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HEMOGLOBIN ATTENUATES THE EFFECTS OF INSPIRED OXYGEN ON PLASMA ISOFURANS IN HUMANS DURING UPPER LIMB SURGERY

Tomas B Corcoran¹, Anne E Barden², Emilie Mas², Sina Grape¹, Viktoria Koren¹, Michael Phillips³, L Jackson Roberts II⁴, and Trevor A Mori²

¹Department of Anaesthesia & Pain Medicine, Royal Perth Hospital, University of Western Australia, Perth

²School of Medicine and Pharmacology, University of Western Australia and the Cardiovascular Research Centre, University of Western Australia, Perth

³Western Australian Institute for Medical Research, University of Western Australia, Perth

⁴Dept of Pharmacology, Vanderbilt University, Nashville, USA

Abstract

Reperfusion injury is characterised by significant oxidative stress. F₂-isoprostanes (F₂-IsoPs) and isofurans (IsoFs), the latter preferentially produced during increased oxygen tension, are recognised markers of *in-vivo* oxidative stress. We aimed to determine whether increasing oxygen tension during reperfusion modified levels of plasma total IsoFs and F₂-IsoPs. Forty five patients undergoing upper limb surgery were randomised to receive inspired oxygen concentrations of 30%, 50% or 80% during the last 15 minutes of surgery. Venous blood samples were taken before the change in inspired oxygen, after 10 minutes (before reperfusion) and after 15 minutes (5 minutes after reperfusion). IsoFs and F₂-IsoPs were measured by gas chromatography-mass spectrometry. Venous oxygen tension and hemoglobin concentrations were also measured. Plasma IsoFs and F₂-IsoP levels in the 50% and 80% O₂ groups were not significantly different from the 30% O₂ group. In secondary analyses, using data combining all groups, levels of IsoFs, but not F₂-IsoPs, associated with higher venous oxygen tension (P=0.038). Hemoglobin negatively modified the influence of oxygen tension on levels of IsoFs (P=0.014). This study has shown for the first time, that plasma IsoFs levels associate with higher oxygen tension in a human model of reperfusion, and this effect is significantly attenuated by hemoglobin.

Keywords

Isofurans; F₂-isoprostanes; oxidative stress; ischemia-reperfusion; limb surgery; oxygen tension

INTRODUCTION

Pneumatic tourniquets are commonly used to exsanguinate extremities to facilitate bloodless limb surgery. There are well-defined endocrine, metabolic and hemodynamic responses to the tourniquet [1, 2], but there is also an associated sub-lethal reperfusion injury. This injury provokes a moderate systemic inflammatory response [3], and depending upon its severity, a

Address for correspondence: Professor Trevor A Mori, School of Medicine and Pharmacology, Medical Research Foundation Building, GPO Box X2213 Perth, Western Australia, AUSTRALIA 6847. Phone: 61 8 9224 0273, Fax: 61 8 9224 0246. trevor.mori@uwa.edu.au.

detectable injury in organs remote from the tourniquet [4, 5]. This is a useful model of reperfusion injury and has been employed to examine the influence of anesthesia interventions on oxidative stress and injury [6, 7]. Free-radical elaboration leading to complement and endothelial cell activation appear to be key central components of this response. The subsequent oxidative stress is central to, and correlates with, the degree of immune system activation [8]. The intensity of this reaction is proportional to the duration of ischemia, the antioxidant reserve of the patient and the nature of the inflammatory response [9]. Later involvement of components of the immune system plays a downstream role and contributes to the local and systemic inflammatory responses [10].

Although oxygen administration under anesthesia is ubiquitous and is predicated on clinical need, the use of higher than clinically indicated concentrations has been recommended for a variety of pathologies, particularly in the settings of cerebral ischemia, stroke, spinal cord ischemia, and cardiac transplantation [11-13]. However, hyperoxia is not without disadvantages [14] and may exacerbate organ injury due to free-radical generation that is believed to account for oxygen toxicity [15, 16].

F₂-isoprostanes (F₂-IsoPs) represent the most reliable currently available biomarkers of oxidative stress status and lipid peroxidation *in vivo* and their measurement can be used for assessing the effect of pharmacological treatment [17-21]. Several F₂-IsoPs isomers are biologically active *in vivo*, acting as vasoconstrictors and altering platelet function [22, 23]. Thus F₂-IsoPs may also act to mediate oxidant injury. Isofurans (IsoFs) are products of lipid peroxidation that contain a substituted tetrahydrofuran ring. The formation of F₂-IsoPs and IsoFs are differentially modulated by oxygen tension. The formation of F₂-IsoPs is favored at reduced oxygen tension whereas the formation of IsoFs is favored at elevated oxygen tensions [24]. Whether the production of these compounds is altered by varying oxygen tension in humans and at the time of reperfusion is unknown. Our hypothesis was that increasing inspired oxygen concentration would increase the production of IsoFs in a human model of upper limb reperfusion.

