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Moderate inhibition of mitochondrial function augments carotid body hypoxic sensitivity

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Abstract

A functional role for the mitochondria in acute O₂ sensing in the carotid body (CB) remains undetermined. Whilst total inhibition of mitochondrial activity causes intense CB stimulation, it is unclear whether this response can be moderated such that graded impairment of oxidative phosphorylation might be a mechanism that sets and modifies the O₂ sensitivity of the whole organ. We assessed NADH autofluorescence and [Ca²⁺]_i in freshly dissociated CB type I cells and sensory chemoafferent discharge frequency in an intact CB preparation, in the presence of varying concentrations of nitrite (NO₂⁻); a mitochondrial nitric oxide (NO) donor and competitive inhibitor of mitochondrial complex IV. NO₂⁻ increased CB type I cell NADH in a manner that was dose-dependent and rapidly reversible. Similar concentrations of NO₂⁻ raised type I cell [Ca²⁺]_i and increased chemoafferent discharge frequency. Moderate inhibition of the CB mitochondria by NO₂⁻ augmented chemoafferent discharge frequency during graded hypoxia, consistent with a heightened CB O₂ sensitivity. Furthermore, NO₂⁻ also exaggerated chemoafferent excitation during hypercapnia signifying an increase in CB CO₂ sensitivity. These data therefore provide support for the hypothesis that mitochondria play a key role in setting the hypoxia-stimulus response coupling process in the CB and raise the possibility that NO, acting at the mitochondria, can modulate the CB sensitivity to both hypoxia and hypercapnia.

Key Words: carotid body, mitochondria, hypoxia, nitrite, nitric oxide

Abbreviations: CB – carotid body. NO₂⁻ - nitrite. NO – nitric oxide. SDB – sleep disordered breathing. CHF – Chronic heart failure. CIH – chronic intermittent hypoxia. TASK – TWIK-related acid sensitive K⁺ channel. BK_{Ca} – large conductance Ca²⁺ activated K⁺ channel. SCG – superior cervical ganglion. PMT – photomultiplier tube. NOX-2 – NADPH oxidase 2. ROS – reactive oxygen species. H₂S – hydrogen sulphide. NOS-3 – nitric oxide synthase 3.

Introduction

The type I cell in the mammalian carotid body (CB) responds acutely to small, physiological reductions in arterial O₂ tension. Activation of the CB type I cell leads to the release of stored neurotransmitters and excitation of the adjacent sensory neuronal fibres [37]. The functional consequence of CB stimulation by hypoxia is the induction of a series of well-characterised cardiovascular, respiratory and endocrine reflex responses [28]. Chronic up-regulation of these reflex pathways, secondary to pathological changes in CB function is implicated in a number of clinical conditions including sleep disordered breathing (SDB) [42] and chronic heart failure (CHF) [45]. This promotes neurogenic hypertension [36,39] and heightens cardiovascular related morbidity and mortality [46]. Recently, sensory neuronal output originating from the CB has also been identified as being the main driving force in eliciting spontaneous hypertension in rats [1,31] and in initiating insulin resistance and hypertension in animals fed on a high calorific diet [43].

The development of clinical interventions for CB dysfunction in patients with SDB, CHF and insulin resistance has been restricted by a lack of fundamental knowledge of the mechanism(s) accounting for CB activation by hypoxia. Of the proposed O₂ sensors, the type I cell mitochondria appear particularly sensitive to arterial O₂ tensions (P_aO₂) within the physiological range at which the CB is stimulated [33,15,8]. In particular, the exceptionally low O₂ affinity of complex IV causes mitochondrial electron flux to be more susceptible to small falls in O₂ compared with other cell types [8]. Whether or not the mitochondria have a functional role in establishing the unique O₂ sensitivity of the whole CB organ is the focus of the current investigation.

In support of the mitochondrial hypothesis, all known inhibitors and uncouplers of the mitochondria excite the CB [53]. The degree of stimulation elicited by saturating concentrations of mitochondrial poisons is similar to that evoked by severe hypoxia or anoxia [15,16,53]. The effects of non-competitive inhibitors (azide and CN⁻) on chemoafferent activity also exhibit a degree of dependence on steady state P_aO₂ [35]. Furthermore, type I cell activation induced by mitochondrial poisons shares many of the same chemotransduction processes associated with CB O₂ sensing including the inhibition of outward K⁺ currents, cellular depolarisation, Ca²⁺ influx and neurosecretion [29].

If the mitochondria are functionally involved in CB O₂ sensing, then competitive inhibitors of O₂ binding at complex IV should heighten CB O₂ sensitivity and exaggerate the increased chemoafferent discharge frequency in response to graded hypoxia. Nitric oxide (NO) acts as a competitive inhibitor of cytochrome oxidase through binding to the Cu_B/haem a₃ binuclear centre in complex IV, in direct competition with O₂ [6]. In order to explore the effects of mitochondrial NO we have taken advantage of the observation that mM concentrations of exogenous nitrite (NO₂⁻) can be reduced by the mitochondria to release small quantities (nM/pM) of NO that subsequently inhibit mitochondrial complexes I and IV making electron transport more sensitive to reductions in O₂ [27,9,2,48,47]. We therefore use NO₂⁻ in this study to examine two key questions: 1) Does a competitive inhibitor of complex IV alter the CB O₂ sensitivity and 2) Can NO inhibit the CB mitochondria and modify type I cell and chemoafferent responses to hypoxia?

Materials and Methods

Ethical approval

All surgical procedures were performed in accordance with project and personal licences issued under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Biomedical Services Unit at the University of Birmingham and University of Oxford.

Cell isolation

The carotid bifurcation, the superior cervical ganglion (SCG), vagus nerve and CB were all excised from rats aged 10-14 days under terminal inhalation anaesthesia (2-4% Isoflurane in O₂). Bifurcations were immediately placed in a dissecting dish containing a phosphate buffered saline (PBS) solution. The SCG was removed along with the vagus nerve. The CBs were teased away from the adjacent arterial walls and then transferred to 2 ml of HAMS F-12 nutrient mixture pre-equilibrated with 11% O₂, 5% CO₂ and containing collagenase (Worthington: 0.6 mg/ml) and trypsin (Sigma: 0.4 mg/ml). CBs were incubated at 37°C, 5% CO₂ and 11% O₂, for 25-30 minutes in this solution. To isolate cells, CBs were transferred to an enzyme free culture medium containing trypsin inhibitor (Sigma: 0.5 mg/ml) and triturated using fine bore glass pipettes (200, 100 and 50 µm internal diameter). 30 µl aliquots of the cell suspension were then transferred to individual poly-L-lysine coated 6 mm diameter coverslips. These coverslips were placed in the incubator at 37°C, 5% CO₂ and 11% O₂, for 2-4 hours before use. The enzyme free culture medium was composed of advanced DMEM F-12 nutrient mixture (Gibco), L-glutamine (2 mM), insulin (4 µg / ml) and foetal bovine serum (10% v/v).

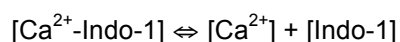
Measurement of NADH and [Ca²⁺]_i

Ca²⁺ and NADH fluorescence measurements were made using an inverted microscope (Nikon Diaphot 200; Nikon) equipped with a 100 W xenon lamp that provided the fluorescence excitation light source. Photomultiplier tubes (PMT; Thorn EMI), cooled to minus 20°C, were used to detect the emitted fluorescence. The output signal was fed through a current-voltage converter and the voltage was recorded using a CED micro1401 (Cambridge Electronic Design) and visualised on a PC with Spike2 (version 7.1) software (Cambridge Electronic Design) as an individual waveform. The voltage signal was sampled at 250 Hz and then averaged over 0.5 sec intervals.

