $-28.8 \pm 10.3\%$, 95% CI $[-25.1, -10.4]$, $P < 0.01$, $d = 2.2$) and Hypocapnia ($-14.3 \pm 4.8 \text{ cm s}^{-1}$ or $-21.7 \pm 6.3\%$, 95% CI $[-9.9, 18.6]$, $P < 0.01$, $d = 3.0$) conditions were greater in comparison to Isocapnia. The change from baseline in HR was greater during Hypocapnia than during Indomethacin ($16.9 \pm 7.1 \text{ beats min}^{-1}$, 95% CI $[10.3, 23.5]$, $P < 0.01$, $d = 2.4$) and Isocapnia ($5.9 \pm 5.6 \text{ beats min}^{-1}$, 95% CI $[0.7, 11.1]$, $P = 0.03$, $d = 1.1$). There was no significant difference in MAP response across all conditions (all $P \geq 0.56$).

Neuromuscular function

MVC. The change in torque production (Fig. 4A) during MVC was similar across all conditions.

Responses to motor cortex stimulation. The change from baseline in peak to peak MEP amplitude (Fig. 4B), expressed as a percentage of M_{max} , was greater during the Hypocapnia condition than during Indomethacin ($13.3 \pm 11.3\%$, 95% CI $[2.8, 23.7]$, $P = 0.01$, $d = 1.17$) and Isocapnia ($11.7 \pm 9.8\%$, 95% CI $[2.6, 20.7]$, $P = 0.01$, $d = 1.20$). Conversely, the change in cVA (Fig. 4C) from baseline was greater during both the Indomethacin ($-11.3 \pm 16.0\%$, 95% CI $[-26.1, 3.6]$, $P = 0.16$, $d = 0.7$) and Hypocapnia ($-12.5 \pm 14.8\%$, 95% CI $[-25.7, 0.7]$, $P = 0.06$, $d = 0.9$) conditions in comparison to Isocapnia. The change in RR_{TMS} of the superimposed twitch (Fig. 4D) and change in cortical silent period (cSP; Fig. 4E) was similar across all conditions.

Responses to peripheral motor nerve stimulation. The change in peak to peak M_{max} amplitude (Fig. 4F), pVA (Fig. 4G) and $\text{RR}_{\text{Tw,Pot}}$ (Fig. 4H) were similar across all conditions.

Discussion

The purpose of this study was to examine neuromuscular function in response to reductions in CBF with and without consequent changes in arterial CO_2 . It was hypothesised that reductions in P_{aCO_2} , independent of changes in CBF, would mediate changes in neuromuscular function. The primary findings of this study were as follows: (1) increased corticospinal excitability (i.e. MEP amplitude) was associated with reductions in P_{aCO_2} , independent of changes in CBF and the mechanical changes (such as increased tidal volume/respiratory rate) associated with hyperventilation; and (2) reductions in cVA were associated with reduced cerebral blood flow, independent of reduced P_{aCO_2} .

