1 **Pulsed high oxygen induces a hypoxic-like response in Human Umbilical**
2 **Endothelial Cells (HUVECs) and in humans**

3 Cimino F.[1]*, Balestra C.[2]*, Germonpré P.[2,4], De Bels D.[2,3], Tillmans F.[2], Saija A.[1],
4 Speciale A[1]§, and Virgili F[5]

5

6 [1]Dep. Farmaco-Biologico, School of Pharmacy, University of Messina, Viale
7 Annunziata, 98168, Messina, Italy
8 [2] Environmental & Occupational Physiology Laboratory, Haute Ecole Paul Henri
9 Spaak, Brussels, Belgium
10 [3] Intensive Care Department, Brugmann University Hospital, Brussels, Belgium
11 [4] Hyperbaric Centre, Queen Astrid Military Hospital, Brussels, Belgium
12 [5] National Research Institute for Food and Nutrition, via Ardeatina 546, 00178,
13 Rome, Italy

14 *Running title: Effects of pulsed high oxygen on human endothelial cells*

15 **F. Cimino and C. Balestra contributed equally to this work.*

16 §Corresponding author: Speciale Antonio, *Dep. Farmaco-Biologico, School of*
17 *Pharmacy, University of Messina, Viale Annunziata, 98168, Messina, Italy.*
18 *specialea@gmail.com*

19

20 ***Abstract***

21 It has been proposed that relative changes of oxygen availability rather than steady
22 state hypoxic or hyperoxic conditions, play an important role in HIF transcriptional
23 effects. According to this hypothesis describing the "normobaric oxygen paradox",
24 normoxia following a hyperoxic event is sensed by tissues as an oxygen shortage,
25 upregulating HIF-1 activity. With the aim of confirming at cellular and at functional
26 level that normoxia following an hyperoxic event is "interpreted" as a hypoxic event,
27 we report a combination of experiments addressing the effects of an intermittent
28 increase of oxygen concentration on HIF-1 levels and the activity level of specific
29 oxygen-modulated proteins in cultured human umbilical vein endothelial cells
30 (HUVECs), and the effects hemoglobin (Hb) levels after intermittent breathing
31 normobaric high (100%) and low (15%) oxygen *in vivo* in humans.

32 Our experiments confirm that during recovery after hyperoxia, an increase of HIF
33 expression occurs in HUVECs, associated to an increase of matrix metalloproteinases
34 activity. These data suggest that endothelial cells "interpret" the return to normoxia
35 after hyperoxia as a hypoxic stimulus. At functional level, our data show that both
36 breathing 15% and 100% oxygen 30 minutes every other day for a period of 10 days,
37 induces an increase of Hb levels in humans. This effect was enhanced after the
38 cessation of the oxygen breathing. These results indicate that a sudden decrease in
39 tissue oxygen tension after hyperoxia, may act as a trigger for EPO synthesis so
40 corroborating the hypothesis that "relative" hypoxia is a potent stimulator of HIF
41 mediated gene expressions.

42

43 **KEYWORDS**: Normobaric oxygen paradox, HIF-1, hyperoxia, endothelial cells.

44 **Introduction**

45 In mammalian cells, basal metabolic processes in the presence of fluctuations of
46 oxygen availability are in large part regulated at the transcriptional level by the
47 transcription factor Hypoxia-inducible factor-1 (HIF-1) (7). This regulation is critical,
48 as cellular metabolic demands must be modulated according to time specific
49 physiological function and needs at any given time.

50 HIF activity is functional to the sensing of changes in oxygen availability and in
51 determining cellular response to relative hypoxia or hyperoxia, allowing a fine tuning
52 of cell adaptation to conditions of different oxygen availability by affecting oxygen
53 transfer, angiogenesis, glycolytic metabolism, proliferation, and apoptosis (14).

54 The importance of oxygen concentration sensing by cells in a wide range of cellular
55 responses, renders the full understanding of HIF activity an attractive tool to open
56 new avenues in the development of therapeutics able to target HIF pathway, either
57 repressing or activating the expression of a large spectrum of genes in turn involved
58 in a wide spectrum of diseases (22, 23).