NADH autofluorescence was excited at 340 nm and the emission was measured at 450 ± 30 nm. A diaphragm placed in the image plane was used to select fluorescent light emanating from chosen cells. Raw traces showing NADH auto-fluorescence are presented without any correction or calibration. Baseline subtraction was achieved by applying a linear filter to account for fluorescence bleaching and data is presented as a proportion of the mean maximum hypoxic response performed before and after addition of NO₂⁻, thus normalising each response to NO₂⁻.

[Ca²⁺]_i was determined by using the fluorescent dye, Indo-1 [21]. Cells were initially loaded by incubation with the Indo-1 acetoxymethyl ester (Indo-1-AM; Invitrogen Molecular Probes) for 1 hour. After

transferring the cells to the recording chamber, cells were illuminated at 340 nm and emission was measured at 405 ± 16 nm (F_{405} ; Ca^{2+} bound Indo-1) and 495 ± 10 nm (F_{495} ; Ca^{2+} free Indo-1). In order to directly quantify the type I cell $[\text{Ca}^{2+}]_i$ the constants R_{\min} , R_{\max} and $F_{495 (\max / \min)}$ were determined in separate recordings. To do this, type I cells loaded with Indo-1 were transferred into a HEPES buffered Krebs Ca^{2+} free solution containing 10 mM EGTA (Sigma-Aldrich) and 10 μM ionomycin for 1 hour at room temperature. This protocol was performed to deplete the cells of calcium as much as possible and de-saturate Indo-1. Cells were subsequently placed in a recording chamber and superfused with a similar solution but containing only 1 μM ionomycin whilst fluorescence measurements were made to determine the R_{\min} from a single type I cell. To determine R_{\max} , the superfusate was replaced with another HEPES buffered Krebs solution containing 2.5 mM Ca^{2+} and 1 μM ionomycin. Using this method the $F_{495 (\max / \min)}$ could also be calculated. The K_d (dissociation constant) of the reaction:



was assumed to be 250 nM as previously reported [21]. In subsequent experimental recordings $[\text{Ca}^{2+}]_i$ was calculated from the fluorescence ratio R according to the equation described by Grynkiewicz and colleagues, as shown below.

$$[\text{Ca}^{2+}]_i = K_d \left(\frac{(R - R_{\min})}{(R_{\max} - R)} \right) / (F_{495 (\max / \min)})$$

Extracellular recordings of single and few-fibre chemoafferent neurones

The carotid bifurcation along with the SCG, vagus nerve, carotid sinus nerve (CSN) and CBs were isolated from adult male rats (50–200 g) under inhalation anaesthesia (2–4% isoflurane in O_2). Following tissue procurement, animals were immediately killed by exsanguination. The tissue was transferred to a small volume (approximately 0.2 ml) dissecting chamber with a Sylgard 184 base (Dow Corning). The tissue was continuously superfused with a bicarbonate buffered extracellular Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4 , 5 Na_2SO_4 , 1.3 MgSO_4 , 24 NaHCO_3 , 2.4 CaCl_2 , 11 D-glucose, equilibrated with 95% O_2 and 5% CO_2 . Connective tissue was removed and the superior cervical ganglion, branches of the vagus nerve and the occipital artery were all individually excised. The CSN was sectioned exposing nerve fibres and axons. To facilitate the later extracellular neuronal recordings, the whole tissue was partially digested by incubation in a bicarbonate buffered enzyme Krebs solution (0.075 mg / ml collagenase type II, 0.0025 mg / ml dispase type I; Sigma Aldrich), equilibrated with 95% O_2 and 5% CO_2 , at a temperature of 37°C, for 20–30 minutes.

Extracellular recordings of single or few-fibre chemoafferent activity were made from the cut end of the CSN using glass suction electrodes as described previously [40,24]. The recorded voltage was amplified using a NeuroLog NL104 AC pre-amplifier (Digitimer), and amplified further with an AC amplifier (NeuroLog 105; Digitimer). Total amplification was x4000. The signal was not filtered. The superfusate PO_2 was continuously measured using an O_2 electrode (ISO2; World Precision Instruments) and O_2 meter (OXELP; World Precision Instruments). The PO_2 and chemoafferent derived voltage were both recorded using a CED micro1401 (Cambridge Electronic Design) and visualised on a PC with Spike2 (version 7.1) software (Cambridge Electronic Design), as two individual waveforms. The chemoafferent voltage signal was sampled at 15000 Hz and the PO_2 at 100 Hz. Using the in-built wavemark analysis in

the Spike2 software (Cambridge Electronic Design), electrical activity originating from a single chemoafferent fibre was determined by its unique 'wavemark' signature based on frequency, shape and amplitude.

Experimental solutions and analysis of functional hypoxic responses

During experimentation, dissociated type I cells were continuously superfused with a standard bicarbonate buffered Krebs solution containing, in mM: 117 NaCl, 4.5 KCl, 1 MgCl₂, 23 NaHCO₃, 2.5 CaCl₂ and 11 D-glucose. For intact CB preparations, an almost identical Krebs solution was used but in addition contained 1.25 mM NaH₂PO₄ and 5 mM Na₂SO₄. All solutions were heated to 37°C using a water bath (Grant W14, Grant Instruments). In experiments using exogenous NaNO₂, osmolality was balanced by appropriate subtraction of NaCl from the superfusate.

The HEPES buffered Krebs solution used for Indo-1 calibrations contained in mM: 140 KCl, 2 MgCl₂, 10 CaCl₂, 11 D-glucose and 20 HEPES, pH 7.4 at 37°C. The Ca²⁺ free solution was deficient in CaCl₂ and contained 10 mM EGTA.

For dissociated cell experiments, normoxic/hyperoxic solutions were equilibrated with 5% CO₂ and 95% air. Mild hypoxic solutions were equilibrated with 5% CO₂, 2% O₂ and 93% N₂ and severe hypoxic solutions with 5% CO₂ and 95% N₂. All solutions had a pH of 7.4 at 37°C.

For whole CB preparations, flow meters with high precision valves (Cole Palmer Instruments) were used in order to gas the superfusate with a desired gas mixture. For normoxia/hyperoxia the superfusate PO₂ was maintained throughout at 300 mmHg and PCO₂ at 40 mmHg to enable a sufficient diffusion gradient [41]. To monitor chemoafferent responses to graded hypoxia the superfusate PO₂ was gradually reduced at constant PCO₂. The single fibre chemoafferent discharge frequency was plotted against the superfusate PO₂, over the range of superfusate PO₂ values. To produce the hypoxic response curves, the data points were fitted to an exponential decay curve with offset, as shown below.

$$y = a + be^{-cx}$$

For the above equation, y is the single fibre discharge frequency in Hz, x is the superfusate PO₂ in mmHg, a is the discharge frequency as the PO₂ tends to infinity (offset), b is the discharge frequency when the PO₂ is 0 mmHg (minus the offset) and c is the exponential rate constant. Specific components of the calculated hypoxic response curves were compared to identify any potential changes in CB hypoxic sensitivity.

Analysis of data

Values are expressed as mean ± standard error of mean unless otherwise stated. Statistical analysis was performed using i) a paired 2-tailed student's t-test ii) an un-paired 2-tailed student's t-test or iii) repeated measures one way Analysis of Variance (ANOVA) with Bonferroni or Dunnett's post hoc analysis where appropriate (StatView version 5). Significance was taken as p<0.05.