MATERIALS AND METHODS

Patients

Forty five fasted, unpremedicated adult patients, American Society of Anesthesiology physical status I or II, participated in this double-blind, randomised controlled trial. All were undergoing planned surgery for isolated upper limb trauma requiring the use of a pneumatic arterial tourniquet. Exclusion criteria included systemic infection, immunosuppressive or disease-modifying medication, non-steroidal anti-inflammatory drugs within the previous 48 hours, morbid obesity or the likely requirement for multiple tourniquet episodes. Patients were randomized with a computer generated sequence and allocated by the opaque sealed-envelope method to three groups; 30%, 50% or 80% inspired oxygen concentrations. Anesthesia was administered by a clinician independent of the study. Anesthesia machines and monitors had the gas administration/monitoring components concealed from the investigators. The study was approved by the Human Research Ethics Committee of Royal Perth Hospital. All participants provided written informed consent.

Anesthesia and study interventions

Induction of anesthesia comprised midazolam (1mg) and fentanyl (1.5 µg/kg) co-induction 3 minutes prior to induction with propofol (3mg/kg) intravenously. An appropriately sized Supreme® laryngeal mask airway was inserted. Anesthesia maintenance comprised inhalation of Sevoflurane in an oxygen/ air mix to achieve end-tidal concentrations of 1.9-2.3%. All patients received intraoperative supplemental fentanyl, morphine and

paracetamol for analgesia. Following the induction of anesthesia an 18 gauge cannula for blood sampling was inserted into the antecubital fossa of the upper limb contralateral to the operative limb. In all patients the inspired oxygen concentration was initially set at 30% (percentage of inspired oxygen) and permitted to stabilise for at least 10 minutes. An arterial tourniquet was inflated to 200-250 mmHg (depending upon systolic blood pressure) to prevent blood flowing to the operative limb during surgery (Figure 1). During the last 15 minutes of surgery inspired oxygen concentration was maintained at 30%, or changed to either 50% or 80%. Venous blood samples were taken prior to anesthesia, before the change in inspired oxygen (T_1), after 10 minutes (prior to tourniquet release) (T_2) and after 15 minutes (5 minutes after tourniquet release) (T_3). The inspired oxygen concentration was maintained at the allocated value until emergence from anesthesia. Patients were then transferred to the recovery room and received oxygen by face mask.

Processing of blood samples

Blood samples for measurement of F_2 -IsoPs and IsoFs were collected into cold tubes containing ethylenediaminetetraacetic acid (EDTA), reduced glutathione and butylated hydroxytoluene, then centrifuged at 4°C and the plasma stored at -80°C. Venous blood samples were drawn into 80 IU of electrolyte-balanced heparin solution (PICO 50®, Radiometer Medical ApS, Copenhagen) and oxygen tension (venous oxygen partial pressure, PvO_2) and hemoglobin (Hb) were analysed on a Radiometer ABL 800FLEX automated point-of-care device (Radiometer Medical ApS, Copenhagen).

Chemicals, Reagents and Chromatography

15- F_{2t} -IsoP (8-iso-PGF $_{2\alpha}$) and 15- F_{2t} -IsoP-d $_4$ (8-iso-PGF $_{2\alpha}$ -d $_4$) were purchased from Cayman Chemicals (Ann Arbor, MI). Pentafluorobenzylbromide and N,N-diisopropylethylamine were purchased from Sigma Chemicals (St Louis, MO). The silylating agent *N,O*-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) was purchased from Pierce Chemicals (Rockford, IL). Certify II cartridges were from Varian (Lake Forrest, CA). All solvents were of HPLC grade. IsoF standards were provided by Prof LJ Roberts II, Department of Pharmacology, Vanderbilt University, Nashville, USA [24].

Measurement of plasma Isoprostanes and Isofurans

Total (free plus esterified) IsoFs and F_2 -IsoPs were measured by gas chromatography-mass spectrometry (GCMS) using electron capture negative ionization and using a modification of our previously reported method [25]. Briefly, internal standard (15- F_{2t} -IsoP-d $_4$) was added to plasma (200 μ l) and samples were hydrolysed with potassium hydroxide in methanol, acidified and applied to pre-washed Certify II cartridges. After washing with methanol/water (1:1) and hexane/ethyl acetate (75:25) the IsoFs and F_2 -IsoPs were eluted with ethyl acetate/methanol (90:10), dried and derivatised. IsoFs and F_2 -IsoPs were quantitated by GCMS using 15- F_{2t} -IsoP-d $_4$ (5ng) as internal standard and monitoring ions at m/z 569, 573 and 585, for F_2 -IsoP, 15- F_{2t} -IsoP-d $_4$ and IsoF, respectively.

