

Linköping University Post Print

Prostaglandins and Radical Oxygen Species Are Involved in Microvascular Effects of Hyperoxia

Andreas Rousseau, Erik Tesselaar, Joakim Henricson and Folke Sjöberg

N.B.: When citing this work, cite the original article.

Original Publication:

Andreas Rousseau, Erik Tesselaar, Joakim Henricson and Folke Sjöberg, Prostaglandins and Radical Oxygen Species Are Involved in Microvascular Effects of Hyperoxia, 2010, JOURNAL OF VASCULAR RESEARCH, (47), 5, 441-450.

<http://dx.doi.org/10.1159/000282667>

Copyright: S. Karger AG

<http://www.karger.com/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-59256>

PROSTAGLANDINS AND RADICAL OXYGEN SPECIES ARE INVOLVED IN MICROVASCULAR EFFECTS OF HYPEROXIA

A. ROUSSEAU¹, E TESSELAAR², J HENRICSON², F. SJÖBERG²

¹ Department of Medicine and Care, ² Department of Biomedicine and Surgery,

Faculty of Health Sciences, University Hospital, Linköping, Sweden

Running title

Microvascular effects of hyperoxia

Correspondence

Dr Andréas Rousseau

Department of Medicine and Care

University Hospital of Linköping

SE-581 85 Linköping, Sweden

Phone: + 46 13 22 20 00

Fax: + 46 13 22 28 36

E-mail: andreas.rousseau@lio.se

ABSTRACT

Hyperoxia causes vasoconstriction in most tissues, by mechanisms that are not fully understood. We investigated microvascular effects of breathing 100% oxygen in healthy volunteers, using iontophoresis to deliver acetylcholine (ACh) and sodium nitroprusside (SNP). Aspirin and vitamin C were used to test for involvement of prostaglandins and radical oxygen species. Forearm skin perfusion was measured using laser Doppler perfusion imaging. Results were analysed using dose-response modelling.

The response to ACh was reduced with 30% during oxygen breathing compared to air breathing (0.98 (0.81 – 1.15) PU vs 1.45 (1.30 – 1.60) PU, $p < 0.001$). ED₅₀ values were unchanged (2.25 (1.84-2.75) vs. 2.21 (1.79-2.74), ns). Aspirin pre-treatment abolished the difference in response between oxygen breathing and air breathing (maximum: 1.03 (0.90-1.16) vs. 0.89 (0.77-1.01), ns; ED₅₀: 1.83 (1.46-2.30) vs. 1.95 (1.65-2.30), ns). ACh-mediated vasodilatation during 100% oxygen breathing was partially restored after pre-treatment with vitamin C. Breathing 100% oxygen did not change the microvascular response to SNP (1.45 (1.28-1.62) vs. 1.40 (1.26-1.53), ns).

These results favour the hypothesis that hyperoxic vasoconstriction is mediated by inhibition of prostaglandin synthesis. Radical oxygen species may be involved as vitamin C, independently of aspirin, partially restored ACh-mediated vasodilatation during hyperoxia.

Key Words: acetylcholine, perfusion, hyperoxia, iontophoresis, prostaglandins, radical oxygen species, sodium nitroprusside

Introduction

Hyperoxia has cardiovascular effects in humans, causing a reduction in cardiac output and heart rate [1-4] and an increase in peripheral resistance and vascular tone [5, 6]. As a result, perfusion is reduced in most tissues [7-9]. It is probable that hyperoxia acts firstly at the microvascular level by inducing peripheral vasoconstriction [5, 7]. The reduction in cardiac output is a secondary effect and works to keep the blood pressure constant.

The mechanisms underlying the effect of oxygen in the microvasculature have still to be elucidated. Several theories have been presented, including the action of red blood cells as an oxygen sensor [10]. Red blood cells release increased amounts of adenosine 5'-triphosphate (ATP) during hypoxia, which result in a vasodilator response. Oxygen may also induce changes in the rheology of red blood cells, which in itself modulates tissue perfusion [11]. In previous findings in rat cremaster arterioles and human umbilical arteries, alterations in endothelium-derived dilator prostanoids have been found to contribute to hyperoxic vasoconstriction [12-14]. Also, the vasoconstricting effect of hyperoxia has been attributed to oxygen free radicals reacting with and inactivating the vasodilator agent nitric oxide (NO) [2,4,15,16].

The primary aim of this study was to test the hypothesis that a brief period of substantial normobaric hyperoxia (100% oxygen breathing during 10 minutes) affects the vasoactive response in the microcirculation of the human forearm skin. The secondary aim was to investigate if any effect of normobaric hyperoxia is dependent on endothelial mechanisms and whether prostaglandins or oxygen free radicals are involved.

To answer these questions, we used a non-invasive technique. A laser Doppler perfusion imager [17] was used to measure perfusion in the skin of healthy subjects and iontophoresis [18] was used as a way of delivering drugs through intact skin. To be able to identify possible involvement of endothelium, acetylcholine (ACh, an endothelium-dependent vasodilator) and sodium nitroprusside (SNP, a direct NO-donor) were used. Acetylsalicylic acid (aspirin) and vitamin C were used to test whether any effect of hyperoxia on microvascular responses in the forearm skin was dependent on prostaglandins or oxygen free radicals. Finally, sodium chloride was

delivered to test for any nonspecific effects of iontophoresis on forearm skin perfusion.

Materials and methods

Subjects

Twenty-two healthy volunteers took part in the study (15 men). The study was divided into five protocols depending on the drugs that were used (acetylcholine alone, acetylcholine with aspirin, acetylcholine with vitamin C, sodium nitroprusside and sodium chloride as a control solution). The volunteers could participate in more than one protocol. Subjects were interviewed and a physical examination was made. Apart from 3 of the women who were taking oral contraceptives, the volunteers were not taking any drugs. Subjects were not included if they had either neurological, cardiovascular, pulmonary, hepatic, renal, haematopoietic, gastrointestinal, or metabolic dysfunction. The volunteers were asked to refrain from coffee, tea, and nicotine products for 10 hours before the experiments. They were all fully informed of the nature and purpose of the study. The study was approved by the local ethics committee. Demographic details of the subjects are presented in table 1.

Table 1. Details of the subjects participating in the study.

Protocol	1	2	3	4	5
Number (Men)	10 (6)	9 (9)	11 (5)	9 (8)	9 (7)
Mean (range) age (years)	28 (22-33)	29 (23-41)	26 (22-29)	28 (23-32)	27 (23-33)
Mean (SD) BMI	22 (2.0)	23 (2.1)	23 (3.3)	22 (1.5)	22 (2.6)
Mean (SD) skin temperature					
air breathing	31 (1.7)	32 (0.7)	31 (1.1)	30 (0.9)	32 (1.0)
100% oxygen breathing	31 (1.6)	32 (0.7)	31 (1.2)	30 (0.9)	32 (0.9)

Laser Doppler perfusion imaging (LDPI)

A laser Doppler perfusion imager (PIM 1.0 LDPI, Lisca Development AB, Linköping, Sweden) was used to measure perfusion in the skin. The laser beam, with a wavelength of 670-nm, penetrates the skin and a fraction of light is backscattered, containing a frequency-shifted part and a non-frequency shifted part. The frequency-shifted part is the light backscattered by mobile red blood cells (the Doppler principle). The relative contribution of the two parts decides the perfusion value, which is defined as the number of blood cells multiplied by the mean cell velocity. At

each measuring site this procedure is repeated (up to 4096 measurements) to form a colour-coded image on a computer screen.