59 According to this pivotal role in metabolism regulation, in the last decade, HIF
60 has been the object of a large number of investigations which addressed the
61 basis of its mechanism of action. It is established that HIF-1 acts as a heterodimer
62 consisting of HIF-1α and HIF-1β subunits. HIF-1α represents the regulatory
63 subunit that is primarily activated under conditions of oxygen deprivation, when
64 hydroxylation by prolyl and asparaginyl hydroxylases (PHD, FIH) is inhibited.
65 This results in stabilization and transactivation of HIF-1α, which induces the
66 expression of about one hundred target genes by binding to the hypoxia-
67 responsive element (HRE) located in the regulatory DNA sequence (23).

68 In spite of such an established understanding of the basic mechanism of action,
69 some aspects of HIF modulation are still unrevealed. Few years ago we have
70 proposed a novel mechanism of regulation of HIF activity based on relative
71 changes of oxygen availability rather than on steady state hypoxic or hyperoxic
72 conditions (4).
73 On the basis of our experimental observations addressing the effect of rebound
74 relative hypoxia after hyperoxia obtained by normo- and hyperbaric oxygen
75 breathing conditions, we hypothesized that the expression of one of the HIF
76 target genes, erythropoietin (EPO), is modulated by the cellular availability of
77 reactive oxygen species (21). Briefly, also according to other evidences
78 published, it has been proposed that rather than the absolute oxygen
79 concentration, tissues respond to relative changes of oxygen availability,
80 upregulating HIF-1 activity, even when returning back to normoxic conditions.
81 In the present paper we report the results of a combination of experiments
82 addressing:
83 1) the effects of an intermittent increase of oxygen concentration on HIF-1
84 levels and the activity level of specific—HRE-regulated proteins in
85 endothelial cultured cells, and
86 2) the effects of intermittent breathing normobaric high (100%) and low
87 (15%) oxygen *in vivo* in humans on hemoglobin levels
88 with the aim of confirming, at cellular and at functional level that normoxia
89 following an hyperoxic event is "interpreted" as a hypoxic event, describing the
90 "normobaric oxygen paradox".
91

92 ***Material and Methods***

93 <u>*Cellular studies.*</u>

94 *Cell Culture and Treatments.*

95 Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from freshly
96 obtained human umbilical cords by collagenase digestion of the interior of the
97 umbilical vein as described elsewhere (25), and were cultured in medium 199,
98 supplemented with 20% of fetal bovine serum (FBS), L-glutamine, hepes,
99 penicillin/streptomycin, endothelial cell growth factor and heparin, in gelatin
100 pretreated flasks. Cells were maintained in a humidified atmosphere containing 5%
101 CO_2 incubator at 37°C. Cells used in this study were from the second to fourth
102 passage.

103 HUVECs were incubated in hyperoxic (32% O_2) or normoxic (21% O_2) conditions at
104 37°C . The 32% oxygen level has been chosen to mimic the relative increase found
105 during 100% of oxygen breathing reported to the cellular level, which is grossly 5
106 times the usual breathed level; moreover, the 32% oxygen enriched air is a common
107 nitrox mixture used by divers, and the reactions of cells submitted to such an
108 environment are presently unknown. Mild hyperoxia was produced using a modular
109 incubator gas chamber (M.I.C.101 modular-incubator, Billups-Rothenberg Co.). The
110 chamber was purged with 32% O_2 for 4 min at a flow rate of 20 L/min and re-flushed
111 after 1 h according Billups-Rothenberg Co. protocol. The chamber was maintained
112 into the incubator at 37°C. All the reagents used to manage cells treated in hyperoxic
113 conditions were also flushed with 32% O_2.

114 Cell exposed to mild hyperoxia for 2 hours were used as positive control. For
115 recovery experiments, cells were exposed to mild hyperoxia for 2 hours, then they
116 were recovered for 4 or 6 hours into normoxic condition with fresh medium. Control

117 cells were exposed to 2 hours of normoxia and then for 4 hours in the same condition
118 with fresh medium.