Results

Nitrite causes dose dependent, carotid body chemostimulation coupled to NADH autofluorescence.

In the intact CB preparation, NO_2^- elevated the chemoafferent frequency at mM concentrations, consistent with those concentrations known to generate NO at the mitochondria (Fig. 1a). Chemostimulation induced by NO_2^- was rapidly induced (less than 1 minute) and well maintained throughout the application and was reversed within 1 to 2 minutes of removal from the superfusate (Fig. 1a). Significant dose dependent increases in mean chemoafferent activity were observed at concentrations of 3.3, 10 and 33 mM NO_2^- with the mean response to 33 mM NO_2^- measuring 69.3 ± 6.5 % of the absolute maximum frequency response to severe hypoxia (superfusate PO_2 approximately 40 mmHg for an intact CB preparation) (Fig. 1b).

In acutely dissociated type I cell clusters (*ca* 2-10 cells) similar concentrations of NO_2^- to those that increased chemoafferent frequency in the intact CB organ also generated significant elevations in NADH autofluorescence, indicative of an attenuation of mitochondrial electron transport (Fig. 1c). Increases in NADH autofluorescence were almost instantaneous upon NO_2^- application and reversed within 1 to 2 minutes of removal from the superfusate (Fig. 1c). Addition of 10 mM NO_2^- caused a 25.5 ± 1.3 % whilst 33 mM NO_2^- caused a 79.2 ± 13.8 % rise in NADH autofluorescence, when measured as the proportion of the peak responses to 0% O_2 (Fig. 1d).

Carotid body type I cell Ca^{2+} is elevated by NO_2^- in mild hypoxia

$[\text{Ca}^{2+}]_i$ was recorded from clusters of type I cells (*ca* 2-10 cells) at two levels of dissolved O_2 : 20% and 2%. An example trace from a single cluster in the presence and absence of 10 mM NO_2^- is shown in Fig. 2a. Addition of 10 mM NO_2^- had little effect on type I cell $[\text{Ca}^{2+}]_i$ in hyperoxia but when the superfusion medium was switched to one equilibrated with 2% O_2 (mild hypoxia), 10 mM NO_2^- stimulated an almost instantaneous and substantial rise in $[\text{Ca}^{2+}]_i$ that was rapidly reversible (Fig. 2a & b), consistent with significant stimulus interaction. At 33mM NO_2^- , the elevation in $[\text{Ca}^{2+}]_i$ in 2% O_2 was more substantial and better maintained (Fig. 2c & d). To maintain osmolality, experiments using NaNO_2 were performed at lower concentrations of NaCl. Therefore, to examine the potential impact of a reduction in Cl^- concentration, experiments were also carried out in the presence and absence of 33 mM sodium nitrate (NO_3^-). Type I cell $[\text{Ca}^{2+}]_i$ was unaffected by the addition of 33 mM NO_3^- , at either 20% or 2% dissolved O_2 (Fig. 2e & f). In view of this, it is unlikely that the response observed in the presence of NO_2^- is a consequence of a reduction in Cl^- ions.

To confirm that the observed rise in $[\text{Ca}^{2+}]_i$ in response to NO_2^- is due to Ca^{2+} entry via voltage gated Ca^{2+} channels, as is the case for equivalent $[\text{Ca}^{2+}]_i$ -responses to hypoxia or other mitochondrial inhibitors [53], we studied the effects of both removal of extracellular Ca^{2+} and of a non-selective Ca^{2+} -channel antagonist Ni^{2+} . Fig. 3a & c show that in a Ca-free Krebs (containing 100 μM EGTA) 30 mM NO_2^- had no discernible effect on $[\text{Ca}^{2+}]_i$. Similarly, in the presence of 2 mM NiCl_2 , NO_2^- again had no effect on $[\text{Ca}^{2+}]_i$.

(see Fig. 3b & d). Thus the $[Ca^{2+}]_i$ -response to NO_2^- would appear to be dependent upon both extracellular Ca^{2+} and the involvement of voltage-gated Ca^{2+} -channels. It is however notable that in both Ca^{2+} -free solutions and in Ni^{2+} solutions there is a rapid rebound increase in $[Ca^{2+}]_i$ when those solutions are washed off. This may reflect a difference in time course between the wash out of extracellular solutions (typical half time for solution exchange in our perfusion chamber is < 2 s) and the washout of intracellular and inner mitochondrial matrix NO_2^- .

Nitrite sensitises the carotid body to acute graded hypoxia

We next sought to investigate whether mild inhibitory targeting of the mitochondria, using NO_2^- , modified the intact CB O_2 sensitivity. The CB was stimulated by hypoxia in the presence and absence of 3.3 mM NO_2^- , a concentration previously shown to evoke only a very small increase in basal activity (0.60 ± 0.10 Hz; control compared with 1.11 ± 0.21 Hz; 3.3 mM NO_2^-) consistent with a very mild level of mitochondrial inhibition. NO_2^- exaggerated the rise in single fibre chemoafferent frequency during hypoxia causing a marked right and upward shift of the CB hypoxic response curve (Fig. 4a & b). The mean right shift was quantified by measuring the PO_2 when the discharge frequency was at 5 Hz (control: 105 ± 7 mmHg PO_2 , NO_2^- : 141 ± 11 mmHg PO_2 , Fig. 4c). This effect was consistent in all the fibres tested (Fig. 4c).

Analysis of the paired differences in discharge frequency calculated at fixed levels of PO_2 , showed that NO_2^- sensitivity was increasingly enhanced by reductions in PO_2 (Fig. 4d), indicative of significant synergistic interaction between NO_2^- and hypoxia.

Nitrite augments the carotid body sensitivity to hypercapnia

Hypoxia causes a multiplicative augmentation of the CB response to hypercapnia, showing that these two stimuli are highly interdependent [40,11,18]. We therefore examined whether the NO_2^- impacts on the CB sensitivity to hypercapnia.

Chemoafferent responses to hypercapnia were performed in the presence and absence of 3.3 mM NO_2^- . An example trace of a single fibre response to hypercapnia in the presence and absence of 3.3 mM NO_2^- is presented in Fig. 5a. NO_2^- significantly enhanced the response to hypercapnia (Fig. 5b). Measurements of the differences between the paired hypercapnic and basal frequencies demonstrate that 3.3 mM NO_2^- augmented the absolute frequency rise induced by hypercapnia in all fibres tested (0.90 ± 0.20 Hz; control, 1.7 ± 0.30 ; nitrite) (Fig. 5c). Accordingly, the calculated CO_2 sensitivity (determined by Δ Hz / mmHg PCO_2) was significantly heightened in the by NO_2^- (Fig. 5d).

Chemoafferent excitation induced by nitrite is not due to a direct action on the nerve ending or secondary to reactive oxygen species generation

To investigate a possible direct action of NO_2^- upon the sensory afferents of the CSN, 10 mM NO_2^- was applied to the intact preparation during hyperoxia (95% O_2 , 5% CO_2), when type I cell activity is effectively silenced. A characteristic example of the responses to NO_2^- during normoxia and hyperoxia is shown in

Fig. 6a. Grouped data shows that the chemoafferent response to 10 mM NO_2^- is abolished in hyperoxia (Fig. 6b). Furthermore, addition of 10 mM NO_2^- in hyperoxia did not augment the response to 1 mM ATP (Fig. 6c), a stimulus that should excite P2X receptors on CSN terminals with minimal involvement of type I cell receptors.