Statistical Analysis

All data was checked for normality and where necessary transformed using a natural logarithm for parametric analysis. Categorical data were examined using chi-squared analysis. Hemolysed samples were excluded from the analysis and there was no significant association between hemolysis and intervention group ($P=0.932$). Between group differences in IsoF and F_2 -IsoP levels were examined over time (T_1 , T_2 and T_3) using linear mixed models with restricted maximum likelihood estimation. T_1 , immediately prior to the change in inspired oxygen concentration (Figure 1), was used as the baseline. The

intervention group was treated as a fixed effect and time was treated as a random effect. This approach to the analysis was chosen because of its flexibility with respect to variable distributions, power and ability to cope with missing data (the hemolysed samples). The linearity of continuous variables was examined using reduced cubic splines. A corrected p value less than 0.05 was taken to indicate a statistically significant result.

Statistical power

Based on the variability of plasma levels of F₂-IsoPs and IsoFs in our laboratory we estimated that 15 patients/group would provide 80% power to detect a 30% difference in plasma F₂-IsoP and IsoF levels.

RESULTS

Forty five patients were enrolled, 15 in each group. Two patients each in the 50% O₂ and 80% O₂ groups were excluded from analysis due to: protocol violations comprising requirement for multiple tourniquet episodes (n=2); intraoperative parecoxib administration (n=1); and dexamethasone administration (n=1); leaving 41 patients in the final analysis. The three groups were comparable in terms of demographics and the details of the surgical procedures (Table 1).

Hemolysis was detected in 7% of plasma samples and found to cause an artefactual increase in plasma IsoFs and F₂-IsoPs (P=0.003 and P=0.042 respectively). The hemolysed samples were equally distributed across the groups and were excluded from subsequent analyses.

Plasma levels of F₂-IsoPs prior to anesthesia were 1491 pmol/L (95% CI, 1312, 1695) in the 30% O₂ group, 1791 pmol/L (1401, 2289) in the 50% O₂ group and 1463 pmol/L (1186, 1803) in the 80% O₂ group. IsoF levels were 3487 pmol/L (2185, 5565), 3878 pmol/L (2290, 6552) and 2411 pmol/L (1564, 3718) in the 30% O₂, 50% O₂ and 80% O₂ groups, respectively. There was no significant change in F₂-IsoP and IsoF levels between the sample taken before anesthesia and T₁ (prior to the change in oxygen) in any of the groups.

Plasma levels of IsoFs and F₂-IsoPs were not different between the groups at T₁ (Table 2). Using longitudinal linear mixed model analyses across time points T₁ to T₃, plasma IsoF and F₂-IsoP levels in the 50% and 80% O₂ groups were not significantly different from the 30% O₂ group (Table 2).

In secondary analyses the relationship between IsoF and F₂-IsoP levels with oxygen tension (PvO₂) was examined in the combined groups over time (T₁ to T₃). There was a significant positive relationship between plasma IsoF levels and PvO₂, such that levels of IsoFs increased by 81 pmol/L with a 1 mmHg increase in PvO₂ (P=0.038) (Table 3). There was no significant relationship between plasma IsoF levels and hemoglobin. However, the relationship between plasma IsoF levels and PvO₂ was attenuated by an interaction of PvO₂ with Hb (P=0.014). These data show that IsoF levels increase with increasing PvO₂, but this is significantly attenuated as Hb concentration increases in the blood. There was no such relationship between plasma F₂-IsoPs and PvO₂ or Hb (Table 3).

DISCUSSION

This is the first study in humans to evaluate the effect of altering inspired oxygen tension in humans on plasma levels of IsoFs. We have shown in a model of upper limb reperfusion, that contrary to our hypothesis, increasing inspired oxygen from 30% to either 50% or 80%, did not result in increased plasma total IsoF levels. However, analysis of the combined groups showed that plasma IsoFs were positively associated with oxygen tension (PvO₂) and

this relationship was significantly attenuated by blood hemoglobin concentration. This is noteworthy given that hemoglobin *per se* did not significantly affect plasma IsoF levels. Plasma total F₂-IsoP levels during reperfusion were also not different between the groups and in analysis of the combined groups, there was no significant relationship between F₂-IsoP levels and PvO₂ or hemoglobin concentration.