119

120 *Western blotting analysis.*

121 For immunoblot analyses, 40 µg of protein lysates per sample were denatured in 4x
122 SDS-PAGE sample buffer (Tris-HCl 260 mM, pH 8.0, 40% (v/v) glycerol, 9.2%
123 (w/v) SDS, 0.04% bromophenol blue and 2-mercaptoethanol as reducing agent) and
124 subjected to SDS-PAGE on 10% acrilamide/bisacrilamide gels.
125 Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF,
126 Amersham Bioscience). Residual binding sites on the membrane were blocked by
127 incubation in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% (w/v)
128 nonfat milk powder overnight at 4°C. Membranes were then probed with specific
129 primary antibodies: rabbit anti-HIF-1α polyclonal antibody (Santa Cruz
130 Biotechnology) (1:200); rabbit anti-cytoskeletal actin (Bethyl Laboratories) (1:5000),
131 followed by peroxidase-conjugated secondary antibody HRP labeled goat anti-rabbit
132 Ig (BD Pharmigen) (1:5000) and visualized with an ECL plus detection system
133 (Amersham Biosciences). The equivalent loading of proteins in each well was
134 confirmed by Ponceau staining and actin control.

135

136 *Gelatin Zymography.*

137 Gel zymography was used to detect the proteolytic activity of MMP2 and MMP9, two
138 matrix metalloproteinases (MMPs) involved in matrix metabolism and vessel
139 maturation, which have been reported to be affected by oxygen levels (5). Following
140 appropriate treatment, media from cell cultures were denaturated in 4X SDS-PAGE
141 sample buffer and loaded onto a 10% acrilamide/bisacrilamide gel containing 0.1%

142 gelatin and then electrophoresed for 5 hours in Tris-glycine SDS running buffer. To
143 enable the enzymes to re-nature, the gel was incubated for 1 hour in 2.5% Triton X-
144 100 at room temperature and incubated in zymogram developing buffer (10mM
145 $CaCl_2$, 50mM Tris-Base pH 8.0) overnight at 37°C. The gel was stained with 0.25%
146 Coomassie Brilliant Blue solution of methanol/acetic acid/water (40:10:50 v/v) for
147 30min at room temperature then destained with methanol/acetic acid/water (40:10:50
148 v/v) for 20 min at room temperature.
149 The presence of clear bands in the gels at the appropriate molecular weights reflects
150 gelatinolytic activity of MMP2 and MMP9.
151
152 *Human studies.*
153 *Subjects.*
154 Two groups of 12 healthy males, physiotherapy students aged of 21.8 ± 2.3 and 21.25
155 ± 2.1 years (Mean±SD, group 1 and 2), height: 179.41 ± 5.6 and 178.3 ± 5.4 cm;
156 weight 73.2 ± 5.5 and 73.16 ± 4.7 Kg; participated in this study after Medical Ethics
157 Committee approval (N° B200-2011-76) and written informed consent was obtained.
158 Subjects were asked not to smoke nor to take any medication or perform strenuous
159 physical exercise 24 hours before and during the entire study protocol, or stay in
160 altitude 2 weeks before experiments.
161
162 *Experimental protocol.*
163 The volunteers received either 15% (n=12) (Group 1) or 100% (n=12) (Group 2) of
164 oxygen by means of oro-facial breathing demand mask (Alduc 2 Drager demand
165 valve breathing inhalator), the oxygen sessions were achieved during 30 minutes
166 every other day for a period of 10 days (5 sessions). Hemoglobin (Hb) levels were

167 measured by a photometric method (Hemocue HB 201) using capillary blood samples
168 withdrawn puncturing the fingertip pulp and analysing the blood drop before every
169 session and were related to the baseline as a percentage.

170

171 *Statistical analysis.*

172 All the experiments conducted in cultured cells were performed in triplicate and
173 repeated three times. Results are expressed as means ± SD from three experiments
174 and statistically analysed by a one-way ANOVA test, followed by Tukey's HSD,
175 using the statistical software ezANOVA. Differences in groups and treatments were
176 considered significant for $p < 0.05$.

177 Differences resulting from hyperoxic treatment in humans were assessed by a
178 repeated measures ANOVA to test the between-and within-subject effect after
179 Kolmogorov Smirnov test for normality. A Dunnet post test was then performed.
180 Individual initial value was considered as 100%, and percent variations were
181 calculated for Hb thereby allowing an appreciation of the magnitude of change rather
182 than the absolute values. The regression lines slopes differences (Hyperoxic vs
183 Hypoxic) were analysed using the ANCOVA procedure. The significance level was
184 set at $p < 0.05$.