To determine whether responses to NO_2^- were mediated by ROS levels within the CB, chemoafferent excitation induced by 10 mM NO_2^- was examined before and during 15 min incubation with 1 mM 4-hydroxy-TEMPO (TEMPOL); a membrane permeable SOD mimetic and free radical scavenger [10,22] previously used to reduce ROS levels in a comparable *in vitro* CB preparation [30]. An example of the responses to 10 mM NO_2^- in the presence and absence of 1 mM TEMPOL is presented in Fig. 6d. TEMPOL did not significantly affect the magnitude of the chemoafferent stimulation induced by 10mM NO_2^- (Fig. 6e). Thus, chemoexcitation induced by NO_2^- does not appear to involve alterations in ROS generation/reduction.

Discussion

Mild mitochondrial inhibition using nitrite increases the carotid body sensitivity to hypoxia.

This study provides evidence supporting a functional role for type I cell mitochondria in establishing the overall sensitivity of the CB to O_2 . Addition of NO_2^- , a type I cell mitochondrial inhibitor, caused greater than additive augmentation in the hypoxic chemoafferent discharge frequency and evoked a reversible right shift in the exponential chemoafferent hypoxic response curve, an effect that is consistent with an alteration in the PO_2 threshold or 'set point' required for CB hypoxic response initiation. Thus we demonstrate that a substance capable of competitively inhibiting mitochondrial complex IV increases the CB O_2 sensitivity. We suggest that CB NO-complex IV interactions may thus provide both a means by which a relatively low O_2 affinity is achieved in the mitochondria of the type I cell and a means by which plasticity could occur during natural development and/or pathology.

NO_2^- was selected in this study as 1) it has been shown to release small quantities (nM/pM) of NO at the mitochondria when applied at mM concentrations and 2) NO (and therefore NO_2^-) is a rapidly reversible competitive inhibitor of complex IV and thus mitochondrial function [27,9,2,48,47]. This allowed us to investigate the effects of NO on CB mitochondrial activity rather more specifically than would be possible by application of NO itself, which is likely to inhibit chemoreceptor function through soluble guanylate cyclase and modulation of L-type Ca^{2+} channels and BK_{Ca} channels [50,49].

With regards to the concentrations of NO_2^- used in this study it is important to note that there is a fundamental physical-chemical difference between NO_2^- and other common inhibitors of complex IV. NO_2^- is a strong ion i.e. in solution it is fully dissociated as the anion NO_2^- , in contrast other inhibitors e.g. cyanide & sulphide are relatively weak ions, or in the case of NO & CO gases. The latter can all readily cross membranes in their lipid soluble uncharged form (e.g. HCN & H_2S) and will reach an equilibrium in which the unionised form is at an equal concentration on either side of the membrane. In contrast NO_2^- is charged and will therefore only be able to cross lipid membranes via ion channels and transporters. Unless there are highly active primary or secondary active transporters for NO_2^- its equilibrium distribution across a membrane will be determined by the Nernst equation. In consequence, as the cell membrane has a resting potential of -60mV, the limiting concentration for intracellular NO_2^- would be 10 fold lower than the extracellular concentration, equally as the inner mitochondrial membrane has a potential of around -180 mV under normal conditions the limiting concentration for inner matrix NO_2^- will be a 1000 fold lower than in the cytosol. Thus, at an extracellular concentration of 30 mM, matrix NO_2^- may be no higher than 3 μ M.

NO levels cannot however be measured here directly because of the small size of the CB (~200 μ g), the fact that NO production is limited to a specific region and the lack of specific fluorescent probes for NO which would allow imaging of localised NO production. In addition, attempts to inhibit responses to NO_2^- with the NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) would be difficult to interpret due to its low cell permeability [20,4]. We were however able to observe the

effects of NO_2^- on mitochondrial function in isolated type I cells by measuring changes in mitochondrial NADH levels [15,8,7]. We did not attempt to measure the effects of NO_2^- on oxygen consumption in carotid body mitochondria due to the lack of suitable instrumentation for working with such a small amount of tissue.

The finding that NO_2^- augmented responses to hypoxia in the intact CB is in striking contrast to recent studies using other complex IV inhibitors. Donnelly et al [14] have reported that the prolonged (mins) application of low levels of cyanide (or azide or sulphide) attenuates the neural response to hypoxia in isolated chemoreceptors. One possible explanation for this is that whereas CN^- , azide and H_2S will indiscriminately inhibit complex IV in all tissue elements within the CB (including nerve endings, type-II cells and blood vessels), NO_2^- would be expected to selectively target type I cell mitochondria. This is because whereas CN^- , azide and H_2S are non-competitive inhibitors of complex IV, NO is a competitive inhibitor and should therefore be much more effective at inhibiting complex IV in the type I cell which has a lower apparent affinity for oxygen [8] than in other cell types. Moreover the reduction of NO_2^- to NO by mitochondria is promoted when hypoxia limits electron transport to oxygen [9]. This is likely to occur at higher levels of tissue oxygenation in the type I cell than in other cells again on account of type I cell mitochondria having a lower apparent O_2 affinity. Thus, mitochondrial NO production and its ability to inhibit cytochrome oxidase should be more favourable in the type I cell compared with other surrounding cell types. Our results with NO_2^- in the intact carotid body may therefore more faithfully represent the effects of mitochondrial inhibition in type I cells free of the confounding effects arising from the metabolic poisoning of nerve endings and other adjacent tissues. In this context it is notable that when Donnelly et al [14] studied the effects of low levels of CN^- in isolated type I cells (as opposed to the intact CB) it also augmented calcium responses to hypoxia.

To eliminate a potential non-selective/alternative action of NO_2^- on chemoreceptor function, we performed a number of additional control experiments. First, experiments using NaNO_2 were performed at reduced NaCl concentrations, in order to maintain osmolality. The finding that NO_3^- had no effect on type I cell $[\text{Ca}^{2+}]_i$ at the same lower concentration of NaCl suggests that the observed excitation evoked by NO_2^- was not subsequent to the reduction in Cl^- ions. Second, as there is evidence that some sensory neurons, often with mitochondrial-rich terminals, may respond to hypoxia [12,5,34], our observations that chemoafferent activity was not elevated by addition of NO_2^- during hyperoxia, or that chemoafferent responses to ATP in hyperoxia were not enhanced by NO_2^- , supports the idea that the chemostimulatory impact of NO_2^- occurs only at the level of the type I cell and has no direct influence on the CSN terminals. Finally, we showed that response to NO_2^- was not altered during administration of TEMPOL, a cell permeable SOD-mimetic and free-radical scavenger [10,22,30], and thus we propose that the excitatory impact of NO_2^- is not a consequence of a modification in levels of mitochondrial/NADPH derived reactive oxygen species (ROS) [3,19,13,38]. We suggest that alternative signaling pathways activated subsequent to type I cell mitochondrial inhibition could include direct inhibition of TASK-like channels due to a fall in $[\text{ATP}]_i$ [52] or activation of the AMP-activated protein kinase [17,54].