To keep a constant distance between the head of the LDPI and the skin, two adjustable laser beams were attached to the head of the scanner. We chose 15 cm as an optimal distance, as this gives a reliable signal [17]. Before starting, the distance between the head of the scanner and the skin was measured with a ruler. Then the two laser beams were set to cross each other at skin level. For each new measurement, the laser beam cross hair was used to set the scanner head quickly at a constant distance.

Perfusion measurements were made with the LDPI in duplex mode, scanning the measuring site continuously. In duplex mode, the measurement value generated by the software (LDPIwin v2.3.13, Lisca, Sweden) is the geometrical mean of the 4×4 measurement points.

Iontophoresis

After the flexor side of one of the forearms had been gently cleaned with an ethanol wipe, a customized iontophoresis electrode chamber (Lisca Development AB, Linköping, Sweden) was attached to the skin by double-sided adhesive Scotch tape. The area of measurement was selected to avoid visible veins and pigmented naevi. The inner diameter of the ring-shaped chamber was 15 mm, and the distance between the silver-silver chloride electrode ring and the skin was 2 mm. The electrode chamber was filled with the drug solution (about 430 μ L) and covered by a thin glass slide. The head of the scanner was tilted somewhat to avoid direct reflection of the laser beam from the glass slide into the head. An indifferent electrode (Perimed AB, Stockholm, Sweden) was applied to the wrist. A battery-powered iontophoresis controller (PeriIont 382, Perimed AB, Stockholm, Sweden) was connected to both electrodes to deliver a constant electrical current to the skin. The subjects were told to hold their arms still during the experiment. Skin temperature (Digital thermometer, Claes Ohlson, Insjön, Sweden) was recorded before and after each measurement on the same forearm, about 5 cm proximal to the measuring site. When more than one measurement was performed in the same subject, different skin sites were used for each measurement.

Drugs

Acetylcholine chloride (Miochol[®]-E 10 mg/ml, Novartis Healthcare, Denmark), sodium nitroprusside (Nitropress[®] 25 mg/mL, Abbott, Chicago, USA), acetylsalicylic acid (Aspirin[®], 500mg, Bayer AB, Solna, Sweden), vitamin C (C-vimin[®] 1g, AstraZeneca, Södertälje, Sweden) and sodium chloride (9 mg/ml), were dispensed by the hospital pharmacy. Acetylcholine (1%) and sodium nitroprusside (1%) were dissolved in a sodium chloride solution such that a final ionic strength of 154 mM was obtained [19]. Drug concentrations were the same throughout the experiments. All drugs were prepared immediately before being used and SNP was kept in the dark.

Air and oxygen

Medical air and 100% oxygen were given through a non-rebreathing system, with the subjects breathing through a facemask. The subjects did not know whether air or oxygen was being given.

The subjects started to breathe air from the facemask 10 minutes before the measurements started, to get used to the mask. Thereafter, air was administered during the period of iontophoresis, after which oxygen was given for the same amount of time. Oxygen was always given last to avoid washout effects of oxygen during the measurements made with air.

Experimental protocol

The subjects rested lying down for 30 minutes in a temperature-controlled room, 23 (0.6) °C. A thin cotton blanket was placed over the trunk and lower extremities to prevent cooling. All measurements were made in a dark room, the only light coming from the screen connected to the LDPI. All measurements were made with the subjects lying down.

In previous studies we have optimised our iontophoresis protocols to reduce the vasodilating effect of the current alone, and to maximise the response to the drugs, with most responses ending as plateaus [19, 20]. For all measurement protocols in this study, we used a single pulse at a constant current. The duration of the pulse was 10 minutes and the current strength was 0.02 mA.

The current study was divided into 5 protocols. In protocol 1 (n=10), acetylcholine was delivered to the skin by anodal iontophoresis. In protocol 2 (n=9), sodium nitroprusside was delivered by cathodal iontophoresis. In protocol 3 (n=11), acetylcholine was delivered after 2x 500 mg of ASA had been given 24 hours before the measurements, followed by another 2 x 500 mg of ASA 2 hours before the measurements. In protocol 4 (n=9), iontophoresis of acetylcholine was preceded by the intake of vitamin C. Vitamin C (2.5 g) was given by mouth every morning for 3 consecutive days. The last dose was taken 1-2 hour before the examination took place [21]. In protocol 5 (n=9), sodium chloride was delivered by cathodal iontophoresis. The protocols were performed on separate days and at different skin sites.

Dose response model

The perfusion response from each individual subject was plotted as a function of the logarithm of electrical charge. The electrical charge is indicative of the dose of drugs given during iontophoresis [22, 23].

The data were analysed by fitting the Emax model, based on classical receptor occupancy theory [24], to the pooled perfusion data in every group of measurements.

The model can be described by the following equation:

$$E(t) = E_{\min} + \frac{(E_{\max} - E_{\min})C(t)^n}{C(t)^n + ED_{50}^n}$$

where E_{\min} is the baseline, E_{\max} is the maximum perfusion, ED_{50} is the iontophoretic charge eliciting half of the maximum response and n is the Hill slope, which defines the steepness of the response. $C(t)$ is the electrical charge as a function of time, defined as the current strength multiplied by the pulse duration: $C(t) = I \cdot t$.

In each protocol, the last 10 seconds before the start of the first iontophoresis period (with air breathing) was considered as baseline perfusion. This baseline value was subtracted from all further data points obtained during both iontophoresis periods (with both air breathing and oxygen breathing).

Statistics

The minimum perfusion (E_{\min}), the maximum response ($E_{\max} - E_{\min}$), the ED_{50} and their respective confidence intervals (95% CI) were estimated from each response by fitting the model to the pooled data in each protocol. Since baseline perfusion was subtracted, E_{\min} was fixed to zero for the air breathing data. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Data in the figures are presented as means \pm SEM. For clarity, a limited number of data points are shown in the figures.

The significance of the difference in these estimated values between air breathing and oxygen breathing was tested using an F-test. Probabilities of less than 0.05 were accepted as significant.

Results

During all protocols where ACh and SNP were delivered, an increase in skin perfusion was found, which almost always resulted in plateaus in the responses. Fitting the model to the obtained perfusion data yielded well-defined dose response curves in all protocols (R^2 –values ranged from 0.55 to 0.95). An overview of the best-fit parameters and comparisons is presented in table 2.

Table 2. Overview of dose response parameters for all protocols (increase in perfusion, ED_{50}). Mean (95% CI) values are presented; p-values are based on the comparison between air breathing and 100% oxygen breathing within each protocol.