Lipids are major targets of free radical attack. After the initial formation of lipid hydroperoxides, a complex series of secondary reactions result in the formation of multiple downstream metabolites. F₂-IsoPs are prostaglandin-like metabolites formed *in vivo* from free radical peroxidation of AA and are produced independently of the cyclooxygenase enzyme system [26], although in some experimental models F₂-IsoPs can be partially derived from cyclooxygenase activity [27]. A closely related but more oxygen-specific family of lipid peroxidation products termed IsoFs are preferentially formed under conditions of high oxygen tension [24, 28]. Measurement of IsoFs may thus provide a sensitive index of oxidative stress at elevated oxygen tension, whereas F₂-IsoPs do not. [24, 28, 29].

The principal clinical benefits of administering inspired oxygen concentrations in excess of that clinically necessary are a decrease in postoperative infection rates [30, 31]. Hyperbaric oxygen administration to reperfused tissue flaps attenuates neutrophil activation [32] and adhesion [33-35]. A common link between oxygen tension and immunomodulatory actions might be explained by altered free-radical elaboration. At least two transcription factors, NF- κ B and AP-1, are regulated by the redox state [36, 37] with important ramifications for the inflammatory response [38]. In contrast, there is evidence of adverse consequences of excessive oxygen administration. Specifically, hyperoxia adversely influences gene-transcription activities in the lung producing alterations in morphology and function [39]. Excessive oxygen concentrations, even for brief but critical periods, may have deleterious effects and produce clinically meaningful differences in outcomes [40]. To date no clear mechanism has been identified to explain these effects. The differential production of IsoFs under the influence of varied oxygen tensions such as we have demonstrated may represent one possible pathway by which altered oxygen concentrations influence such inflammatory processes. This remains speculative, however, as the physiological effects of the IsoFs are currently poorly understood.

In contrast to our hypothesis, increasing inspired oxygen from 30% to either 50% or 80%, did not increase plasma IsoF levels during surgery associated with reperfusion injury. A strength of our study is the use of linear mixed models to analyse the data as this affords maximal power while allowing for missing data that arose from the hemolysed samples. In accordance with previous investigators, hemolysis was shown to artifactually increase both F₂-IsoP and IsoF concentrations [41]. Whilst our study was powered to detect between group differences in F₂-IsoPs and IsoFs of 30%, we cannot exclude that smaller changes in IsoFs and F₂-IsoPs may have occurred with different inspired oxygen levels. Whilst we have shown that plasma IsoFs and F₂-IsoP are not significantly altered in humans over a 15 min period following a change in inspired oxygen, we cannot exclude the possibility that a longer period of exposure to oxygen may alter plasma IsoFs and F₂-IsoPs. For practical reasons blood samples for IsoF and F₂-IsoP measurement were obtained from the arm contralateral to the surgical procedure and it is possible that this may have diluted any effect of oxygen tension on IsoFs and F₂-IsoPs produced at the site of the reperfusion injury.

Although a previous report has identified a correlation between free hemoglobin and plasma IsoF levels in patients undergoing cardiopulmonary bypass surgery [42], our study highlights for the first time, a complex relationship between oxygen tension and plasma IsoF levels that is modified by hemoglobin concentration. This finding is worth noting in view of

the fact that hemoglobin did not *per se* significantly affect plasma IsoF levels. These findings are consistent with the comprehensively understood biology of the hemoglobin molecule. At atmospheric pressure, the presence of hemoglobin in blood permits the carriage of 30-100 times as much oxygen bound to the hemoglobin molecule than could be transported simply in the form of dissolved oxygen in blood without hemoglobin. In this study, hemoglobin concentration did not have an independent effect, but rather, negatively modified the influence of oxygen tension on plasma IsoF levels. This highlights the fact that *in vivo* systems are prone to multiple influences that are difficult to model *in vitro*.

To date, few studies have examined IsoFs as markers of oxidative stress. IsoFs were shown to be elevated in the substantia nigra of patients with Parkinson's disease and with dementia with Lewy body disease [43] and following general anesthesia during ischemia/reperfusion of the leg in patients undergoing knee replacement surgery [44]. Studies examining the effect of increasing oxygen tension on IsoFs have been confined to *in vitro* oxidation of AA [24] or animal models that have measured tissue IsoF levels [24, 45-47]. The biological effects of IsoFs have not been comprehensively studied, but there is some evidence that IsoFs can affect platelet function [22]. The interaction between oxygen tension and hemoglobin that modifies plasma IsoF levels may serve as a homeostatic mechanism to limit systemic oxidative stress under conditions of increased inspired oxygen.