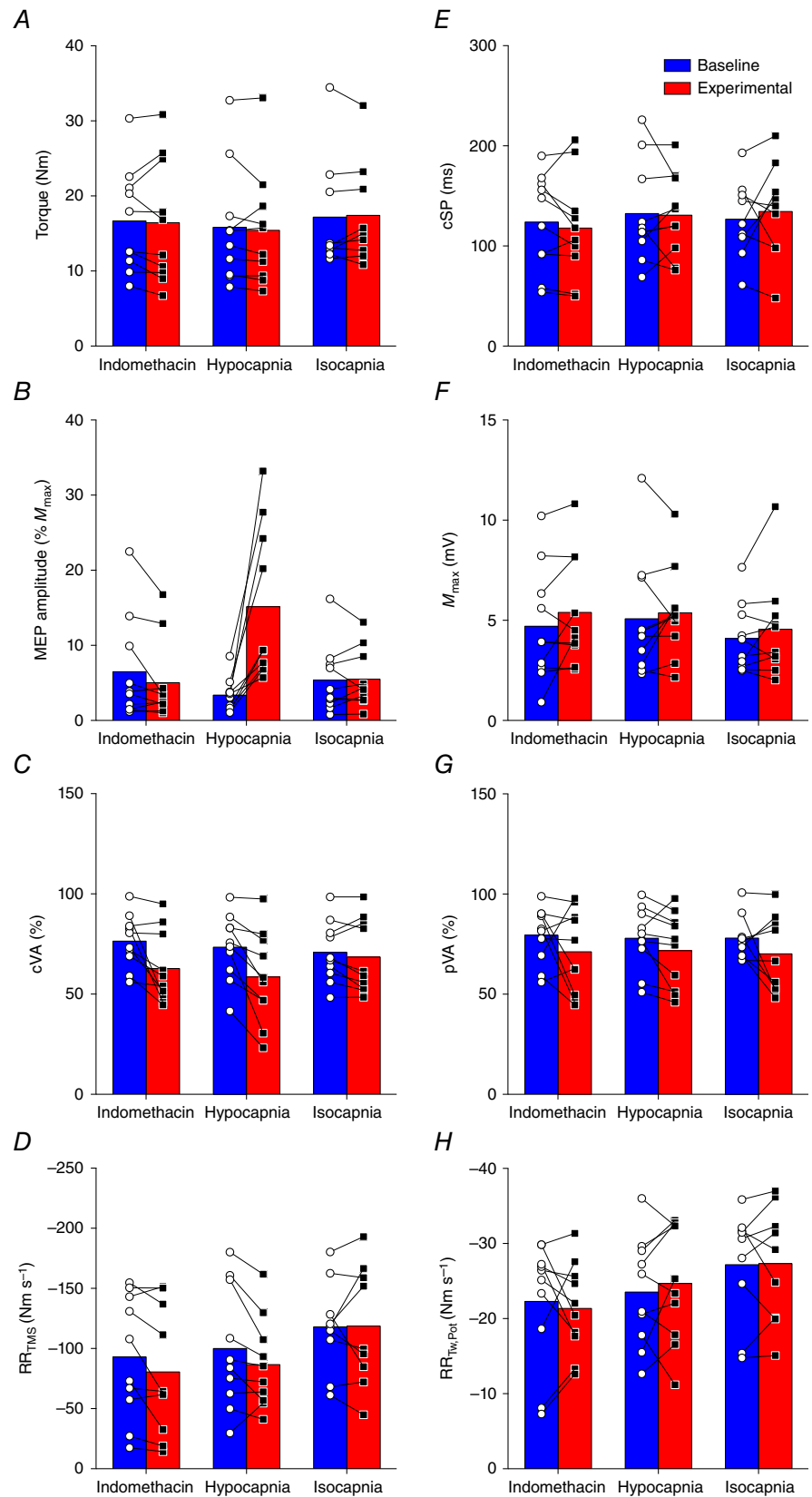


Figure 4. Neuromuscular response to Indomethacin, Hypocapnia, and Isocapnia
A, voluntary torque production; *B*, motor evoked potential (MEP) amplitude; *C*, cortical voluntary activation (cVA); *D*, maximal relaxation rate of the superimposed TMS (RR_{TMS}); *E*, cortical silent period (cSP); *F*, maximal M-wave (M_{max}); *G*, peripheral voluntary activation (pVA); *H*, maximal relaxation rate of the potentiated twitch ($RR_{TW, Pot}$). Connected pairs of open circles (○) and closed squares (■) represent individual baseline and experimental data, respectively. Underlying bars represent mean values for baseline (blue) and experimental (red) measurements.

Peripheral neuromuscular function

Reduced CBF and P_{aCO_2} associated with controlled hyperventilation were not associated with significant changes within the muscle fibre itself. The amplitude of M_{max} elicited by stimulation of the median nerve was unaffected across all conditions (Fig. 4F), suggesting that neuromuscular propagation remained intact. These findings are consistent with data obtained (i.e. no change in M_{max} amplitude) following highly selective cyclooxygenase-2 inhibition using acetaminophen (Mauger & Hopker 2013) and extends this lack of peripheral response to indomethacin or any other non-selective cyclooxygenase non-steroidal anti-inflammatory drug supplementation. Also consistent with our findings, previous research (Hilbert *et al.* 2012) indicates that hyperventilation-induced reductions in P_{aCO_2} (P_{ETCO_2} 16 mmHg below baseline) have no effect on M_{max} amplitude, despite significant reductions in arterial pH obtained via direct blood sampling.