Maximum response [PU]			
Protocol	Air	100% O ₂	Air vs. 100% O ₂
1 ACh	1.45 (1.30-1.60)	0.98 (0.81-1.15)	p < 0.001
2 SNP	1.45 (1.28-1.62)	1.40 (1.26-1.53)	ns
3 ACh + ASA	1.03 (0.90-1.16)	0.89 (0.77-1.01)	ns
4 ACh + Vitamin C	1.58 (0.88-2.28)	0.79 (0.66-0.91)	p < 0.001
5 NaCl	0.01 (0.00-0.02)	0.05 (0.00-0.11)	ns
3 (Air) vs. 1 (Air)	p = 0.006		
3 (100% O ₂) vs. 1 (Air)		p < 0.001	
ED_{50} [mC]			
Protocol	Air	100% O ₂	Air vs. 100% O ₂
1 ACh	2.25 (1.84-2.75)	2.21 (1.79-2.74)	ns
2 SNP	4.21 (3.71-4.77)	4.21 (3.84-4.62)	ns
3 ACh + ASA	1.83 (1.46-2.30)	1.95 (1.65-2.30)	ns
4 ACh + Vitamin C	5.00 (2.35-10.6)	2.09 (1.72-2.53)	p = 0.002

Baseline perfusion

In protocol 1 (ACh) and 2 (SNP), a decrease in mean baseline skin perfusion was found during breathing of oxygen (p<0.001). In the other protocols, mean baseline perfusion was similar during breathing of air and oxygen. There was no significant difference in baseline perfusion at the start of the air-breathing period between the protocols.

Protocol 1: Acetylcholine

In protocol 1 (ACh), the maximum perfusion response was significantly lower while breathing oxygen than while breathing air. (oxygen: 0.98 (0.81-1.15) vs. air: 1.45 (1.30-1.60), $p < 0.001$). The ED_{50} values did not differ significantly between results with breathing oxygen and air (figure 1).

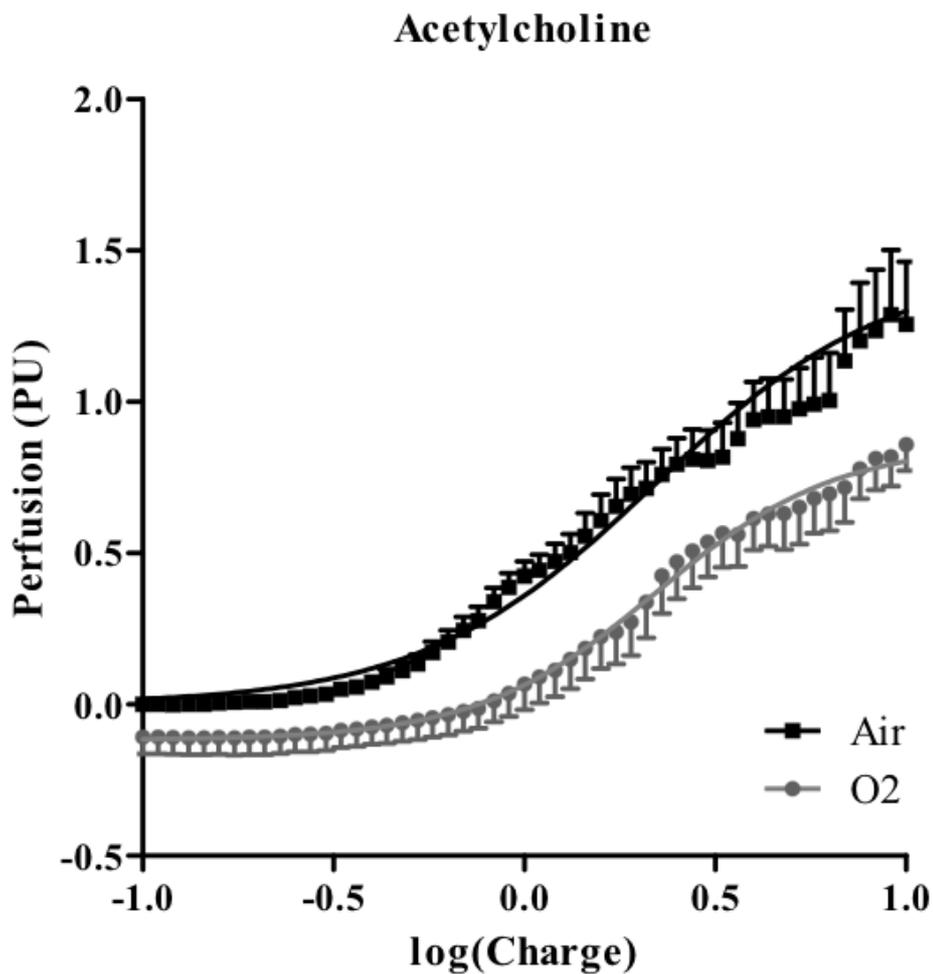


Figure 1. Perfusion response to acetylcholine delivered by anodal iontophoresis during 10 minutes of air breathing and 100% oxygen breathing (protocol 1). A reduction of 30% in maximum perfusion increase was found during 100% oxygen breathing ($p < 0.001$), while ED_{50} values were similar for the two responses.

Protocol 2: Sodium nitroprusside

During iontophoresis of SNP, the maximum responses were similar for breathing of oxygen and air (oxygen: 1.40 (1.26-1.53) vs air: 1.45 (1.28-1.62)). Also no difference was found between the ED₅₀ values during breathing of oxygen and air (oxygen: 4.21 (3.84-4.62) vs. air: 4.21 (3.71-4.77)). See figure 2.