In conclusion, this study has not demonstrated an effect of increasing inspired oxygen from 30% to either 50% or 80% on plasma levels of IsoFs and F₂-IsoPs in a human model of reperfusion injury. However, in analysis of the combined groups we have identified a positive influence of oxygen tension on the production of plasma IsoFs and this relationship was negatively modified by hemoglobin concentration. This study serves to highlight the difficulty in modeling complex biological systems *in vitro*. Future studies examining IsoFs under conditions of varying oxygen tension will need to control for hemoglobin concentration.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
F₂-IsoPs	F ₂ -isoprostanes
GCMS	gas chromatography mass spectrometry
HPLC	high performance liquid chromatography
IsoFs	Isofurans
PvO₂	Oxygen tension / venous oxygen partial pressure

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Figure 1.
Study design outlining the timing of blood sampling in relation to surgery and changes in oxygen levels.

TABLE 1
BASELINE CHARACTERISTICS OF THE STUDY GROUPS

	Intervention Group		
	30% O ₂ (n=15)	50% O ₂ (n=13)	80% O ₂ (n=13)
Age (years)	34.8 ± 12.6	34.1 ± 15.8	33.5 ± 13.8
Gender (M/F)	11/4	11/2	9/4
BMI (kg.m ⁻²)	25.4 ± 3.3	24.1 ± 3.1	25.1 ± 4.6
Tourniquet time (mins)	87 ± 26	86 ± 29	71 ± 24
Smoker (n, %)	7 (47)	8 (62)	6 (46)
Surgical subgroups, (n, %)			
Wound exploration	2 (13.3)	1 (7.7)	1 (7.7)
Kirschner-wire	2 (13.3)	1 (7.7)	1 (7.7)
ORIF Hand	6 (40)	5 (38.5)	5 (38.5)
ORIF Radius/Ulna	2 (13.3)	5 (38.5)	3 (23.1)
Tendon repair	3 (42.9)	1 (7.7)	3 (23.1)

There were no significant differences between the intervention groups for any of the variables shown. Open Reduction Internal Fixation (ORIF).
 Values are mean ± SD

TABLE 2
Changes In Venous Oxygen Partial Pressure, Plasma IsoFs And F2-IsoPs, And Blood Hemoglobin In The Groups

Time	T ₁ Before the change in inspired oxygen			T ₂ 10 mins after inspired oxygen changed (prior to tourniquet release)			T ₃ 15 mins after inspired oxygen changed (5 mins after tourniquet release)		
Group	30	50	80	30	50	80	30	50	80
PvO ₂ (mmHg)	108 (98, 120)	83 (74, 93)	86 (67, 109)	96 (81, 114)	105 (87, 128)	212 (156, 288)	92 (72, 117)	100 (75, 135)	133 (84, 211)
Plasma IsoFs (pmol/L)	6105 (4131, 9022)	6651 (4060, 10895)	5937 (3649, 9661)	4985 (3404, 7300)	8255 (4927, 13832)	5093 (3175, 8168)	6019 (3882, 9330)	5703 (3642, 8933)	4618 (2908, 7333)
Plasma F ₂ -IsoPs (pmol/L)	1410 (1196, 1662)	1835 (1223, 2754)	1376 (1156, 1639)	1196 (1026, 1394)	1372 (1178, 1597)	1384 (1098, 1746)	1373 (1060, 1778)	1421 (1145, 1763)	1268 (1067, 1507)
Hb (g/L) *	130±13	134±13	129±11	130±13	134±12	126±18	131±13	132±14	130±13

T₁, before the change in inspired oxygen; T₂, after 10 minutes (prior to tourniquet release); T₃, after 15 minutes (5 minutes after tourniquet release); PvO₂, venous partial pressure of oxygen; Hb, hemoglobin concentration; IsoFs, isofurans; F₂-IsoPs, F₂-isoprostanes.

* Values are geometric mean (95% CI) or mean ± SD.

TABLE 3**PLASMA ISOFURAN AND F²-ISOPROSTANE CONCENTRATIONS**

PLASMA ISOFURANS	Coefficient	95% CI	P
PvO ₂	80.7	4.4, 156.9	0.038
PvO ₂ × Hb interaction	−0.7	−1.3, −0.2	0.014
Constant	9505	7233, 11777	<0.001

PLASMA F₂-ISOPROSTANES	Coefficient	95% CI	P
PvO ₂	−9.2	−23.8, 5.3	0.214
PvO ₂ × Hb interaction	0.06	−0.1, 0.2	0.300
Constant	1723	1289, 2158	<0.001