185

186 **Results**

187 **Experiments on cultured HUVECs**

188 Given the ability of the cells to sense and respond to changes in oxygenation through
189 the involvement of HIF, we measured the level of HIF-1α protein in HUVECs
190 exposed to alternate oxygen concentration (mild hyperoxia and then recovery to
191 normoxia).

192 MMPs genes contains an HRE and are therefore modulated by HIF-1α playing critical
193 roles in several aspects of tissue growth, development and remodelling, wound
194 healing, and angiogenesis in response to oxygen availability (24). Therefore, we
195 evaluated the effects of oxygen sensing by measuring the expression of matrix
196 metalloproteinases (MMP-2 and -9).

197

198 *Normoxia after hyperoxia is associated with an increase of HIF-1 levels.*

199 In our experimental condition the level of HIF-1α in HUVEC cells is down-regulated
200 by a mild hyperoxic treatment. Figure 1 shows that increased oxygen levels inhibit
201 HIF-1α and -1β dimerization. Interestingly, during recovery, when oxygen
202 concentration was brought back to the initial level of 21%, we observed that HIF-1
203 was upregulated in a time-dependent way. 6 hours after the administration of
204 hyperoxia HIF-1α levels were about twice the baseline level.

205 As HIF-1α acts as an oxygen sensor and is expected to be upregulated during hypoxia
206 (7), these results suggest that endothelial cells "interpreted" the return to the baseline
207 oxygen concentration during post hyperoxia recovery, as a relative oxygen shortage
208 and then as hypoxia.

209

210 [Figure 1]

211

212 *Normoxia after hyperoxia is associated with an increased activity level of HIF-1-*
213 *regulated proteins.*

214 The cellular reaction indicated by the changes of HIF-1α levels were confirmed by
215 assessing MMP activity. Figure 2 shows that the activity of both MMP-2 and MMP-9
216 follows the same trend of HIF-1 being significantly down-regulated (about 50% of the
217 baseline) during hyperoxia and rapidly reaching a level significantly higher than that
218 monitored at the baseline after 4 hours of normoxic treatment. At 6 hours from the
219 cessation of high oxygen treatment, MMP-2 and MMP-9 protein levels were 2.5 and
220 2.6 fold higher than at the baseline. The upregulation of the catalytic activities of
221 MMP-2 and MMP-9 after the return to normoxia, supports the hypothesis that
222 normoxia following a hyperoxic event is sensed by endothelial cells as an oxygen
223 shortage.

224

225 [Figure 2]

226

227 *Human studies.*

228 Since the first report presenting the possibility to increase Erythropoietin synthesis
229 with a single non hypoxic stimulus (4), the clinical use of this acute effect of oxygen
230 breathing has been proposed to be an expedient treatment in neuroprotection and
231 cardioprotection in preoperative treatment and beneficial for septic patients (9).
232 Similarly, we reported potential advantages of normobaric high oxygen treatment in
233 cardiac surgery patients (10).

234 In order to corroborate the observations hereby reported on cultured cells, we have
235 exposed human healthy volunteers to pulsed hyperoxia and hypoxia to evaluate the

236 effects at functional level by measuring blood Hb concentration as the final outcome
237 of the induction of erythropoietin synthesis via HIF activation.
238
239 [Figure 3]
240
241 Figure 3 shows that both temporary hypoxic and hyperoxic treatment are associated
242 with an increase of Hb levels in humans. In comparison to the background, Hb levels
243 after breathing 100% oxygen reached 105.53 ± 7.65 % (p=0.016). Similarly, in the
244 hypoxic group, Hb levels were 116.75 ± 9.58 % (p=0.0002) of the baseline after 10
245 days (15% oxygen breathing). A further increase of Hb concentration was observed
246 after the cessation of the oxygen breathing eventually followed by a rapid decrease to
247 basal levels after few days.
248
249 [Figure 4]
250
251 The hypoxic stimulus was expected to be associated with a significant increase of Hb
252 levels since this is a well established stimulus for erythropoietin (EPO) expression
253 and subsequent Hb increase as shown in voluntary breath-hold studies (13).
254 Conversely, the increase of Hb associated with the hyperoxic stimulus was
255 unexpected and in agreement with our previous observations and with cultured cells
256 data. Comparing the increase tendencies of both groups with linear regressions and
257 then comparing them by means of an ANCOVA analysis, no differences were found
258 between the slopes. Therefore, one single regression line can be drawn expressing the
259 increase of Hb in both groups (see Fig. 4).