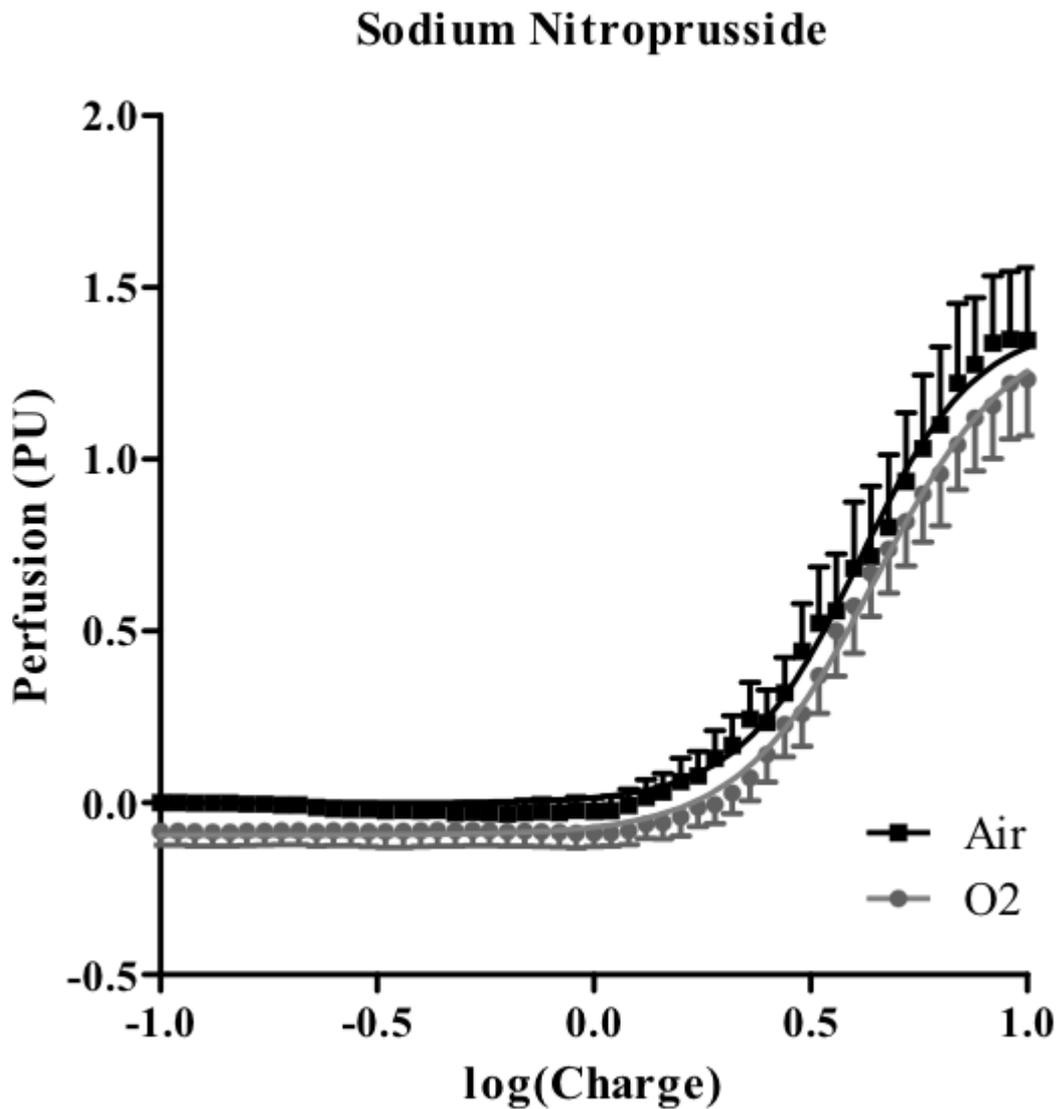


Figure 2. Perfusion response to sodium nitroprusside delivered by cathodal iontophoresis during 10 minutes of air breathing and 100% oxygen breathing (protocol 2). No significant difference was found in dose response parameters between air breathing and 100% oxygen breathing.

Protocol 3: Acetylcholine + aspirin

When ACh iontophoresis was preceded by intake of aspirin, no difference in the maximum responses between breathing oxygen and air was found (oxygen: 0.89 (0.77-1.01) and air: 1.03 (0.90-1.16)). The ED₅₀ values did not differ significantly between results with breathing oxygen and air (figure 3). Maximum responses during air and oxygen breathing were significantly lower compared with untreated, air-breathing subjects ($p = 0.006$ resp. $p < 0.001$).

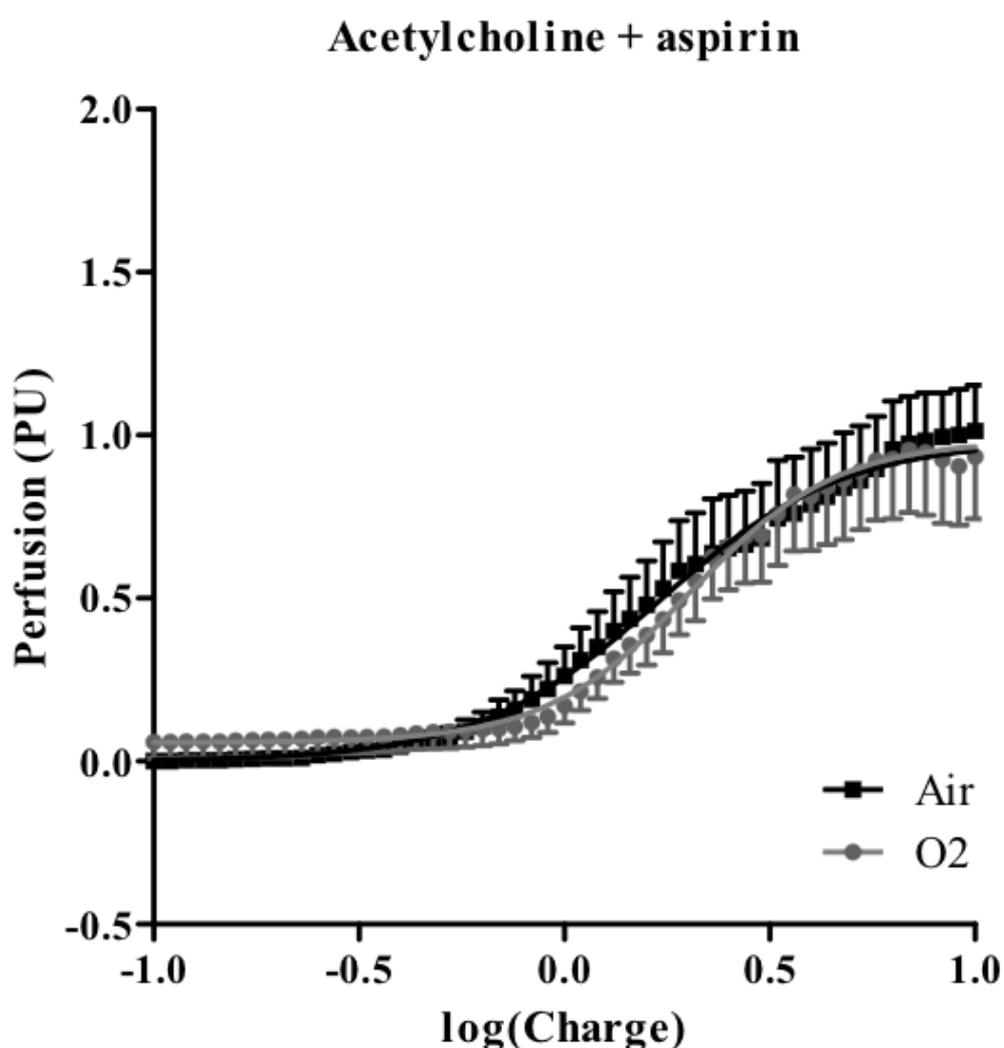


Figure 3. Perfusion response to acetylcholine delivered by anodal iontophoresis during 10 minutes of air breathing and 100% oxygen breathing. Synthesis of prostaglandins was inhibited by pre-treatment with aspirin (protocol 3). No significant difference was found in dose response parameters between air breathing and 100% oxygen breathing. The maximum increase in perfusion was similar to the maximum perfusion increase to acetylcholine during 100% oxygen breathing without pre-treatment (figure 1).

Protocol 4: Acetylcholine + vitamin C

When ACh iontophoresis was preceded by intake of Vitamin C, the perfusion response was reduced during oxygen breathing, but only at a charge of 6 mC and above (oxygen: 0.79 (0.66-0.91) vs. air: 1.58 (0.88-2.28)). As a result of the absence of a plateau in the data obtained during breathing of air, the respective maximum response and ED₅₀ value could not be estimated with high accuracy. However, the mean ED₅₀ value was significantly lower during oxygen breathing than during air breathing (oxygen: 2.09 (1.72-2.53) vs air: 5.00 (2.35-10.6)). See figure 4.

