Hypoxia Selectively Activates the CREB Family of Transcription Factors in the *In Vivo* Lung

Martin O. Leonard¹, Katherine Howell¹, Stephen F. Madden¹, Christine M. Costello¹, Desmond G. Higgins¹, Cormac T. Taylor¹, and Paul McLoughlin¹

¹University College Dublin, School of Medicine and Medical Science, and Conway Institute of Biomolecular and Biomedical Research, Dublin, Ireland

Rationale: Pulmonary hypertension is a common complication of chronic hypoxic lung diseases and is associated with increased morbidity and reduced survival. The pulmonary vascular changes in response to hypoxia, both structural and functional, are unique to this circulation.

Objectives: To identify transcription factor pathways uniquely activated in the lung in response to hypoxia.

Methods: After exposure to environmental hypoxia (10% O₂) for varying periods (3 h to 2 wk), lungs and systemic organs were isolated from groups of adult male mice. Bioinformatic examination of genes the expression of which changed in the hypoxic lung (assessed using microarray analysis) identified potential lung-selective transcription factors controlling these changes in gene expression. In separate further experiments, lung-selective activation of these candidate transcription factors was tested in hypoxic mice and by comparing hypoxic responses of primary human pulmonary and cardiac microvascular endothelial cells *in vitro*.

Measurements and Main Results: Bioinformatic analysis identified cAMP response element binding (CREB) family members as candidate lung-selective hypoxia-responsive transcription factors. Further *in vivo* experiments demonstrated activation of CREB and activating transcription factor (ATF)1 and up-regulation of CREB family-responsive genes in the hypoxic lung, but not in other organs. Hypoxia-dependent CREB activation and CREB-responsive gene expression was observed in human primary lung, but not cardiac microvascular endothelial cells.

Conclusions: These findings suggest that activation of CREB and ATF1 plays a key role in the lung-specific responses to hypoxia, and that lung microvascular endothelial cells are important, proximal effector cells in the specific responses of the pulmonary circulation to hypoxia.

Keywords: hypoxia; cAMP response element binding; pulmonary hypertension; transcription factor binding site

Pulmonary hypertension is a common complication of chronic hypoxic lung diseases, which is associated with increased morbidity and reduced survival. Moreover, cor pulmonale is an independent predictor of increased mortality, suggesting that pulmonary hypertension contributes directly to reduced survival (1, 2). The increased pulmonary vascular resistance underlying chronic hypoxic pulmonary hypertension is accompanied by characteristic changes in the blood vessels, which include remodel-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chronic hypoxia causes increased pulmonary vascular resistance and hypertension, changes that are unique to the lung. The mechanisms responsible for the underlying lung-specific changes in gene expression are poorly understood.

What This Study Adds to the Field

The transcription factors CREB and ATF1 are selectively activated in the hypoxic lung, in particular the pulmonary microvascular endothelium, but not in systemic organs, suggesting a central role for these transcription factors in the pulmonary vascular responses to hypoxia.

ing and thickening of the walls of precapillary vessels and sustained vasoconstriction (3–8). These long-term structural and functional changes in the pulmonary vessels in response to hypoxia are unique to this circulation, and are not observed in the vasculature of other organs of the body.

The important roles of the vascular endothelium in the local control of vascular smooth muscle tone and in modulating changes in the structure of the vessel wall are now well recognized (9). It is also well recognized that endothelial cells from different organs show considerable heterogeneity, and that their roles in the control of the vasculature differ from organ to organ (10–12). These observations suggest that the pulmonary endothelium probably plays a central role in producing the distinct responses of the pulmonary circulation to hypoxia.

The lung displays a further unique aspect in its responses to reductions in alveolar oxygen, that is, marked responses are observed at higher partial pressures of oxygen than would elicit responses in any other organ. For example, hypoxic pulmonary vasoconstriction and pulmonary hypertension develop when alveolar PO₂ is 40–60 mm Hg (2, 5, 13), a value that is substantially higher than that in other organs under conditions of normal oxygenation (14–16). This implies that there are specific mechanisms within the lung that are responsible for sensing and responding to changes in PO₂, which are different from those found in other organs and tissues. At present, these mechanisms are poorly understood.