260 **Discussion**

261 Numerous evidences demonstrate that the hypoxia-inducible factor-1 (HIF-1) is one
262 of the major factors controlling cellular adaptive response to hypoxia (20). HIF-1 was
263 originally identified as a regulator of hypoxia-induced erythropoietin expression, and
264 eventually found as an essential global regulator of oxygen homeostasis (27). The
265 generation of the heterodimeric transcription factor composed of HIF-1α and HIF-1β
266 subunits is well known to induce the expression of genes (including EPO) bearing the
267 hypoxia responsive element (HRE) at the promoter level.

268 In a previous report, we hypothesized that relative changes of oxygen availability
269 rather than on steady state hypoxic or hyperoxic conditions and that reactive oxygen
270 species play an important role in HIF transcriptional effects. The repetition of such a
271 stimulus has been used to increase hemoglobin and reticulocytes in anemic patients
272 (8, 3), the possible doping effect of such a method has also been recently discussed
273 (1).

274 We used a cellular approach in order to reach a deeper insight of the Normobaric
275 Oxygen Paradox (4). Experiments conducted on cultured endothelial cells (HUVECs)
276 confirm that an increase of HIF during recovery occurs and corroborate the
277 hypothesis of a relative hypoxic environment in the presence of 21% oxygen (Figure
278 1). Furthermore, MMPs, known to be modulated by the hypoxic stimulus in
279 endothelial cells (6), were upregulated after the cell recovery to normoxia. Our
280 cellular experiments indicate that MMPs activity levels paralleled oxygen-induced
281 modulation of HIF and strongly indicate that endothelial cells sense the return to
282 normoxia after hyperoxia as an hypoxic stimulus (Figure 5).

283 In order to confirm the results obtained on endothelial cells, we studied the effects of
284 pulsed hyperoxic and hypoxic treatment in healthy human volunteers. In fact, in

285 response to hypoxia, the capacity of red blood cells to transport oxygen is up-
286 regulated by the expression of genes involved in erythropoiesis and iron-metabolism.
287 Hypoxia increases the expression of EPO, which is required for the formation of red
288 blood cells (19) as an increase in the number of erythrocytes enhances the delivery of
289 oxygen to tissues. As reported in figure 3, both breathing 15% oxygen and hyperoxia
290 induced an increase of Hb levels in humans. This effect was enhanced after the
291 cessation of the oxygen breathing. These results indicate that a sudden and sustained
292 decrease in tissue oxygen tension after hyperoxia, may act as a trigger for EPO
293 synthesis (Figure 5).
294

295 [Figure 5]
296

297 Althought our *in vivo* study only addressed the final outcome of oxygen effects, we
298 can speculate that our observation are underlined by two different pathways: one
299 related to the increase of the glutathione synthesis (possibly due to a NRF-2 mediated
300 signalling) coping with the increase of reactive oxygen species generated during
301 hyperoxia, as suggested by studies on human subjects supplemented with N-Acetyl
302 Cysteine (28, 18), and the other one due to an increase of HIF availability shown for
303 the first time in this study in pulsed hyperoxic conditions (see Fig. 1 and 2). Data
304 were already available in intermittent hypoxic conditions (17). It is evident that a
305 concomitant intervention of the two pathways is clearly possible.
306 It seems that, according to our findings the "relative" hypoxia is a potent stimulator of
307 the HIF mediated gene expressions, medical potential uses can be considered since a
308 real hypoxia is difficult to be proposed as a treatment for patients. Some applications
309 have been proposed in several clinical settings (10, 11, 12, 2, 15, 16) . Moreover,

310 increased expression of HIF 1 has been demonstrated in ICU patients with shock

311 (26). Our cellular data warrant the future clinical approaches.