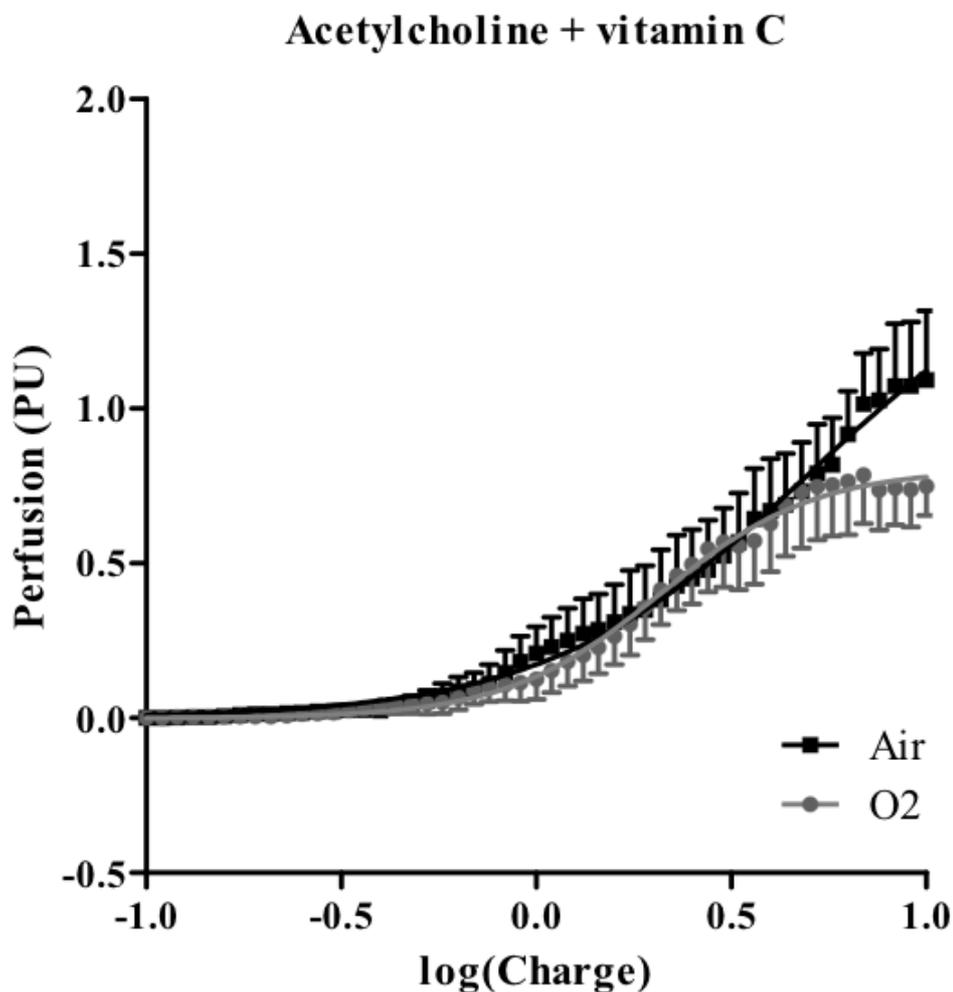


Figure 4. Perfusion response to acetylcholine delivered by anodal iontophoresis during 10 minutes of air breathing and 100% oxygen breathing after pre-treatment with vitamin C (protocol 4). No significant difference was found in dose response parameters between air breathing and 100% oxygen breathing up to a charge of 6 mC (5 minutes of oxygen breathing). At higher charges (longer time), the response to acetylcholine during 100% oxygen breathing did not increase further, while the response during air breathing continued to increase. Thus, the maximum increase in perfusion after pre-treatment with vitamin C was significantly higher for air breathing ($p = 0.0018$).

Protocol 5: Sodium chloride

When sodium chloride was delivered with iontophoresis to test for nonspecific effects, no significant increase in perfusion was found during breathing of air or oxygen (figure 5).

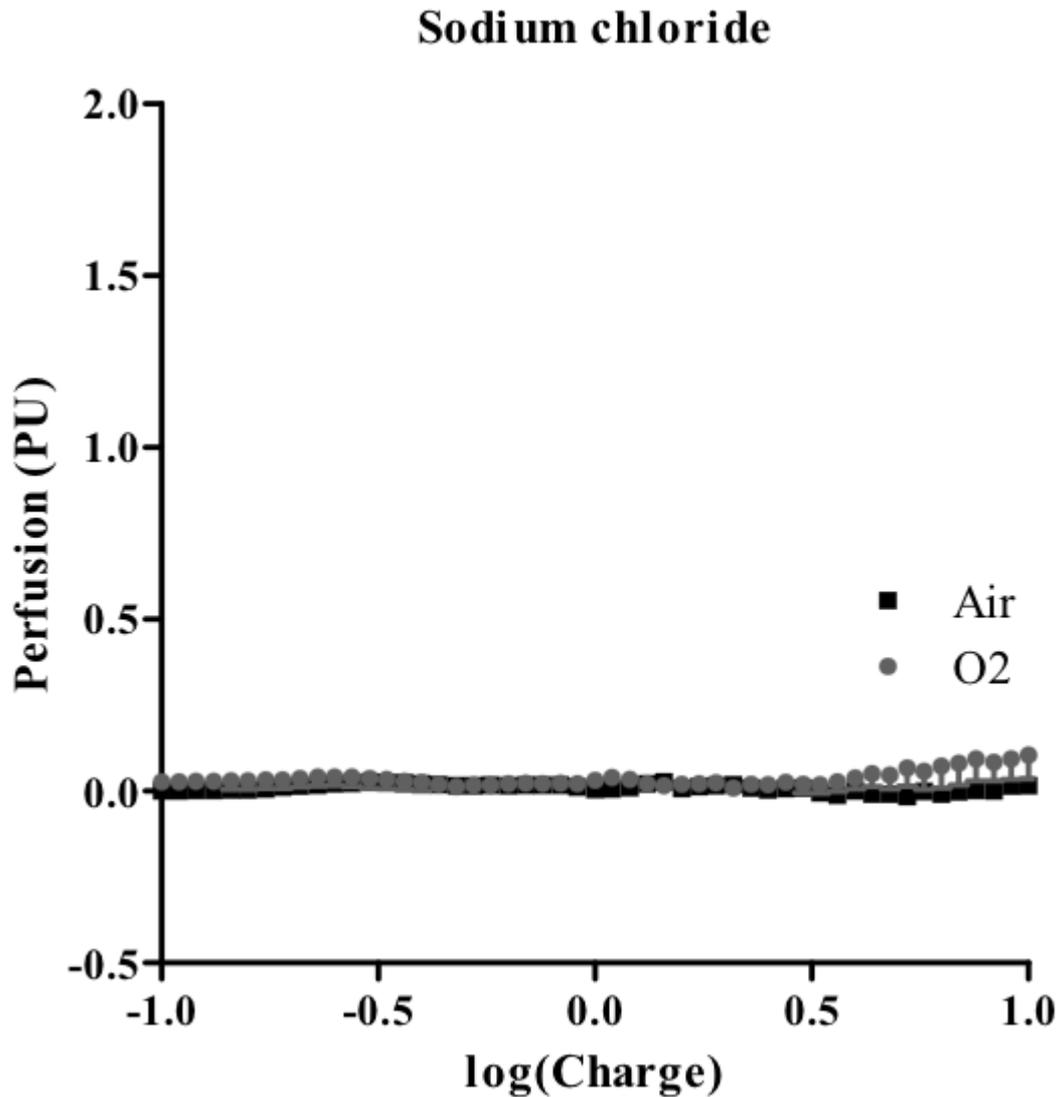


Figure 5. Perfusion response to sodium chloride delivered by cathodal iontophoresis during 10 minutes of air breathing and 100% oxygen breathing. The perfusion did not change significantly, indicating that the protocols used in this study (single 10-min pulse, 0.02 mA, total charge 12 mC), do not induce any nonspecific responses.

In none of the protocols was there any difference in ED₅₀ or maximum response between male and female subjects ($p > 0.32$).