Whether there is a role for endogenous NO (and/or NO₂⁻) in mediating CB mitochondrial function and hypoxic sensing remains to be more clearly defined. Measurable amounts of NO have been identified in mitochondrial membranes of type I cells [55]. The more likely source of endogenous NO is nitric oxide synthase 3 (NOS-3) given that this is the only NOS isoform to be detected within the type I cell [55]. Selective inhibitors of NOS-3 are currently unavailable. However, mice deficient in NOS-3 do exhibit a significantly diminished ventilatory response to hypoxia coupled with a downgraded CB function [26]. The absence of significant type I cell hyperplasia in these mice, suggests that the reduced CB excitability is not mediated by chronic hypoxia [51,32]. Rather, it points towards an alternative explanation and perhaps a tonic excitatory action of NO on CB chemoreceptor activity. Future investigations could test whether endogenous NO generation from NOS-3 or NO₂⁻ is sufficient to reduce type I cell mitochondrial O₂ affinity. A sufficiently high mitochondrial production of NO may well help explain the exceptionally low O₂ affinity of the mitochondria in the CB type I cell [8].

Mitochondrial inhibition with nitrite sensitises the CB to hypercapnic stimulation

Significant stimulus interaction between hypoxia and hypercapnia is well documented in the CB [40,11]. Our own findings demonstrated that mitochondrial targeting with NO₂⁻ increased CB hypercapnic sensitivity. Identification of the synergy between these two stimuli further substantiates the proposal that hypoxia and mitochondrial inhibition have similar effects on CB chemoreceptor function.

The exact site of convergence between mitochondrial inhibition and hypercapnic stimuli is still to be detected. These stimuli do share a number of key transduction processes including attenuation of background TASK-like conductance, activation of L-type Ca²⁺ channels, neurosecretion and stimulation of the CSN afferents [29]. The observed stimulus interaction could be a consequence of up-regulation of any or all of these processes and additional examination was beyond the scope of this study. The data does however support a role for the mitochondria in setting not only the CB threshold for the response to hypoxia but also to hypercapnia. Whether it may set the threshold to other putative stimuli of the CB remains to be investigated.

Relevance to carotid body pathology.

The mechanisms underpinning pathological CB activation in CHF and SDB are as yet unknown but it is thought that reactive oxygen species (ROS) production, possibly by an NADPH oxidase [30,25], may play a role. Downstream targets/effectors for ROS mediated signalling/damage and chemoreceptor activation are yet to be identified. Mitochondrial function could well be such a target. Electron transport complexes are known to be susceptible to ROS damage [23] (as is mitochondrial DNA [44]) and, as shown here, inhibition of electron transport has the capacity to dramatically alter chemoreceptor activity. It is therefore important that the effects of CHF and SDB on type I cell mitochondrial function are evaluated along with other potential effectors.

Conclusions

NO_2^- , a NO donor and mitochondrial inhibitor, produces dose dependent rises in CB type I cell NADH autofluorescence combined with a similar degree of $[\text{Ca}^{2+}]_i$ and chemoafferent stimulation. Inhibitory targeting of the mitochondria using NO_2^- sensitises the CB to hypoxia, thus supporting the hypothesis that mitochondrial function is important in establishing the CB sensitivity to O_2 . Manipulation of CB mitochondrial energy metabolism and downstream metabolic signalling pathways may prove to be important in reducing the CB sensory output that drives hypertension in certain pathologies.

Ethical standards

The authors declare no competing interests.

All surgical procedures were performed in accordance with project and personal licences issued under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Biomedical Services Unit at the University of Birmingham and University of Oxford.

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Figure captions

Fig. 1 Exogenous nitrite acts as a chemostimulant and mitochondrial inhibitor in the carotid body. **a)** An example trace showing the impact of nitrite (NO_2^-) on the frequency recorded from a single chemoafferent fibre. Raw discharge is shown (upper) along with frequency histograms (lower) grouped in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination. **b)** Mean frequencies induced by different concentrations of NO_2^- , expressed as a percentage of the paired peak frequency response to severe hypoxia. Data presented is from 9 fibres from 6 different CB preparations. Error bars indicate \pm S.E.M. * denotes $p < 0.05$ compared with initial NO_2^- dose; one way repeated measures ANOVA with Dunnett's post hoc analysis. **c)** Example recordings demonstrating the effect of 10 mM and 33 mM NO_2^- on NADH autofluorescence in two different type I cell clusters, measured in 2% O_2 . **d)** Mean increase in NADH autofluorescence induced by 10 mM NO_2^- (4 clusters, 4 CB preparations) and by 33 mM NO_2^- (6 clusters from 4 CB preparations), expressed as a percentage of the paired mean peak NADH autofluorescence responses evoked by severe hypoxia (0% O_2). Error bars indicate \pm S.E.M. * denotes $p < 0.05$ compared with 10 mM NO_2^- ; unpaired t-test.

Fig. 2 The carotid body type I cell $[\text{Ca}^{2+}]_i$ response to nitrite is dose dependent and influenced by the background steady state level of dissolved O_2 . **a)** Example recording from a type I cell cluster demonstrating the impact of 10 mM nitrite (NO_2^-) on $[\text{Ca}^{2+}]_i$ measured in 20% and 2% dissolved O_2 . **b)** Mean $[\text{Ca}^{2+}]_i$ measured in 4 type I cell clusters from 3 CB preparations. **c)** and **d)** As for a) and b) but in the presence of 33 mM NO_2^- (n=9 cell clusters from 4 preparations). **e)** An example trace showing the absence of any augmentation in $[\text{Ca}^{2+}]_i$ upon addition of 33 mM nitrate (NO_3^-). **f)** Mean $[\text{Ca}^{2+}]_i$ in the presence and absence of NO_3^- (n=9 cell clusters from 3 preparations). Error bars indicate \pm S.E.M. * denotes $p < 0.05$ compared with 2% O_2 ; one way repeated measures ANOVA with Bonferroni post hoc analysis test.

Fig. 3 Effects of removal of extracellular calcium and calcium channel block on nitrite response. Figure shows intracellular Ca^{2+} measurements in isolated rat type I cells. All cells were confirmed as being oxygen sensitive by brief application of a severe hypoxic stimulus (95% $\text{N}_2/5\%$ CO_2 ; PO_2 approximately 2 mmHg) prior to commencement of the experiment. Responses to NO_2^- were tested in the presence of a mild hypoxic stimulus i.e. 2% O_2 . **a)** Effects of 30 mM NO_2^- in the presence and absence of extracellular Ca^{2+} (Ca-free Krebs also contained 100 μM EGTA). **b)** Effects of 30 mM NO_2^- in the presence and absence of extracellular NiCl_2 (2 mM). **c)** Comparison of average $[\text{Ca}^{2+}]_i$ under control conditions (20% O_2), mild hypoxia (2% O_2), 30 mM NO_2^- in mild hypoxia and 30 mM NO_2^- (2% O_2) in Ca-free media. (n= 8, * $p < 0.001$). Statistical comparisons were performed between NO_2^- and NO_2^- in Ca-free media using a paired t-test. **d)** Comparison of average $[\text{Ca}^{2+}]_i$ under control conditions (20% O_2), mild hypoxia (2% O_2), 30 mM NO_2^- in mild hypoxia and 30 mM NO_2^- (2% O_2) in the presence of 2 mM NiCl_2 . (n=9, * $p < 0.001$). Statistical comparisons were performed between NO_2^- and NO_2^- in the presence of Ni^{2+} using a paired t-test.