312

313 **References**

314 1. **Balestra C and Germonpré P.** EPO and doping. *Eur J Appl Physiol* 109: 1001-
315 1002, 2010.
316 2. **Balestra C and Germonpré P.** Hypoxia, a multifaceted phenomenon: the
317 example of the "Normobaric Oxygen Paradox". *Eur J Appl Physiol* (March 31,
318 2012). doi: 10.1007/s00421-012-2392-y.
319 3. **Balestra C, Germonpré P, Lafere P, Ciccarella Y, and Van Der Linden P.**
320 The 'normobaric oxygen paradox': a simple way to induce endogenous
321 erythropoietin production and concomitantly raise hemoglobin levels in anemic
322 patients. *Transfus Altern Transfus Med* 11: 39-42, 2010.
323 4. **Balestra C, Germonpré P, Poortmans JR, and Marroni A.** Serum
324 erythropoietin levels in healthy humans after a short period of normobaric and
325 hyperbaric oxygen breathing: the "normobaric oxygen paradox". *J Appl Physiol*
326 100: 512-518, 2006.
327 5. **Ben-Yosef Y, Lahat N, Shapiro S, Bitterman H, and Miller A.** Regulation of
328 endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. *Circ Res* 90:
329 784-791, 2002.
330 6. **Ben-Yosef Y, Miller A, Shapiro S and Lahat N.** Hypoxia of endothelial cells
331 leads to MMP-2-dependent survival and death. *Am J Physiol Cell Physiol* 289:
332 C1321-C1331, 2005.
333 7. **Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M,**
334 **Scarpulla RC, and Chandel NS.** Oxygen sensing requires mitochondrial ROS
335 but not oxidative phosphorylation. *Cell Metab* 1: 409-414, 2005.
336 8. **Burk R.** Oxygen breathing may be a cheaper and safer alternative to exogenous
337 erythropoietin (EPO). *Med Hypotheses* 69: 1200-1204, 2007

338 9. **Calzia E, Asfar P, Hauser B, Matejovic M, Ballestra C, Radermacher P, and**
339 **Georgieff M.** Hyperoxia may be beneficial. *Crit Care Med* 38: S559-S568, 2010.
340 10. **Ciccarella Y, Balestra C, Valsamis J, and Van der Linden P.** Increase in
341 endogenous erythropoietin synthesis through the normobaric oxygen paradox in
342 cardiac surgery patients. *Br J Anaesth* 106: 752-753, 2011.
343 11. **De Bels D, Corazza F, and Balestra C.** Oxygen sensing, homeostasis, and
344 disease. N *Engl J Med* 365: 1845, 2011.
345 12. **De Bels D, Corazza F, Germonpré P, and Balestra C.** The normobaric oxygen
346 paradox: a novel way to administer oxygen as an adjuvant treatment for cancer?
347 *Med Hypotheses* 76: 467-470, 2011
348 13. **de Bruijn R, Richardson M, and Schagatay E.** Increased erythropoietin
349 concentration after repeated apneas in humans. *Eur J Appl Physiol* 102: 609-613,
350 2008.
351 14. **Dong Z, Venkatachalam MA, Wang J, Patel Y, Saikumar P, Semenza GL,**
352 **Force T, and Nishiyama J.** Up-regulation of apoptosis inhibitory protein IAP-2
353 by hypoxia. Hif-1-independent mechanisms. *J Biol Chem* 276: 18702-18709,
354 2001.
355 15. **Khalife M, Wiams K, Ben Aziz M, Paesmans M, Balestra C, and Sosnowsky**
356 **M.** Effects of induced relative hypoxia during the postoperative period of
357 abdominal oncologic surgery, on hemoglobin and reticulocyte levels: a
358 prospective, randomized controlled clinical trial. *Critical Care* 16: P262, 2012.
359 16. **Lafère P, Schubert T, De Bels D, Germonpré P, and Balestra C.** Could the
360 Normobaric Oxygen Paradox (NOP) reduce allogenic transfusions after hip
361 surgery? A pilot study. *J Clin Anes* (in press).