Given that the lung shows distinct phenotypic responses to hypoxia, at uniquely high partial pressures of oxygen, we postulated that there must be activation of specific transcription factors within the lung that mediate these responses. The aim of this study was to identify transcription factors that are activated in the lung in response to alveolar hypoxia similar to that seen in lung disease, but are not activated in the systemic organs by the same stimulus. In addition, given the important role of endothelial cells in pulmonary vascular remodeling and vasoconstriction, we wished to determine whether hypoxic activation of lung-specific transcription factors was observed in these cells.

(Received in original form December 25, 2007; accepted in final form July 17, 2008)

Supported by grants from the Health Research Board of Ireland (P.M.), an unrestricted grant from Actelion (P.M.), the Higher Education Authority of Ireland, PRTL (P.M.), the Wellcome Trust (C.T.T.), and the Science Foundation of Ireland (C.T.T.).

Correspondence and requests for reprints should be addressed to Paul McLoughlin, M.B., Ph.D., University College Dublin, School of Medicine and Medical Sciences, Conway Institute, Belfield, Dublin 4, Ireland. E-mail: paul.mcloughlin@ucd.ie

This article has an online supplement, which is available from the issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 178, pp 977–983, 2008

Originally Published in Press as DOI: 10.1164/rccm.200712-1890OC on August 8, 2008
Internet address: www.atsjournals.org

METHODS

With institutional ethics approval, adult male-specific, pathogen-free C57BL/6J mice (20–25 g) were exposed to normoxic (F_IO₂ = 0.21) or hypoxic conditions (F_IO₂ = 0.10) for varying periods, from 3 hours to 2 weeks (n = 4–8 per group) using a servo-controlled environmental chamber (Biospherix, Lacona, NY), as previously described (13). After exposure, mice were killed under anesthesia and organs were immediately removed for isolation of RNA.

Gene expression within lung tissue was analyzed using microarray (U430Av2 array; Affymetrix, Santa Clara, CA) and Taqman real-time polymerase chain reaction (PCR) techniques. For the microarray analysis, RNA from mice at each time point of normoxia and hypoxia was pooled (n = 6) before cRNA synthesis. Genespring software (Agilent Technologies, Stockport, UK) was used to identify genes, the expression of which was changed by more than 1.5-fold from control normoxic values after hypoxic exposure. These genes were then clustered using k-means analysis. Candidate lung-selective transcription factors were identified based on the rationale that binding sites for these transcription factors would occur with higher-than-expected frequency in the promoters of the genes contained in the hypoxia-regulated clusters. These binding sites were identified using the approach of Ho Sui and colleagues (17) and a recently developed database of transcription factor binding sites that are conserved within the promoters of orthologous genes across the human, mouse, rat, and dog genomes (18, 19). The Fischer exact test (*P* < 0.001) and the *z* score statistic (>10) were used to identify overrepresented binding sites within a specific cluster.

In separate groups of control and hypoxic mice, nuclear protein was extracted and analyzed for selected transcription factors from the cAMP response element binding (CREB) family (CREB, activating transcription factor [ATF]1, and ATF2) by Western blotting of both total and serine-133 phosphorylated forms.

For *in vitro* experiments, human primary lung (human lung microvascular endothelial cells [HMVECs]-L) and cardiac HMVECs (HMVEC-Cs) were cultured according to the supplier's recommendations (Lonza Bioscience, Basel, Switzerland). Cells between passage six and nine were maintained under hypoxic (5% O₂) or normoxic (21% O₂) conditions for varying periods of time up to 24 hours. Nuclear extracts from cells were analyzed for activated, ser133 phosphorylated CREB using a specific CREB response element DNA binding assay (TransAm; Active Motif, Carlsbad, CA). Cells were also harvested, processed for RNA extraction, and analyzed for changes in gene expression by Taqman real-time PCR. All data were presented as mean (±SEM) for n independent experiments. The statistical significance of the differences between means was evaluated using Student's unpaired *t* test or analysis of variance with a Dunnett post hoc test (GraphPad, San Diego, CA). Where data were nonnormally distributed, Mann-Whitney *U* test with the Bonferroni correction was used. Further details of experimental procedures can be found in the online supplement.