Fig. 4 Nitrite enhances the carotid body chemoafferent response to hypoxia **a)** An example of the sensory neuronal frequency recorded during graded hypoxia in the presence and absence of 3.3 mM nitrite (NO_2^-). The continuous superfusate PO_2 is shown along with frequency histograms and demonstrate an augmented discharge in the presence of 3.3 mM NO_2^- . Overdrawn action potentials are shown inset to illustrate the single fibre discrimination. For this same fibre, PO_2 response curves are plotted (right), displaying a reversible 'right shift' induced by 3.3 mM NO_2^- , characteristic of an heightened O_2 sensitivity. **b)** Mean single fibre frequencies recorded during graded hypoxia in the presence and absence of 3.3 mM nitrite (NO_2^-). † denotes $p < 0.05$, 3.3 mM NO_2^- vs control; two way repeated measures ANOVA. **c)** For all fibres the PO_2 was measured at 5 Hz in the presence and absence of 3.3 mM NO_2^- in order to quantify the mean 'right shift'. + denotes $p < 0.05$, 3.3 mM NO_2^- compared with control; paired t-test. **d)** The calculated augmentation in single fibre discharge frequency induced by 3.3 mM NO_2^- was plotted over a range of defined superfusate PO_2 values and shows an enhanced impact in hypoxia, consistent with stimulus interaction. Error bars indicate \pm S.E.M. * denotes $p < 0.05$ compared with the frequency difference at 300 mmHg PO_2 ; one way repeated measures ANOVA with Dunnett's post hoc analysis. For b), c) and d) data is from 9 fibres from 7 different CB preparations.

Fig. 5 Nitrite enhances the carotid body sensitivity to hypercapnia. **a)** Example of the sensory neuronal response to hypercapnia in the presence and absence of 3.3 mM nitrite (NO_2^-). Raw discharge is shown (upper) along with frequency histograms (lower) that collate single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure the frequency. **b)** Mean discharge frequencies recorded under normocapnic ($\text{PCO}_2 = 40$ mmHg) and hypercapnic ($\text{PCO}_2 = 80$ mmHg), in control conditions and following addition of 3.3 mM NO_2^- . Error bars indicate \pm S.E.M.* denotes $p < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. **c)** Discharge frequency differences (80 – 40 mmHg PCO_2) for each fibre in the presence and absence of 3.3 mM NO_2^- . * denotes $p < 0.05$ compared with control group; paired t-test. **d)** Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following NO_2^- application. Error bars indicate + S.E.M. * denotes $p < 0.05$ compared with control group; paired t-test. For b), c) and d) mean data is from 10 fibres from 6 CB preparations.

Fig. 6 Responses to nitrite are not a consequence of a direct action on the carotid sinus nerve ending or dependent on reactive oxygen species. **a)** Example chemoafferent response to 10 mM nitrite (NO_2^-) in normoxia and following CB silencing with hyperoxia (95% O_2). Raw neuronal discharge is presented along with frequency histograms. Overdrawn action potentials are shown inset to demonstrate single fibre discrimination. **b)** Mean frequency responses to 10 mM NO_2^- in normoxia and hyperoxia. * denotes $p < 0.05$; one way repeated measures ANOVA with Bonferroni post hoc analysis, 10 fibres from 5 CB preparations. **c)** Mean responses to 1mM ATP (excitatory neurotransmitter) in hyperoxia, in the presence and absence of 10mM NO_2^- , 6 fibres from 3 CB preparations. **d)** Example chemoafferent response to 10 mM NO_2^- before and during addition of 1mM TEMPOL (free-radical scavenger). **e)** Mean