Discussion

The main findings in this study are that in healthy subjects, 100% oxygen breathing reduces the endothelium-dependent microvascular response to acetylcholine with 30% compared to the response during normal air breathing. When subjects had been pre-treated with aspirin, there was no difference in response between oxygen breathing and air breathing. Instead, both responses were reduced with 30% compared to the response in untreated, air-breathing subjects. Acetylcholine-mediated vasodilatation during 100% oxygen breathing was partially restored after pre-treatment with the antioxidant vitamin C. Breathing 100% oxygen did not change the microvascular response to the NO-donor sodium nitroprusside.

Dose response modelling

The application of the E_{\max} model to perfusion response data obtained from a single-pulse iontophoresis protocol has been validated in previous studies by our group [22, 23]. This approach offers important advantages over more qualitative descriptions of responses. It enables a more powerful assessment of the dynamics of perfusion responses, as responses can be interpreted in terms of conventional pharmacodynamic parameters such as ED_{50} and maximum response. Also, modelling in tests of microvascular mechanisms may avoid confounding factors in the interpretation of responses [25].

Effect of oxygen on the response to acetylcholine

When the subjects breathed 100% oxygen, the maximum response to acetylcholine was reduced with 30% compared to the response during breathing of air, while the ED_{50} value was unchanged. This reduction was not found in the response to sodium nitroprusside. This finding confirms the results of a study by Yamazaki, in which breathing of 100% oxygen reduced the vasodilatation in response to iontophoretically applied acetylcholine [13], and suggests that the effect of hyperoxia is mediated by a non-competitive, endothelium-dependent mechanism.

Effect of aspirin on the response to acetylcholine

The results of the present study indicate that prostaglandins take part in the acetylcholine-mediated vasodilatation in the skin, since pre-treatment with aspirin

resulted in a 30% decrease in maximum response to the iontophoresis of acetylcholine, when the subjects breathed air. Aspirin (acetylsalicylic acid) is a non-competitive, irreversible antagonist of the enzyme cyclooxygenase that inhibits the synthesis of prostaglandins by permanent acetylation [26]. This finding is in line with a number of other studies, which have shown that the response to acetylcholine in the cutaneous microvasculature is the result of at least two different mechanisms [13, 27-31]. The first mechanism is that acetylcholine stimulates the activation of nitric oxide synthase (NOS, both neuronal and endothelial), by promoting the binding of a calmoduline-Ca²⁺ complex to NOS. The conversion of L-arginine to form citrulline and NO is then catalysed by NOS. The second mechanism is the increased production of prostaglandins from the conversion of arachidonic acid by cyclooxygenase (COX). Apart from the two mechanisms above, other mechanisms, such as endothelium-derived hyperpolarizing factor (EDHF), may be responsible for the acetylcholine-mediated vasodilatation.

Effect of oxygen on the response to acetylcholine with aspirin

Firstly, when aspirin was given before the experiment and when subjects breathed air, a similar reduction (30%) in maximum response was found compared to the acetylcholine response during oxygen breathing, without pre-treatment with aspirin. Secondly, under aspirin pre-treatment, 100% oxygen breathing did not further reduce the response to acetylcholine. Instead, the both the maximum responses and the ED₅₀ values for air and oxygen breathing were similar. These two results together strongly favour the hypothesis that the effect of hyperoxia on the endothelium-dependent vasodilatation involves prostaglandins.

Whether the interaction between hyperoxia and prostaglandins takes place during the synthesis or the action of prostaglandins is unclear. The role of prostaglandins in hyperoxia has been put forward by a number of previous studies. On the cell level, hyperoxia has been found to reduce cyclooxygenase activity [32]. In an in vitro model of isolated rat cremaster muscle arterioles, it was found that increasing oxygen tensions cause endothelium-dependent vasoconstriction, but that this was completely abolished in the presence of indomethacin, a cyclooxygenase inhibitor [12]. Furthermore, it has been shown that vasoconstricting prostanoids are released from the endothelium of the human umbilical vein at hyperoxia [14]. In healthy volunteers,

hyperoxia significantly reduced the vasodilatation in response to isometric forearm contraction, but no further reduction was found after pretreatment with aspirin [33]. These studies together with our present findings suggest that hyperoxia modulates the synthesis rather than the action of prostaglandins by inhibition of the cyclooxygenase pathway.

Effect of oxygen on the response to acetylcholine with vitamin C

In the present study, vitamin C inhibited the effects of hyperoxia that were seen during iontophoresis of acetylcholine up to an electrical charge of 5 mC (or up to 250 seconds of iontophoresis). At higher charges, the maximum response was significantly lower during 100% oxygen breathing. Due to the absence of a clear plateau in the perfusion response during air breathing, it is difficult to compare the response to the corresponding responses in the untreated group, especially the ED₅₀ values. However, the maximum responses were not significantly different from the maximum responses without vitamin C pre-treatment, for both air breathing and 100% oxygen breathing.

Vitamin C is a potent aqueous phase antioxidant and it has been shown to lower oxidative stress in humans [34-36]. The molecular basis for its antioxidant effect is not fully understood but there is some evidence that it increases the activity of superoxide dismutase (SOD) [37]. Extracellular SOD (SOD3) catalyzes dismutation of superoxide to hydrogen peroxide (H₂O₂) and O₂. By activating SOD3, vitamin C could reduce concentrations of superoxide and so augment the bioavailability of NO. Another plausible way for vitamin C to have an antioxidant effect is by lowering the activity of NADPH-oxidase, the enzyme responsible for the production of superoxide. This has been shown recently in animal models [38].

The finding that vitamin C partly abolishes the reduced endothelium-dependent vasodilatation during hyperoxia suggests that reactive oxygen species (ROS) are involved. Hyperoxia has been associated with increased production of reactive oxygen species. Supporting evidence mainly comes from in vitro preparations [15, 39, 40] and animal studies [41, 42]. Recently, human studies also have been published in favour of a hyperoxic-ROS linkage [43, 44]. In vitro studies have shown that hyperoxia results in the production of up to 2.5 times the normal concentrations of superoxide, which is probably the most important of the oxygen radicals [39].

From the present study it is impossible to determine by which mechanism reactive oxygen species reduce the endothelium-dependent vasodilatation. The increase in superoxide may reduce the efficacy of the NO-producing chain, by either inhibiting activation of NOS or by lowering the availability of L-arginine through increased action of its degrading enzyme, L-arginase. This will lead to lower concentrations of NO and hence, less vasodilatation. However, the fact that during cyclooxygenase inhibition, 100% oxygen breathing did not further reduce the perfusion response compared with air breathing, suggests that there was no difference in the bioavailability of NO. On the other hand, based on the present results it could well be possible that reactive oxygen species are mediators in the inhibiting effect of hyperoxia on prostaglandin production. In that case, vitamin C is indeed expected to reduce the inhibition of prostaglandin production during hyperoxia, through scavenging of these reactive oxygen species.

In general, our observations are consistent with the result of a previous human study by Mak et al [16]. In their study, ACh-mediated vasodilatation, measured by venous occlusion plethysmography in healthy subjects, was blunted by hyperoxia (inhalation of 100% oxygen for 10 minutes). The hyperoxic vasoconstriction was then reversed by infusion of vitamin C. When an endothelium-independent vasodilator was used (verapamil), hyperoxia had no effect.