RESULTS

Identification of Overrepresented Transcription Factor Binding Sites

After isolation of RNA from hypoxic lungs, its integrity was checked and only samples that met the highest-quality standard were used in the subsequent microarray analysis. Initial analysis of the microarray data confirmed that well known hypoxia-responsive genes were up-regulated, including solute carrier family 2 (facilitated glucose transporter) member 1, adrenomedullin, hexokinase 2, and endothelin (EDN)-1. K-means cluster analysis (K = 24) was then used to group genes, the expression of which was altered by hypoxia (total no. = 1,534) based on similar temporal expression profiles. One cluster showed prominent early up-regulation, suggesting that these genes were central to initiating the pulmonary hypoxic responses (Figure 1). In this cluster, there was significant overrepresentation of 20 specific transcription factor binding sites (Table 1). Note that only known transcription factors are shown; six overrepresented unknown

Figure 1. K-means clustering of hypoxia-regulated genes in the lung. Microarray RNA expression analysis of the hypoxic mouse lung. Microarray analysis of mRNA from lungs of mice exposed to normobaric normoxia (21% O₂) or hypoxia (10% O₂) for 3 hours, 6 hours, 1 day, 1 week, and 2 weeks. A total of 1,534 genes, the expression of which was altered by 1.5-fold or more at any time point of hypoxia compared with normoxia, are shown. Results are expressed so that each row represents an individual gene, and the intensity of color (*red* is up-regulated and *green* down-regulated) is directly proportional to the level of change. The number of genes in each cluster is indicated on the left of the panel. Specific cluster of genes up-regulated by hypoxia is outlined by a yellow box.

(predicted) transcription bindings sites were also found, but are not shown here. The most significantly overrepresented binding site was a binding site for the hypoxia-inducible factor (HIF)-1β (aryl hydrocarbon receptor nuclear translocator) homodimer (Table 1). However, of major interest was the presence in this list of specific binding sites for the cAMP response element binding protein (CREB) family of transcription factors including CREB, ATF1/ATF3, ATF3, and CREBp1CJUN. Because these were the most abundant of the transcription factor binding sites present (five motifs identified) and were associated with genes, the expression of which was up-regulated early in the course of hypoxic exposure, we focused our further investigations on this family. We next asked which of the 137 genes in this cluster had promoters that contained putative CREB and CREBp1CJUN motifs, and identified 21 of these (Table 2). Literature review revealed previously published direct biological evidence of hypoxia-induced changes in gene expression for nine of these genes. CREB-dependent control of expression had been previously documented for seven of these 21 genes, while five of this

TABLE 1. CONSERVED TRANSCRIPTION FACTOR BINDING SITES IDENTIFIED IN GENES THAT SHOWED EARLY HYPOXIA-INDUCED INCREASES IN EXPRESSION (CLUSTER 1)

Transcription Factor	Fisher <i>P</i> Value	z Score	Transcription Factor Binding Motif
ARNT (HIF1 β)	0.000016	14.1	MA0004*
CREBP1CJUN	0.000095	15.9	V\$CREBP1CJUN†
Fos	0.000122	17.5	MA0099*
ATF-1/ATF-3	0.000122	16.1	c14_M_TGACGTCA‡
CREB1	0.000122	16.1	c14_M_TGACGTMR‡
PAX2	0.000139	15.4	MA0067*
TAL1 β E47	0.000153	12.4	c15_M_ACASRTGGY‡
ATF3	0.000159	15.7	c9_M_TGAYRTCA‡
LMO2COM	0.000278	12.2	V\$LMO2COM_02†
TATA_01	0.000299	11.1	V\$TATA_01†
RSRFC4	0.000348	13.7	c28_M_CTAWWWATA‡
USF	0.000742	13.2	V\$USF_C†
CREB1	0.000871	14.0	V\$CREB1_01†
TAL1 β E47	0.000959	14.6	c35_M_MCAGATGK‡

* Transcription factor binding site information was derived from Jaspard database.

† Transcription factor binding site information was derived from Transfac 6.2 Professional database.

‡ Transcription factor binding site information was derived from Xie and colleagues (25).

group were known to be HIF-dependent (i.e., responsive to the HIF-1 α /HIF- β heterodimer) (Table 2).