In support of maintained peripheral neuromuscular function, $RR_{Tw,Pot}$ and RR_{TMS} were unaffected across conditions (Fig. 4H, 4D). These findings are supported by Morrow *et al.* (1988) who reported no significant changes in performance outcome measures following respiratory alkalosis. Similarly, Spriet *et al.* (1986) found no significant changes in isometric force production following sciatic nerve stimulation in mice and, furthermore, no significant differences in muscle oxygen uptake, glucose utilisation and total lactate production following induced respiratory alkalosis. This evidence suggests that peripheral neuromuscular function was maintained despite marked reductions in P_{aCO_2} and related respiratory alkalosis.

Corticospinal excitability and reduced P_{aCO_2}

A central finding of the present study is that MEP amplitude ($\%M_{max}$) was increased during Hypocapnia (Fig. 4B). Our data are in agreement with several previous reports (Seyal *et al.* 1998; Sparing *et al.* 2007) using controlled hyperventilation to induce reductions in P_{aCO_2} ; however, this finding is not universally accepted (Kong *et al.* 1994; Priori *et al.* 1995). Seyal *et al.* (1998) reported an inverse relationship between P_{ETCO_2} and MEP amplitude during graded hyperventilation and noted significant enhancement of the MEP response when reductions in P_{ETCO_2} were greater than 10 mmHg below eucapnia. Similarly, Sparing *et al.* (2007) observed a significant decrease in the motor threshold and an enhancement of the MEP stimulus–response curve to TMS after hyperventilation resulting in a 15 mmHg reduction in P_{ETCO_2} . In contrast to these findings, Kong *et al.* (1994) and Priori *et al.* (1995) reported no significant alterations in MEP response during hyperventilation-induced reductions in P_{aCO_2} . A potential explanation for these conflicting results

may be related to methodological issues. For example, Kong *et al.* (1994) suggested that their measure of motor cortex excitability (namely, post-stimulus time histograms) may be too insensitive to detect the modest changes associated with reductions in P_{aCO_2} . Similarly, the TMS stimulus intensity utilised by Priori *et al.* (1995) may have been too high to discriminate differences in MEP amplitude, despite significant reductions in P_{ETCO_2} (Seyal *et al.* 1998). This hypothesis has since been corroborated by Sparing *et al.* (2007), who reported a significant enhancement in the TMS stimulus–response curve only at intensities lower than 60% of stimulator output.

The mechanistic influence of reduced P_{aCO_2} on motor cortex excitability has been elucidated in rat hippocampal slice preparations (Dulla *et al.* 2005). The increase in extracellular pH associated with reductions in P_{aCO_2} inhibits ecto-ATPase activity – a trans-membrane enzyme that is involved in the hydrolysis of extracellular ATP – causing the accumulation of extracellular ATP and the decrease in extracellular adenosine. Increased extracellular ATP concentrations enhance purinergic receptors, causing an increase in excitatory inputs on interneurons (Khakh *et al.* 2003), modulation of excitatory post-synaptic potentials (Pankratov *et al.* 1998) and facilitation of long-term potentiation (Almeida *et al.* 2003). Conversely, decreased extracellular concentrations of adenosine impair A_1 receptor activity, reducing the tonic inhibition of glutamate, a neurotransmitter that is responsible for mediating excitatory signals (Johansson *et al.* 2001). Collectively, these changes are associated with increased neuronal excitability and therefore increased MEP amplitude.

Similar research studies (Priori *et al.* 1995; Sparing *et al.* 2007) examining supra-spinal responses to reductions in P_{aCO_2} reported a 4–15% decrease in cortical inhibition; however, the present data fail to support this observation as cSP was unaffected by the experimental manipulations (Fig. 4E). It is plausible that the duration of the hyperventilation protocol utilised in the present study was too short to induce significant changes in cortical inhibition. For example, Priori *et al.* (1995) reported that shortening of the cSP was significant only after 10 min of hyperventilation and lagged significantly behind changes in P_{ETCO_2} . Hyperventilation is associated with respiratory alkalosis and a reduction in serum ionised calcium concentration (Davies *et al.* 1986). The latter factor may modulate the primary inhibitory neurotransmitter GABA and NMDA receptors (Chesler & Kaila 1992; Lee *et al.* 1996). Specifically, shortening of the cSP is probably mediated through the $GABA_B$ receptor (Korchounov *et al.* 2005), a metabotropic trans-membrane receptor that is characterised by its coupling to G-proteins (Bowery *et al.* 2002) and, consequently, slow time-course response in motor cortex inhibition (Kohl & Paulsen 2010).