362 17. **Malec V, Gottschald OR, Li S, Rose F, Seeger W, and Hanze J.** HIF-1 alpha
363 signaling is augmented during intermittent hypoxia by induction of the Nrf2
364 pathway in NOX1-expressing adenocarcinoma A549 cells. *Free Radic Biol Med*
365 48: 1626-1635, 2010.
366 18. **Momeni M, De Kock M, Devuyst O, and Liistro G.** Effect of N-acetyl-cysteine
367 and hyperoxia on erythropoietin production. *Eur J Appl Physiol* 111: 2681-2686,
368 2011.
369 19. **Semenza GL, Koury ST, Nejfelt MK, Gearhart JD, and Antonarakis SE.**
370 Cell-type-specific and hypoxia-inducible expression of the human erythropoietin
371 gene in transgenic mice. *Proc Natl Acad Sci U S A* 88: 8725-8729, 1991.
372 20. **Semenza GL.** HIF-1: mediator of physiological and pathophysiological responses
373 to hypoxia. *J Appl Physiol* 88: 1474-1480, 2000.
374 21. **Semenza GL.** O2-regulated gene expression: transcriptional control of
375 cardiorespiratory physiology by HIF-1. *J Appl Physiol* 96: 1173-1177, 2004
376 22. **Semenza GL.** Oxygen sensing, homeostasis, and disease. *N Engl J Med* 365: 537-
377 547, 2011a.
378 23. **Semenza GL.** Regulation of metabolism by hypoxia-inducible factor 1. *Cold*
379 *Spring Harb Symp Quant Biol* 76: 347-353, 2011b.
380 24. **Shyu KG, Hsu FL, Wang MJ, Wang BW, and Lin S.** Hypoxia-inducible factor
381 1alpha regulates lung adenocarcinoma cell invasion. *Exp Cell Res* 313: 1181-
382 1191, 2007.
383 25. **Speciale A, Anwar S, Ricciardi E, Chirafisi J, Saija A, and Cimino F.** Cellular
384 adaptive response to glutathione depletion modulates endothelial dysfunction
385 triggered by TNF-α. *Toxicol Lett* 207: 291-297, 2011.

386 26. **Textoris J, Beaufils N, Quintana G, Ben Lassoued A, Zieleskiewicz L,**
387 **Wiramus S, Blasco V, Lesavre N, Martin C, Gabert J, and Leone M.**
388 Hypoxia-inducible factor (HIF1alpha) gene expression in human shock states.
389 *Crit Care* 16: R120, 2012.
390 27. **Wenger RH and Gassmann M.** Oxygen(es) and the hypoxia-inducible factor-1.
391 *Biol Chem* 378: 609-616, 1997.
392 28. **Zembron-Lacny A, Slowinska-Lisowska M, Szygula Z, Witkowski Z, and**
393 **Szyszka K.** Modulatory effect of N-acetylcysteine on pro-antioxidant status and
394 haematological response in healthy men. *J Physiol Biochem* 66: 15-21, 2010.
395

396 **Figure Legends**

397 **Figure 1.** Modulation of HIF-α in HUVECs exposed to hyperoxia (32% O_2) for 2h
398 and then recovered to normoxia for the following 6 h.

399 Cultures exposed to normoxic conditions for 6h were used as controls. The figure
400 shows a representative image out of at least three independent experiments. Results
401 by densitometry are reported as fold change against control (6h of normoxia) and
402 expressed as mean ±SD of at least three independent experiments. *$p < 0.05$ vs cells
403 exposed to hyperoxia for 2h.

404 **Figure 2.** Zymographic analysis of MMPs in the culture medium of HUVECs
405 exposed to hyperoxia (32% O2) for 2h and then recovered to normoxia in the
406 subsequent 6 h. Cultures exposed to normoxic conditions for 6h were used as
407 controls. The picture represents the inverted image of the zymography gel from one
408 representative experiment out of three independent experiments. The dark bands
409 represent lytic zones. The activities were abolished by the addition of EDTA (5 mM),
410 confirming that the detected gelatinase activities were specifically due to the MMPs
411 (not shown). The histogram displays the mean ± SD of three independent
412 experiments. Results by densitometry are reported for the active form of MMP-2 and
413 MMP-9 as fold change against control cultures. *$p < 0.05$ vs normoxic control cells.

414 **Figure 3**. Percent variation of Hb after breathing low (15%) and high (100%) oxygen
415 (see methods for subjects details). Each subject acted as his own control.

416 **Figure 4**. regression line drawn for both experimental groups. (see text for furher
417 details). The pulsed hypoxic and hyperoxic stimulus is leading to similar
418 physiological reactions.

419 **Figure 5**. tentative sketch of a mechanisms for oxygen sensing leading to the
420 activation of HIF and transcription of HRE genes (EPO, MMPs). Our results support
421 the hypothesis that normoxia following a hyperoxic event is sensed by endothelial
422 cells as an oxygen shortage. This represent a logical explanation for the sustained
423 decrease in EPO production after hyperbaric oxygen breathing, as opposed to the
424 normobaric oxygen paradox.