CREB/ATF1 Activation in the Hypoxic Lung

Activation of CREB requires protein phosphorylation at serine-133; ATF1 is also activated in a similar manner. Western blotting

TABLE 2. GENES FROM CLUSTER 1 (EARLY HYPOXIA-INDUCED INCREASES IN EXPRESSION) THE PROMOTERS OF WHICH CONTAIN PUTATIVE CREB BINDING SITES

Gene Name	Gene Symbol	Hypoxia	CREB	HIF-1/HRE
A kinase (PRKA) anchor protein (gravin) 12	AKAP12	No	No	No
Activating transcription factor 3	ATF3	Yes	Yes	No
Arginine vasopressin-induced 1	AVP11	No	Yes	No
Brain-derived neurotrophic factor	BDNF	Yes	Yes	No
Cyclin-dependent kinase inhibitor 1A (P21)	CDKN1A	Yes	Yes	Yes
Cholesterol 25-hydroxylase	CH25H	No	No	No
DNA damage-inducible transcript 4	DDIT4	Yes	No	Yes
Endothelin-1	EDN1	Yes	No	Yes
Eukaryotic translation initiation factor 2-3	EIF2S3X	No	No	No
Eukaryotic translation initiation factor 4A	EIF4A1	No	No	No
FMS-like tyrosine kinase 1 (VEGFR-1)	VEGFR1	Yes	Yes	Yes
Follistatin	FST	No	Yes	No
V-MAF musculoaponeurotic fibrosarcoma F	MAFF	Yes	No	No
Myocardin	MYOCD	Yes	No	No
N-MYC downstream-regulated gene 1	NDRG1	Yes	No	Yes
Plasminogen activator, tissue (tPA)	PLAT	No	Yes	No
Polo-like kinase 2 (<i>Drosophila</i>)	PLK2	No	No	No
Riken CDNA 1300003H02 gene	QSCN6	No	No	No
T cell lymphoma invasion and metastasis 1	TIAM1	No	No	No
Tumor necrosis factor receptor member 12A	TNFRSF12A	No	No	No
Tubulin, alpha 8 (PEX26)	TUBA8	No	No	No

Definition of abbreviations: CREB = cAMP response element binding; HIF = hypoxia-inducible factor; HRE = hypoxia response element; VEGFR = vascular endothelial growth factor receptor.

The column headed hypoxia indicates whether (Yes) or not (No) there is previously published evidence that gene expression is altered by hypoxia. The column headed CREB indicates whether (Yes), or not (No) there is previously published evidence that gene expression is altered by CREB activation. The column headed HIF-1/HRE indicates whether (Yes) or not (No) there is previously published evidence that gene expression is altered by HIF-1 binding to a hypoxia response element in its promoter. See online supplement for detailed bibliography.

of lung nuclear lysates revealed CREB phosphorylation after hypoxia when compared with normoxic tissues (Figure 2A). This activation was observed at 3 hours, was more marked at 1 day of hypoxia, and had returned to control levels by 1 week. Total CREB was unchanged by hypoxic exposure. Levels of the constitutively expressed nuclear protein, octamer-binding transcription factor (OCT)1, were analyzed as a control for protein loading (Figure 2A). Densitometric analysis of phosphorylated CREB expression over total CREB levels demonstrated a biphasic pattern of statistically significant increase thereafter 3 hours, and again after 1 day, of hypoxic exposure as compared with normoxic controls (Figure 2B). We also observed a significant increase in phosphorylation of a second member of the CREB family, ATF1, after 3 hours of exposure (Figure 2). The phosphorylation-dependent activation of a further CREB family transcription factor, ATF2, was also assessed, but did not show any significant hypoxia-induced alterations (data not shown). Interestingly, there were no significant changes in CREB or ATF1 phosphorylation in three systemic organs (kidney, liver, and spleen) isolated from the same hypoxic animals (Figure 3).

Confirmation of CREB-dependent Gene Expression within the Hypoxic Lung

From the list of 21 potentially CREB-dependent genes, we selected four genes in which to confirm increased expression using real-time PCR; namely, ATF3, EDN-1, vascular endothelial growth factor receptor (VEGFR)-1, and follistatin (FST). Three of these, ATF3, EDN-1, and FST, showed significant up-regulation early in hypoxia (6 h and/or 1 d), and had returned to baseline values by 1 week (Figure 4). In the case of VEGFR1, a significant change in gene expression was also observed over time ($P < 0.025$, analysis of variance), with the highest values seen after 1 day of hypoxic exposure. However, on post hoc testing, the difference between the peak and baseline values was not statistically significant. Three of the four genes demonstrated progressive increases between 6 and 24 hours, suggesting that peak expression had occurred at some point between the first day and 1 week of hypoxic exposure.