Novel to the present study is the ability to delineate the influence of reduced P_{aCO_2} versus the modulation of the sensory attention effects of hyperventilation. Increased MEP amplitude during Hypocapnia in comparison to Isocapnia suggests that reduced P_{aCO_2} , rather than confounds associated with hyperventilation and potential activation of the CNS respiratory control centres (Neubauer *et al.* 1983), is associated with the increase in corticospinal excitability. For example, Ruge *et al.* (2014) provided evidence that attention demands placed on internal or external sources (in the context of the present study, focus on f_{br} or tidal volume represents internal attention, whereas focusing on the sound of the metronome represents external focus of attention) increases corticospinal excitability and reduces GABA-mediated inhibition in humans. This finding has been postulated elsewhere (Sparing *et al.* 2007); however, to the best of our knowledge, the present study is the first to systematically investigate this question.

Voluntary activation and cerebral blood flow

Although there were no significant reductions in voluntary torque production, cVA was reduced in comparison to baseline during the Indomethacin and Hypocapnia conditions (Fig. 4C). Reduced cVA during voluntary contractions occurs because of suboptimal output from the motor cortex, causing a decrease in motor neuron firing frequency and/or motor neuron recruitment (Peters & Fuglevand 1999). As cVA was selectively lower during reductions in CBF independent of reduced P_{aCO_2} (i.e. the Indomethacin condition), these findings suggest that CBF may be an important mechanism regulating voluntary activation in the present study.

Previous research indicates that reductions in voluntary activation occur in response to a variety of stressors, such as fatiguing exercise (Todd *et al.* 2003), hyperthermia (Morrison *et al.* 2004; Todd *et al.* 2005; Thomas *et al.* 2006; Ross *et al.* 2012) and hypoxia (Goodall *et al.* 2010). During passive heating, Ross *et al.* (2012) observed a progressive decrease in voluntary activation that was associated with thermal hyperpnoea and concomitant reductions in P_{ETCO_2} and CBF. Furthermore, breathing a hypercapnic gas mixture (5% CO_2) at the point of thermal intolerance caused the partial restoration of voluntary activation, suggesting that cerebrovascular mechanisms, rather than the direct effects of hyperthermia, mediate neuromuscular impairments. The present study supports these findings, suggesting that cerebrovascular mechanisms mediate reductions in voluntary activation and, furthermore, delineate the primary role of CBF (rather than reduced P_{aCO_2} *per se*) as a modulator of descending drive from the motor cortex.

Previous research (Rasmussen *et al.* 2007; Nybo & Rasmussen 2007; Secher *et al.* 2008; Seifert *et al.* 2009)

suggests that impaired cerebral oxygenation, rather than the direct influence of reduced CBF, mediates central neuromuscular fatigue. For example, Rasmussen *et al.* (2007) reported that reduced maximal handgrip strength occurs in conjunction with lactate spill-over from cerebral tissue, suggesting that oxygen levels are inadequate to support aerobic metabolism. Impaired oxygenation results in reductions in (estimated) mitochondrial oxygen tension and consequently may impair neuronal function (Rasmussen *et al.* 2007; Lewis *et al.* 2014). It is important to note that the present study did not measure cerebral oxygenation, but rather cerebral blood velocity (a surrogate measure of blood flow). Therefore, we cannot exclude the possibility of increased oxygen extraction (e.g. Lewis *et al.*, 2014) in response to cerebral hypoperfusion as a possible compensatory mechanism in the present study.

In addition to the independent influence of changes in CBF on neuromuscular function, increased perception of effort (Berchicci *et al.* 2013; Lampropoulou & Nowicky 2014) or discomfort during Indomethacin and Hypocapnia may have contributed to the reductions in cVA. Both indomethacin and hyperventilation, via their impact on causing cerebral vasoconstriction, are associated with similar neurological side effects, such as dizziness, nervousness and headache – factors that contribute to an overall sensation of distress. Recently, Lampropoulou & Nowicky (2014) indicated that perception of effort is a primary regulator of voluntary motor control during isometric contractions. Furthermore, Baweja *et al.* (2011) indicated that high-amplitude respiration contributes to a lack of motor control during MVC. Although the association between reductions in CBF and increased perception of effort or distress remain speculative at best, the present study excludes the possible psychosomatic modulating effect of elevations in ventilation, rather than reduced P_{aCO_2} or CBF *per se*, given that reductions in voluntary activation were not observed during the Isocapnia condition (Fig. 4C).