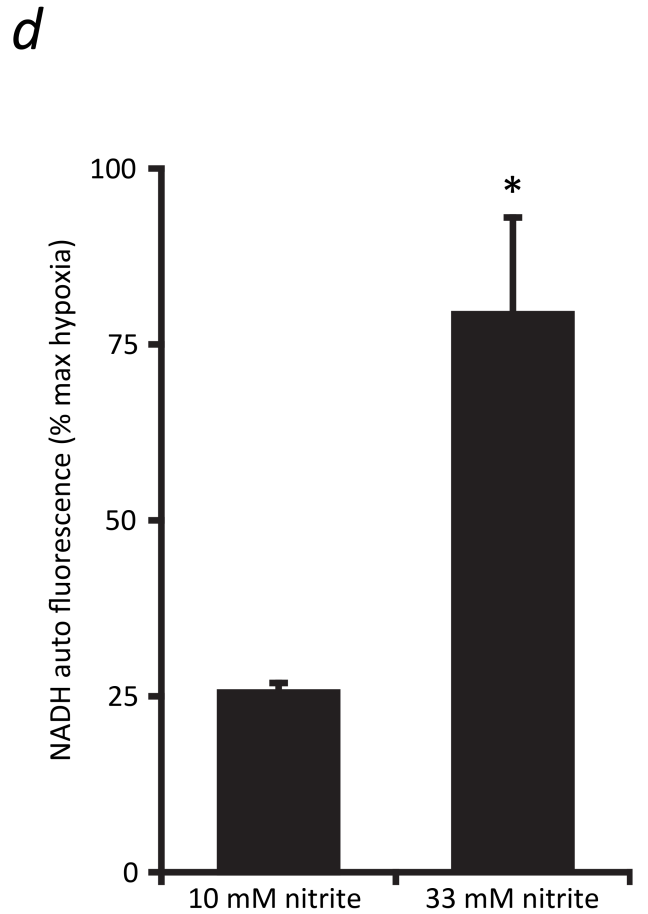
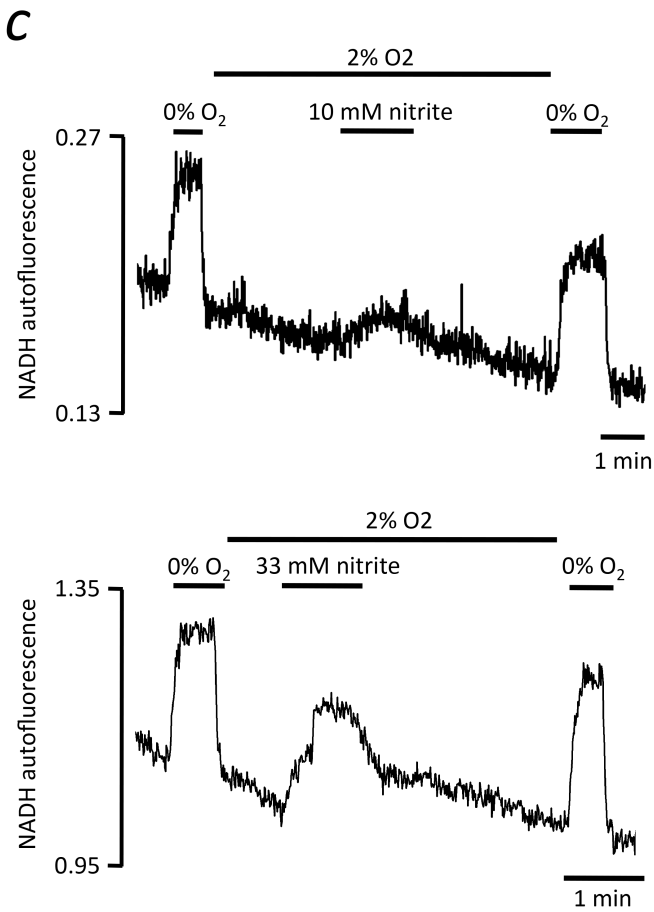
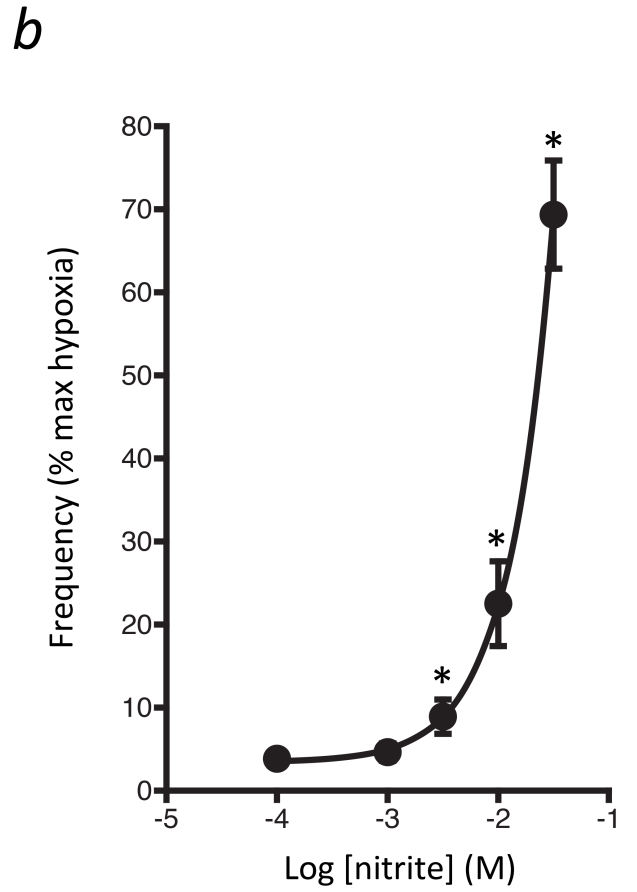
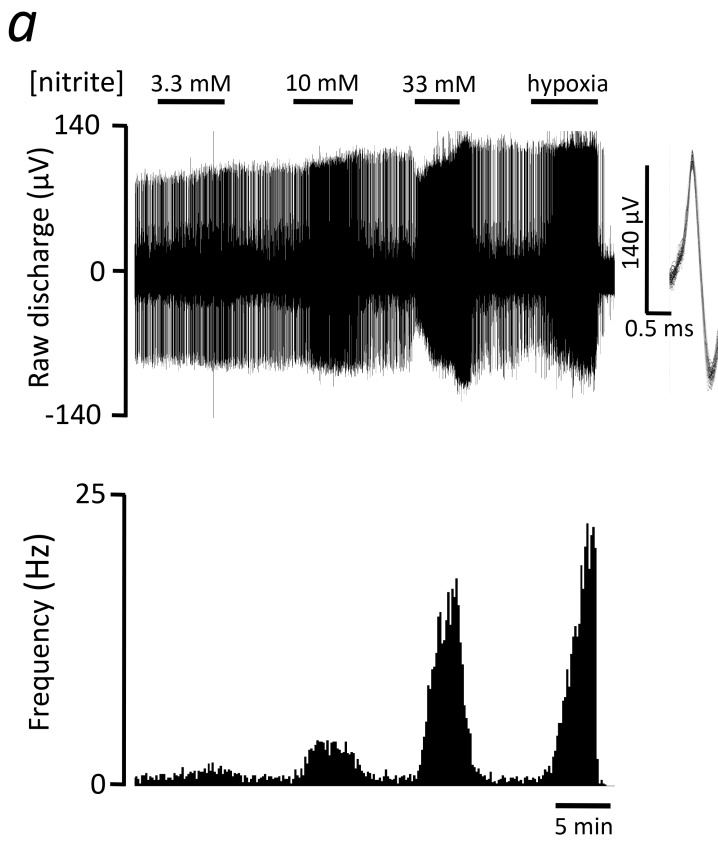
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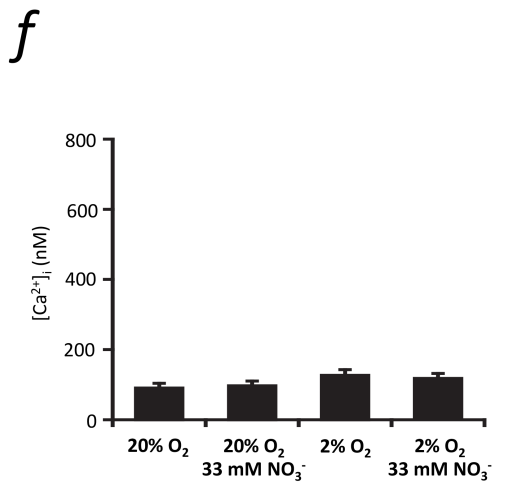
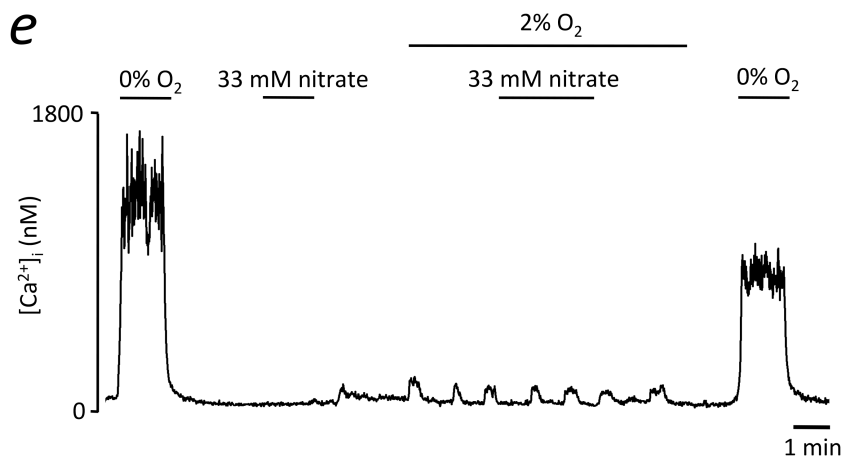
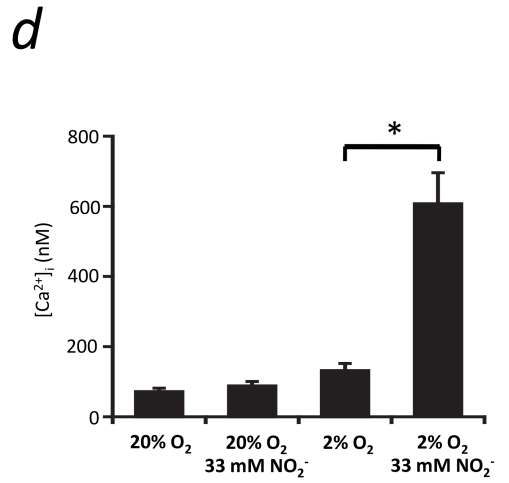