Lung-selective CREB-dependent Gene Expression

To confirm that the lung-selective activation of CREB family transcription factors caused an organ selective pattern of gene expression in hypoxia, we undertook a separate, independent series of experiments in which we examined ATF3, EDN-1, FST, and VEGFR1 mRNA in the lung, and a panel of five systemic organs in groups of control normoxic and hypoxic mice ($n = 4$). This showed up-regulation of these genes in the lung without significant alterations in any other organ examined (Figure 5).

Selective Activation of CREB and ATF-1 in the Pulmonary Microvascular Endothelium

Given the central role that the endothelium plays in hypoxia-induced pulmonary vascular remodeling and the development of pulmonary hypertension, we compared responses in primary HMVECs to those in cardiac microvascular endothelial cells. Hypoxia (5% O₂) treatment resulted in the activation of CREB in a time-dependent manner in lung, but not cardiac cells (Figure 6A), showing a biphasic pattern of activation similar to that observed within the *in vivo* lung. Examination of CREB-dependent gene expression revealed an up-regulation of ATF3, EDN-1, and VEGFR-1 that occurred only in the pulmonary, but not in the cardiac cells (Figure 6B). FST mRNA was undetectable in the pulmonary endothelial cells, both under normoxic conditions and after hypoxic exposure.

Figure 2. Hypoxia-dependent activation of cAMP response element binding (CREB) in the mouse lung. (A) Western blot showing serine-133 phosphorylation of CREB (pCREB) and activating transcription factor (pATF) in lungs isolated from mice exposed to normobaric normoxia (21% O₂) or hypoxia (10% O₂) for 3 hours, 6 hours, 1 day, 1 week, and 2 weeks. Total CREB (tCREB) and total ATF1 (tATF1) were also analyzed after stripping and reprobing. Analysis of total octamer-binding transcription factor 1 (Oct1) expression was performed as a loading control. Blots show results of four samples from a total of eight mice exposed at each time point. (B) Results of densitometric analysis of phosphorylated CREB and phosphorylated ATF1 showing mean $\pm$ SEM values at each time point of hypoxic exposure (n = 8). Results in each lung were normalized to total CREB protein in that lung, and then expressed as fold over the mean control normoxic value. *P < 0.01 and **P < 0.001, significant difference from corresponding control normoxic value (analysis of variance).

DISCUSSION

In undertaking this study, we set out to identify specific transcription factors, the activation of which could be responsible for the changes in protein expression that underpin the well known lung-specific responses to hypoxia, including hypoxic pulmonary vascular remodeling and pulmonary hypertension. From the genes identified as hypoxia responsive in the lung in our initial gene array studies, we selected for transcription factor binding site analysis one cluster in which genes showed early increases in response to hypoxia. This early response suggested that genes

within this cluster played a key role in initiating the pulmonary hypoxic response and, for this reason, we focused our further investigations on these. Bioinformatic analysis identified a list of transcription factor binding sites that had a putative role in the pulmonary responses to hypoxia; among them, some that were not previously known to be hypoxia responsive (Table 1). The most significantly overrepresented binding site was one that binds the HIF-1 β (aryl hydrocarbon receptor nuclear translocator) homodimer (Table 1). Although it is likely that this homodimeric transcription factor plays a role in controlling gene expression (20), there is little evidence for its involvement in

Figure 3. Effect of hypoxia on cAMP response element binding (CREB) activation in liver, kidney, and spleen. Western blot showing serine-133 phosphorylation of CREB (pCREB) and activating transcription factor (pATF) in liver, kidney, and spleen isolated from mice exposed to normobaric normoxia (21% O₂) or hypoxia (10% O₂) for 3 hours, 6 hours, 1 day, 1 week, and 2 weeks. Total CREB (tCREB) and tATF1 were also analyzed after stripping and reprobing. Blots show results of four representative samples from a total of eight mice exposed at each time point.