Quantification of voluntary activation was assessed using both electrical stimulation of the peripheral motor nerve (pVA) and TMS of the motor cortex (cVA). Although both measures represent the magnitude of neural drive to the muscle, comparing the stimulation responses obtained at different locations throughout the neuromuscular system may help to localise the site of impairment (Todd *et al.* 2004). In the present study, reductions in voluntary activation, assessed as cVA, were greater during Indomethacin and Hypocapnia (Fig. 4C); however, pVA failed to detect a significant difference in change in voluntary activation across these conditions. Collectively, this evidence suggests that the mechanism(s) that contribute to neuromuscular impairments in response to decreased CBF reside at or above the level of the motor cortex.

Methodological considerations

In the present study, we were limited to measurements of P_{ETCO_2} for the estimation of P_{aCO_2} , but this assumption appears tenable, as previous research (Peebles *et al.* 2007; Willie *et al.* 2012) demonstrated that P_{ETCO_2} accurately reflects measurements of P_{aCO_2} throughout progressive reductions in P_{aCO_2} ($P_{\text{ETCO}_2} \pm 25$ mmHg baseline). Measurements of MCAv using TCD provide an index representation of CBF; however, this relationship is only valid assuming the diameter of the MCA does not change during the intervention (Ainslie & Hoiland 2014; Coverdale *et al.* 2014; Verbree *et al.* 2014). The stability of the MCA has been confirmed via magnetic resonance imaging during prostaglandin inhibition using a 100 mg oral dose of indomethacin (Xie *et al.* 2006). Changes in flow are adequately reflected by changes in flow velocity only when conduit artery diameter is unchanged (Valdúeza *et al.* 1997), which appears to be true during moderate changes in MAP (Giller *et al.* 1993). Recent evidence indicates that MCA diameter may constrict in response to reductions in P_{aCO_2} (Ainslie & Hoiland 2014; Coverdale *et al.* 2014; Verbree *et al.* 2014), although small differences in MCAv and flow during reductions in P_{aCO_2} are unlikely to detract from our primary findings; if anything, MCA constriction would underestimate our findings. Also, we only measured blood flow velocity in the territory of the MCA; whether the observed blood flow response was homogenous across all vascular territories in the brain is unknown (Willie *et al.* 2012; Skow *et al.* 2013).

The indomethacin dose was supplemented with ranitidine, an H_2 -antagonist that inhibits the activity of histamine. Previous research suggests that histamine may have a role in post-exercise vasodilatation of human skeletal muscle (Martins *et al.* 1980; Halliwill *et al.* 2013) and, furthermore, animal models suggest that histamine may act directly on the cerebral vasculature to increase CBF. Therefore, H_2 -receptor antagonists alter CBF and its response to different stressors (Audibert *et al.* 1991), and probably potentiate the reduction in CBF when supplemented with indomethacin.

Note that the present study did not include female participants and therefore we cannot examine the influence of oestrogen and progesterone on corticospinal excitability in response to changes in CBF. Previous research (Brackley *et al.* 1999; Inghilleri *et al.* 2004) highlights significant differences in intracranial cerebral circulation throughout the menstrual cycle, where reductions in CBF were associated with the luteal phase in comparison to the follicular phase. Furthermore, hormonal changes associated with the menstrual cycle have been shown to have a direct influence on motor cortex excitability. Therefore, the present results should be interpreted with caution when extrapolating to females.

Conclusions

We demonstrated that increased corticospinal excitability appears to be exclusive to reductions in P_{aCO_2} , but not reductions in CBF itself. In contrast, reductions in cVA were moderately associated with decreased CBF and the loci of fatigue were identified at or above the level of the motor cortex. Our findings further delineate the mechanisms that contribute to central fatigue by demonstrating that reduced P_{aCO_2} versus the normal associated reductions in CBF have distinct effects on the neuromuscular system.

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Additional information

Competing interests

The authors have no competing interests to disclose.

Author contributions

The study was performed in the Environmental Ergonomics Laboratory in the Department of Kinesiology and Brock University. (1) Conception and design of the experiment: G.L.H., P.N.A., M.J.G., S.S.C.; (2) collection, analysis and interpretation of the data: G.L.H., C.L.W., P.N.A., C.D.T., D.A.G., D.D.O., S.S.C.; (3) drafting the article or revising it critically for important intellectual content: G.L.H., C.L.W., P.N.A., C.D.T., M.J.G., D.A.G., D.D.O., S.S.C. G.L.H. developed the end-tidal forcing system (available upon request).

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