Author's response to reviews

Title: A Long-term memory of HIF Induction in Response to Chronic Mild Decreased Oxygen after Oxygen Normalization

Authors:

Chandrashekar D Kamat (shekhar-kamat@ouhsc.edu)
Jessica E Thorpe (jessica-thorpe@ouhsc.edu)
Satyendra S Shenoy (satyendra-shenoy@ouhsc.edu)
Antonio Ceriello (antonio.ceriello@warwick.ac.uk)
Dixy E Green (dixy-green@ouhsc.edu)
Linda A Warnke (linda-warnke@ouhsc.edu)
Michael A Ihnat (michael-ihnat@ouhsc.edu)

Version: 2 Date: 13 December 2006

Author's response to reviews: see over
Response to the review of “A Long-term “memory” of HIF Induction in Response to Chronic Mild Decreased Oxygen after Oxygen Normalization.”

Because we have received review with no criticisms, only comments and one review with many major and minor criticisms, which if completely addressed would lead to enough data for at least one additional paper, we would like the opportunity to reply to the reviewers comments.

**Dr. Fung:**

**Suggestions:**
1. We have tempered our conclusions with respect the direct link of our findings to endothelial dysfunction by using words such as speculate, surmise, etc.
2. We have described experiments with varying levels of oxygen to examine which percentage was the lowest without resulting in cell death (see Dr. Haddad, major point 8) and have added additional citations for the use of 15% oxygen in our studies as a clinically relevant oxygen level.

**Dr. Haddad:**

**MAJOR**
1. We have tempered our conclusions with respect to the findings in this paper and their link to clinical pathogenesis. However, based on extensive literature searches, our work is the first to show that such mild decreased oxygen can act through the HIF system and that this HIF induction persists a week after oxygen normalization. In terms of folks with COPD, heart failure, etc. leading to chronic exposure to mild decreased oxygen this could be important because oxygen levels previously thought too low to induce the HIF stress pathway have now been shown to result in induction.
2. We agree that the exploration of the regulation of basal (i.e., normoxic) HIF levels in our system and their relation to HIF ubiquitylation is a very interesting topic, however we believe that these studies go well beyond the goal of this paper to examine HIF signaling in response to chronic mild decreased oxygen and whether these effects persist after oxygen normalization.
3. As mentioned in the discussion, we were surprised that only GLUT-1 had a firm link to reactive species and that in our system HIF was being post-translationally regulated. The last figure with HIF-1α ubiquitylation begins to address how this regulation at the protein level could be happening after oxygen levels are normalized and ongoing studies to look further at the proteasome, HPHs, etc. are ongoing as described in the discussion section and could alone constitute another manuscript. It is possible that HIF-3α could be down regulated in response to mild decreased oxygen in our model and remain down after oxygen levels are normalized and we have added a statement in the discussion addressing this as a future direction of this work, but again these studies could result in a manuscript by themselves.
4. In Fig. 3, we are making no arguments regarding absolute levels of HIF and its regulated proteins in HUVEC, ASMC and HMEC-1 (in Fig. 1), although we want to focus the reader’s attention to the novel “memory” phenomenon by indicating that HIF and its target proteins remained induced post-oxygen normalization as compared to their respective normoxic controls.

5. The goal of this paper was to determine whether HIF signaling remains induced in vascular cells in response to very mild decreased oxygen and whether levels would remain induced one week after oxygen levels were normalized. The observation that GLUT-1 levels are similar in response to acute hypoxia and decreased oxygen followed by oxygen normalization are simply that, an observation without any link between the two stressors (i.e., acute hypoxia and mild decreased oxygen). It could well be that the re-oxygenation is inducing HIF signaling instead of a prolongation of HIF stress signaling in response to chronic mild decreased oxygen and in order to address this, we have added a statement in the discussion section. Most importantly, based on literature and the chronic nature of cardiovascular disease, the week length in which HIF is induced is itself a very novel finding. To add cytokines as inducers of ROS to elucidate the mechanistic basis behind this at this apparent similarity would not only dilute the paper significantly from its goals but may also not answer this issue.

6. As mentioned in the text, we titrated the dose of YC-1 to get around 60% inhibition of HIF-1α without any apparent death. We have also used extensive literature searches to define the doses of each antioxidant and reactive species scavenger. In addition, we tested each dose for a lack of cell killing in our system. The doses of the reactive species inhibitors and scavengers are well cited for use in many cell systems including HUVEC. We also made certain that these doses of inhibitors and scavengers did not result in any decrease in cell proliferation. We agree that esterified glutathione would be a great way to ascertain the further role of ROS on propagation of HIF induction and this was added to the discussion section of the paper as a part of suggestive future work.

7. We agree that exogenous ROS generating system could answer the role of ROS in the cellular “memory” of HIF signaling induction. However, it was found only the cellular “memory” of GLUT-1 induction was found to be ROS-dependent while the other HIF regulated factors were not (Fig. 4). A point in this regard was added to the discussion section of the paper.

8. A dose-response relation of oxygen 2.5%, 5.0%, 10% and 17.5% oxygen was done with the low oxygen levels resulting in significant cell death making it unable to carry out long term studies and frankly not mimicking the clinical situation of endothelial dysfunction where endothelial cell death is not observed until terminal stages of cardiovascular disease.

MINOR:
1. Typo mistakes were checked and changed.
2. Introduction was slightly reduced in size.
3. The number of references was reduced in the introduction, however additional references added in addressing reviewers’ concerns have resulted in an increased number of total references.
4. If desired, we can change the "data not shown" to supplemental figures, which, conservatively would number 6 at this point. Further, no positive data was omitted from the paper, only data showing lack of cell death, etc.

5. The top gel of Figure 5 was sharpened, however in other articles where HIF-1α was immunoprecipitated and probed for ubiquitin, a similar broad band/banding pattern was observed.

6. Although the term proof-of-principle might be trite, it is our view that we have proven our two goals for the paper using isolated cells of the vasculature – that chronic exposure to mild decreased oxygen can induce HIF signaling and that this induction can persist after oxygen normalization.

Please contact me should you require any additional information and thank you so very much for your time and consideration.

Michael Ihnat