Figure 4. Results of real-time polymerase chain reaction (PCR) analysis of four genes from cluster 1. Real-time PCR analysis of ATF3, endothelin (EDN)-1, follistatin (FST), and vascular endothelial growth factor receptor (VEGFR)-1 mRNA from lungs of mice exposed to normobaric normoxia (21% O₂) or hypoxia (10% O₂) for 3 hours, 6 hours, 1 day, 1 week, and 2 weeks. Results were expressed as fold over control normoxic levels (n = 5–7 in each groups) and values are shown as means (±SEM). **P* < 0.05 and ***P* < 0.01, significant difference from corresponding control normoxic value (analysis of variance). †*P* < 0.05 and ††*P* < 0.01, significant difference from corresponding control normoxic value (Mann-Whitney *U* test).

hypoxia-induced responses. Interestingly, five separate binding sites for members of the CREB family were found (Table 1). To test the putative role of CREB during pulmonary hypoxia suggested by our bioinformatics analysis, we examined CREB and

ATF1 phosphorylation in a separate series of experiments. Both transcription factors showed a basal level of phosphorylation under normoxic conditions that was increased after hypoxic exposure (Figure 2), with peak values observed after 24 hours. This finding

Figure 5. Analysis of gene expression in the lungs and a panel of systemic organs from hypoxic mice. Real-time polymerase chain reaction analysis of ATF3, endothelin (EDN)-1, follistatin (FST), and vascular endothelial growth factor receptor (VEGFR)-1 mRNA from lungs, hearts, livers, kidneys, spleens, and thymus of mice exposed to normobaric normoxia (21% O₂) or hypoxia (10% O₂) for 2 days. Results are expressed normalized to 18S RNA (n = 4 in each group) and values are shown as means ± SEM. **P* < 0.05, significant difference from corresponding control normoxic value (analysis of variance).

Figure 6. Hypoxia-dependent activation of cAMP response element binding (CREB) and CREB-dependent gene expression in primary human lung microvascular endothelial cells. (A) CRE-specific DNA binding of serine-133 phosphorylation of CREB (pCREB) from human primary lung and cardiac microvascular endothelial cells exposed for various time points (2–24 h) to normoxic or hypoxic (5% O₂) conditions. Results were expressed as fold over normoxic levels (n = 3–7 groups), with values displayed as means ± SEM. (B) Real-time polymerase chain reaction analysis of ATF3, EDN-1, and VEGFR-1 mRNA from primary human lung and cardiac microvascular endothelial cells exposed for 6 and 24 hours to normoxic or hypoxic (5% O₂) conditions. Results are expressed as fold over control normoxic levels (n = 4 groups), and values are displayed as means ± SEM. *P < 0.05 and **P < 0.01, significant difference from corresponding control normoxic value (analysis of variance).

directly validated the central role of CREB family members suggested by the initial bioinformatics analysis. Moreover, in three systemic organs harvested from the same mice (liver, kidney, and spleen), neither of these transcription factors showed increased phosphorylation, suggesting that the response was lung specific.

Within the early responding cluster, 21 of the 137 genes had putative CREB family binding sites (Table 2), of which nine were previously reported to be hypoxia responsive, including EDN-1, which plays a key role in the development of hypoxic pulmonary hypertension (21). For seven of these genes, there was previously published evidence indicating CREB responsiveness (Table 2). We chose four genes from this list for validation by real-time PCR; two of these, EDN-1 and VEGFR-1, were chosen because they were known to be HIF responsive (HIF-1 α /HIF-1 β heterodimer) and up-regulated by hypoxia in the lung (21, 22). Two further genes, ATF3 and FST, were chosen because they were known to be CREB responsive, but had not previously been shown to be HIF responsive (23, 24). In the data presented here, all four of these exemplary genes showed significant increases in expression early in the time course of hypoxic exposure (Figure 4), confirming these important responses *in vivo*.