The results of a previous study by Waring et al [4] are partially conflicting with our present findings. Waring et al. investigated the effects of hyperoxia on perfusion after local application of ACh, SNP and a nitric oxide synthase inhibitor (L-NMMA) in normal subjects using plethysmography. In their study there was a tendency towards a reduced response to acetylcholine when breathing oxygen, but this was not significant. However, breathing oxygen did not alter perfusion in response to SNP or L-NMMA, suggesting that there was no significant involvement of nitric oxide [4], which is in agreement with our present results.

The reason why pre-treatment with vitamin C did not fully restore the reduced response during 100% oxygen breathing at higher charges remains unclear. It is possible that the local concentration of vitamin C in the skin was not sufficient to fully prevent the effect of reactive oxygen species. The vitamin C dose, 2.5 g daily for 3 days, was chosen as higher doses do not increase the bioavailability of vitamin C,

but only increase urinary excretion [21]. Also, single doses of 2 g have been shown to induce endothelial effects in man [45]. Therefore, it is unlikely that higher doses would have resulted in a fully restored perfusion response during 100% oxygen breathing. Neither is it likely that vitamin C had any direct systemic effects of its own, as short-term intake (hours to several days) is known to leave the cardiovascular system unaffected [16, 46]. Apart from the possibility of incomplete inhibition of oxygen radicals, other effects may be responsible for the dependency of the effect of vitamin C on the iontophoretic charge, including more complex physiological interactions between vitamin C, reactive oxygen species and prostaglandins.

Study limitations

Limitations of this study include the fact that the effect of hyperoxia on microvascular responses was studied only indirectly, by observing the perfusion response in the forearm skin to 100% oxygen breathing during iontophoresis with endothelium-dependent and –independent drugs. A possible source of a systematic error in our study is that oxygen was given in all cases after air had been breathed. However, this was done to avoid the need for washout periods.

Because the iontophoresis methodology itself has potent vasodilator effects, especially at the cathode, this could obviously be a confounding factor [19]. Our study protocol was chosen to minimize the effects of current, as has been earlier recommended by our group [19, 20]. However, to investigate the importance of any nonspecific effects, we used cathodal iontophoresis with sodium chloride, using the same settings as during iontophoresis of SNP. We found no significant increase in perfusion, which makes it unlikely that our results are affected by any nonspecific effects of the iontophoresis current.

It should be stressed that the effects of hyperoxia found in this study may not be present at more modest hyperoxia levels, as the formation of superoxide may be more likely at higher oxygen concentrations. Further studies are required to elucidate the effects at modest hyperoxia levels. We did not measure concentrations of oxygen and carbon dioxide in blood. The model of oxygen delivery has, however, been used by our group with high reproducibility in which concentrations increased to a mean oxygen arterial partial pressure of 74 kPa while breathing 100 % in this non-rebreathing setup [47].

Studies have shown that the concentration of carbon dioxide decreases during hyperoxia, probably as a result of hyperventilation [48], which could have effects on vascular tone. The effects seem, however, to be modest, and possibly affect all oxygen measurements equally [3].

We did not consider the phase of the menstrual cycle of the female participants and the male/female balance was different for the different protocols. We have tested if ED₅₀ and maximum response differed significantly between men and women in protocols 1 and 3 where the number of female subjects was largest. This was not the case. Also, we found that exclusion of all female subjects does not affect the conclusions drawn in this study. Nevertheless, an effect of gender has been suggested in different previous studies [49, 50] and we cannot entirely exclude the possibility that gender is of influence on the vascular mechanisms proposed in this study. Further investigations on a larger group of male and female subjects are needed to elucidate this issue.

Conclusion

This is the first completely non-invasive study that investigates the involvement of prostaglandins, nitric oxide and oxygen free radicals in the microvascular vasoreactivity during hyperoxia in healthy humans. We found that breathing 100% oxygen significantly attenuated that endothelium-dependent vasodilatation elicited by iontophoresis of acetylcholine in the forearm skin of healthy subjects and that prostaglandins are involved in this attenuated response, as suggested by the lack of effect of hyperoxia on the response after pretreatment with aspirin. Vitamin C partially abolished the attenuating effect of hyperoxia on acetylcholine-mediated vasodilatation. Hyperoxia did not have any effect on endothelium-independent vasodilatation. These results indicate that hyperoxia inhibits the synthesis or action of vasodilator prostanoids by a mechanism that may involve reactive oxygen species, and that hyperoxia is not likely to affect the production or the bioavailability of nitric oxide.

References

1. Daly WJ, Bondurant S: Effects of oxygen on the heart rate, blood pressure, and cardiac index of normal men-resting, with reactive hyperemia, and after atropine. *J Clin Invest* 1962;41:126-134.
2. Milone SD, Newton GE, Parker JD: Hemodynamic and biochemical effects of 100% oxygen breathing in humans. *Can J Physiol Pharmacol* 1999;77:124-130.
3. Rousseau A, Bak Z, Janerot-Sjoberg B, Sjoberg F: Acute hyperoxemia-induced effects on regional blood flow, oxygen consumption and central circulation in man. *Acta Physiol Scand* 2005;183:231-240.
4. Waring WS, Thomson AJ, Adwani SH, Rosseel AJ, Potter JF, Webb DJ, Maxwell SR: Cardiovascular effects of acute oxygen administration in healthy adults. *J Cardiovasc Pharmacol* 2003;2:245-250.
5. Crawford P, Good PA, Gutierrez E, Feinberg JH, Boehmer JP, Silber DH, Sinoway LI: Effects of supplemental oxygen on forearm vasodilation in humans. *J Appl Physiol* 1997;2:1601-1606.
6. Kenmure AC, Beatson JM, Cameron AJ, Horton PW: Effects of oxygen on myocardial blood flow and metabolism. *Cardiovasc Res* 1971;4:83-89.
7. Joachimsson PO, Sjoberg F, Forsman M, Johansson M, Ahn HC, Rutberg H: Adverse effects of hyperoxemia during cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1996;112:812-819.
8. Kiss B, Polska E, Dorner G, Polak K, Findl O, Mayrl GF, Eichler HG, Wolzt M, Schmetterer L: Retinal blood flow during hyperoxia in humans revisited: concerted results using different measurement techniques. *Microvasc Res* 2002;64:75-85.
9. Watson NA, Beards SC, Altaf N, Kassner A, Jackson A: The effect of hyperoxia on cerebral blood flow: a study in healthy volunteers using magnetic resonance phase-contrast angiography. *Eur J Anaesthesiol* 2000;17:152-159.
10. Ellsworth ML, Forrester T, Ellis CG, Dietrich HH: The erythrocyte as a regulator of vascular tone. *Am J Physiol* 1995;269:H2155-2161.
11. Amin HM, Camporesi EM, Cohen D, Hakim TS, Kaniewski WS: Effects of acute exposure to hyperbaric oxygen on the rheology and morphology of the red blood cells in the rat. *Microvasc Res* 1995;50:417-428.