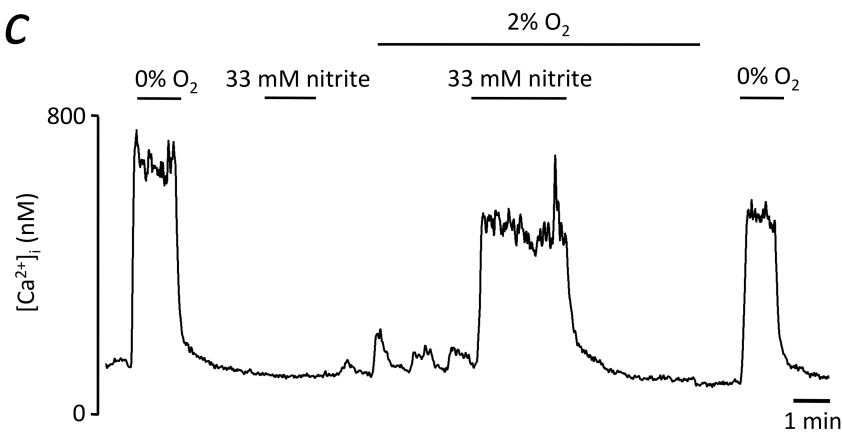
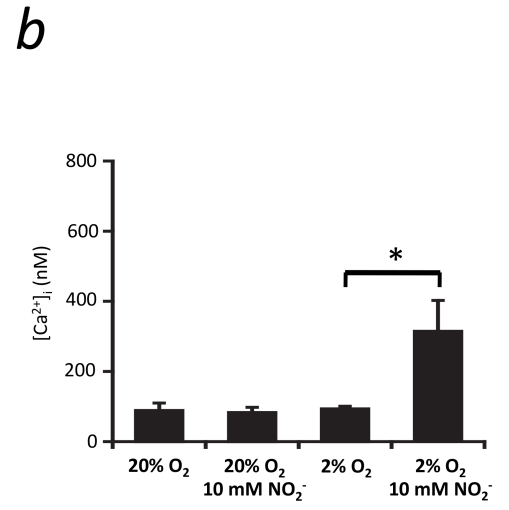
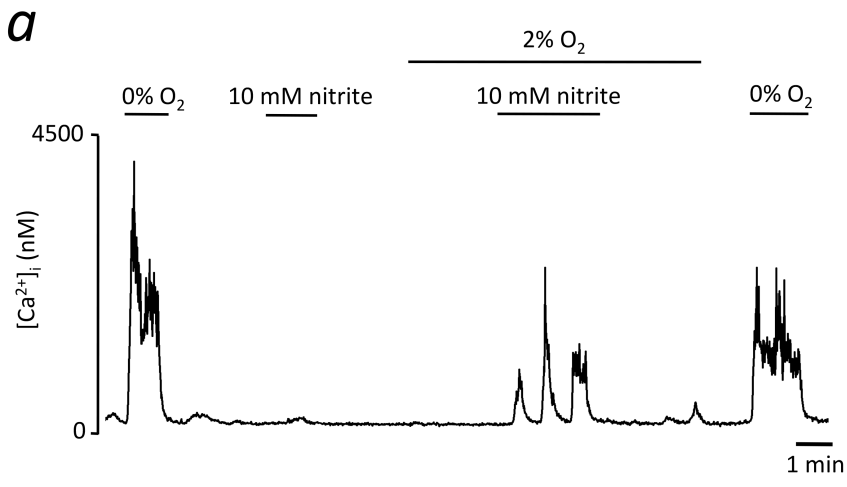
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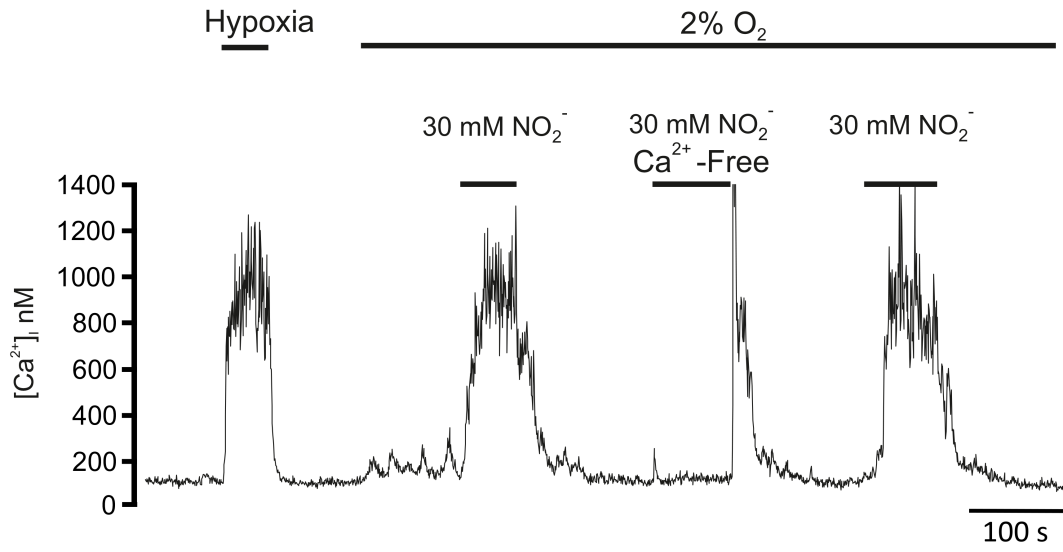
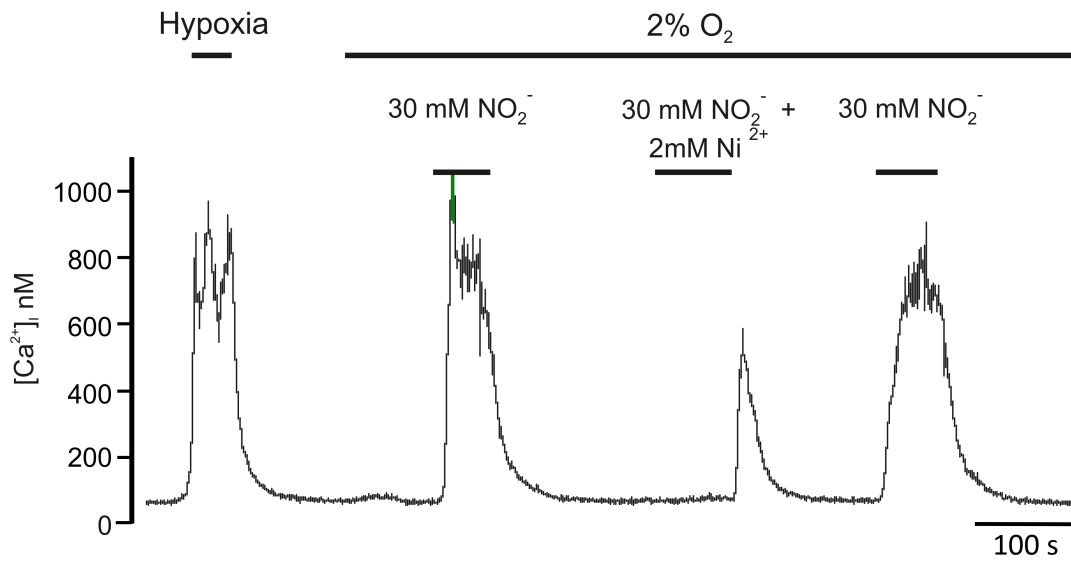
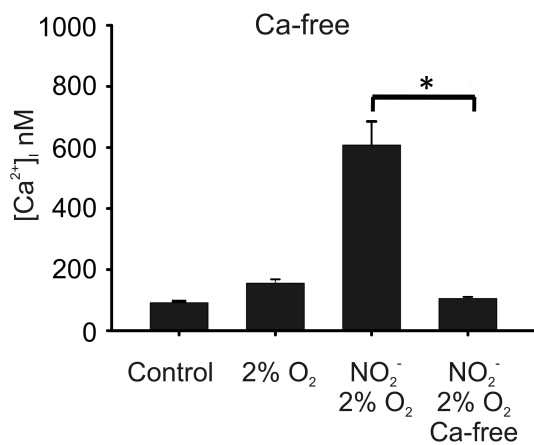
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