12. Messina EJ, Sun D, Koller A, Wolin MS, Kaley G: Increases in oxygen tension evoke arteriolar constriction by inhibiting endothelial prostaglandin synthesis. *Microvasc Res* 1994;48:151-160.
13. Yamazaki F: Hyperoxia attenuates endothelial-mediated vasodilation in the human skin. *J Physiol Sci.* 2007;57(1):81-84
14. Mildenerger E, Siegel G, Versmold HT: Prostanoids contribute to the oxygen-dependent regulation of vascular tone of human umbilical vein. *J Perinat Med.* 2004;32(2):149-54.
15. Rubanyi GM, Vanhoutte PM: Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *J Appl Physiol* 1986;250:H822-H827.
16. Mak S, Egri Z, Tanna G, Colman R, Newton GE: Vitamin C prevents hyperoxia-mediated vasoconstriction and impairment of endothelium-dependent vasodilation. *Am J Physiol Heart Circ Physiol* 2002;282:H2414-H2421.
17. Wardell K, Jakobsson A, Nilsson GE: Laser Doppler perfusion imaging by dynamic light scattering. *IEEE Trans Biomed Eng* 1993;40:309-316.
18. Morris SJ, Shore AC: Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. *J Physiol* 1996;496:531-542.
19. Droog EJ, Henricson J, Nilsson GE, Sjöberg F: A protocol for iontophoresis of acetylcholine and sodium nitroprusside that minimises nonspecific vasodilatory effects. *Microvasc Res* 2004;67:197-202.
20. Droog EJ, Sjöberg F: Nonspecific vasodilatation during transdermal iontophoresis-the effect of voltage over the skin. *Microvasc Res* 2003;65:172-178.
21. Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR: Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* 1996;93:3704-3709.
22. Henricson J, Tesselaar E, Persson K, Nilsson G, Sjöberg F: Assessment of microvascular function by study of the dose-response effects of iontophoretically applied drugs (acetylcholine and sodium nitroprusside)--methods and comparison with in vitro studies. *Microvasc Res* 2007;73(2):143-9.
23. Tesselaar E, Henricson J, Jonsson S, Sjöberg F: A Time-Response Model for Analysis of Drug Transport and Blood Flow Response during Iontophoresis of Acetylcholine and Sodium Nitroprusside. *J Vasc Res* 2008;46(4):270-277.

24. Wagner JG: Kinetics of pharmacologic response. I. Proposed relationships between response and drug concentration in the intact animal and man. *J Theor Biol* 1968;20(2):173-201.
25. Clough GF, Gush R: Is it important to model the impact of blood flow on the dose of drugs delivered transcutaneously? *J Vasc Res* 2009;46:267–269.
26. Harker L, Fuster U: Pharmacology of platelet inhibitors. *J Am Coll Cardiol* 1986;8:21B–32B.
27. Durand S, Tartas M, Bouyé P, Koitka A, Saumet JL, Abraham P: Prostaglandins participate in the late phase of the vascular response to acetylcholine iontophoresis in humans. *J Physiol* 2004;561(Pt 3):811-9.
28. Holowatz LA, Thompson CS, Minson CT, Kenney WL: Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol* 2005 Mar 15;563(Pt 3):965-73.
29. Kellogg DL Jr, Zhao JL, Coey U, Green JV: Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *J Appl Physiol* 2005;98(2):629-32.
30. Medow MS, Glover JL, Stewart JM: Nitric oxide and prostaglandin inhibition during acetylcholine-mediated cutaneous vasodilation in humans. *Microcirculation*. 2008;15(6):569-79.
31. Lenasi H, Strucl M: The effect of nitric oxide synthase and cyclooxygenase inhibition on cutaneous microvascular reactivity. *Eur J Appl Physiol*. 2008;103(6):719-26.
32. Ishii Y, Morita I, Murota S, Kitamura S: Hyperoxia decreases cyclooxygenase activity in endothelial cells. *Prostaglandins Leukot Essent Fatty Acids* 1993;48(6):455-61.
33. Win TS, Marshall JM: Contribution of prostaglandins to the dilation that follows isometric forearm contraction in human subjects: effects of aspirin and hyperoxia. *J Appl Physiol* 2005;99(1):45-52.
34. Aghdassi E, Wendland BE, Steinhart AH, Wolman SL, Jeejeebhoy K, Allard JP: Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress: a randomized controlled trial. *Am J Gastroenterol* 2003;98:348-353.
35. Huang HY, Appel LJ, Croft KD, Miller ER 3rd, Mori TA, Puddey IB: Effects of vitamin C and vitamin E on in vivo lipid peroxidation: results of a randomized controlled trial. *Am J Clin Nutr* 2002;76:549-555.

36. Sanchez-Moreno C, Cano MP, de Ancos B, Plaza L, Olmedilla B, Granado F, Elez-Martinez P, Martin-Belloso O, Martin A: Pulsed electric fields-processed orange juice consumption increases plasma vitamin C and decreases F2-isoprostanes in healthy humans. *J Nutr Biochem* 2004;15:601-607.
37. Chen X, Touyz RM, Park JB, Schiffrin EL: Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. *Hypertension* 2001;38:606-611.
38. Ulker S, McKeown PP, Bayraktutan U: Vitamins reverse endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. *Hypertension* 2003;41:534-539.
39. Parinandi NL, Kleinberg MA, Usatyuk PV, Cummings RJ, Pennathur A, Cardounel AJ, Zweier JL, Garcia JG, Natarajan V: Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L26-L38.
40. Yusa T, Crapo JD, Freeman BA: Hyperoxia enhances lung and liver nuclear superoxide generation. *Biochim Biophys Acta* 1984;798:167-174.
41. Freeman BA, Crapo JD: Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *Biol Chem* 1981;256:10986-10992.
42. Jaensch S, Cullen L, Raidal SR: Normobaric hyperoxic stress in budgerigars: enzymic antioxidants and lipid peroxidation. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;128:173-180.
43. Carpagnano GE, Kharitonov SA, Foschino-Barbaro MP, Resta O, Gramiccioni E, Barnes PJ: Supplementary oxygen in healthy subjects and those with COPD increases oxidative stress and airway inflammation. *Thorax* 2004;59:1016-1019.
44. Vento M, Asensi M, Sastre J, Lloret A, Garcia-Sala F, Vina J: Oxidative stress in asphyxiated term infants resuscitated with 100% oxygen. *J Pediatr* 2003;142:240-246.
45. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF, Vita JA: Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 1996;93:1107-1113.
46. Eskurza I, Monahan KD, Robinson JA, Seals DR: Ascorbic acid does not affect large elastic artery compliance or central blood pressure in young and older men. *Am J Physiol Heart Circ Physiol* 2004;286:H1528-H1534.

47. Rousseau A, Abdiu A, Sjoberg F: Hyperoxaemia does not change concentrations of serotonin and beta-thromboglobulin in blood of healthy humans. *Scand J Clin Lab Invest* 2004;64:81-85.
48. Dean JB, Mulkey DK, Henderson RA 3rd, Potter SJ, Putnam RW: Hyperoxia, reactive oxygen species, and hyperventilation: oxygen sensitivity of brain stem neurons. *J Appl Physiol* 2004;96:784-791.
49. Hendry RG, Marshall JM: Vasoconstrictor products of cyclo-oxygenase activity limit acetylcholine-induced cutaneous vasodilatation in young men. *Clin Sci (Lond)*. 2004;107(3):323-30.
50. Algotsson A, Nordberg A, Winblad B: Influence of age and gender on skin vessel reactivity to endothelium-dependent and endothelium-independent vasodilators tested with iontophoresis and a laser Doppler perfusion imager. *J Gerontol A Biol Sci Med Sci*. 1995;50(2):M121-7.