Our initial hypothesis predicted that the CREB-dependent genes that we had identified should show selective, or at the least preferential, up-regulation in the hypoxic lung *in vivo*. To test this prediction independently of the data set (and mRNA) that had initially identified the CREB family of transcription factors as important, we undertook further independent experiments using separate groups of mice (i.e., not those used in the initial gene

expression studies) and confirmed hypoxia-induced up-regulation in the lung in the absence of changes in expression in five systemic organs: the heart, liver, kidney, spleen, and thymus (Figure 5). These data strongly support the central role of this family of transcription factors in mediating the lung-specific hypoxia responses.

Given the key role of the endothelium in hypoxic pulmonary vascular remodeling, hypoxic vasoconstriction, and the development of pulmonary hypertension, we examined CREB and ATF1 activation and the expression of CREB-dependent genes in human primary microvascular endothelial cells in response to hypoxia and compared these responses to those observed in cardiac microvascular endothelial cells. Our finding that CREB and ATF1 activation were observed only in the pulmonary endothelial cells supports the *in vivo* observation that this response is specific to the pulmonary tissue. Moreover, three of the four exemplary CREB-responsive genes were selectively up-regulated by hypoxia in the pulmonary endothelial cells (Figure 6), suggesting that CREB family activation was functionally important. These findings in isolated endothelial cells show that the responses were not secondary effects *in vivo*, but that they were due to a direct effect of hypoxia on the cells.

Lung-selective CREB Activation

It has been previously established that CREB is a hypoxia-responsive transcription factor, although in ways that differ very substantially from the activation that we report here. In cell culture models, levels of total CREB are reduced by hypoxia with consequent change in CREB-responsive gene expression (25, 26).

That effect is in marked contrast to the serine-133 phosphorylation and activation of CREB that we report here (in the absence of any change in total CREB levels).

The data presented here expand in another important way upon previously published work. Previous *in vivo* work has shown hypoxia-induced CREB phosphorylation in nonpulmonary tissues, but this has only been shown in response to the extreme hypoxia caused by vascular occlusion (ischemia) (27). The present findings are markedly different in that we have demonstrated lung-selective phosphorylation of CREB on serine-133 at very modest levels of hypoxia (40–60 mm Hg), typical of those seen in the pulmonary alveoli during the course of lung diseases (2). In addition, our data also demonstrate, for the first time in any *in vivo* tissue, phosphorylation of ATF1 induced by hypoxia. These findings show that there are lung-specific mechanisms responsible for CREB and ATF1 activation in the conditions of relatively mild hypoxia characteristic of lung disease.

Conclusions

In summary, by combining a novel modification of a bioinformatics approach with the use of an *in vivo* model of pulmonary hypoxia, the results of the present study identify a set of transcription factor binding sites that are overrepresented within the promoters of hypoxia-responsive genes in the lung. We show that one family of these transcription factors, the CREB family, was selectively activated in the lung at levels of hypoxia that mimic those found in lung diseases. Furthermore, we demonstrate that CREB-responsive genes were up-regulated in the lung in response to such hypoxia, whereas their expression was unchanged in five systemic organs. Members of the CREB family of transcription factors were also activated, and CREB-dependent genes up-regulated, in hypoxic primary human microvascular endothelial cells *in vitro*, but not in cardiac microvascular endothelial cells cultured under the same hypoxic conditions.

These findings suggest that the CREB family of transcription factors plays a key role in mediating the specific responses of the pulmonary circulation to hypoxia. These data also suggest that pulmonary microvascular endothelial cells are important, proximal effector cells in the specific hypoxic responses of the pulmonary circulation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Hopkins N, McLoughlin P. The structural basis of pulmonary hypertension in chronic lung disease: remodelling, rarefaction or angiogenesis? *J Anat* 2002;201:335–348.
- Kessler R, Faller M, Weitzenblum E, Chaouat A, Aykut A, Ducoloné A, Ehrhart M, Oswald-Mammoser M. “Natural history” of pulmonary hypertension in a series of 131 patients with chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:219–224.
- Stenmark KR, McMurtry IF. Vascular remodeling versus vasoconstriction in chronic hypoxic pulmonary hypertension: a time for reappraisal? *Circ Res* 2005;97:95–98.
- McLoughlin P, McMurtry I. Counterpoint: chronic hypoxia-induced pulmonary hypertension does not lead to loss of pulmonary vasculature. *J Appl Physiol* 2007;103:1451–1453. [Discussion, pp. 1453–1454.]
- Hyvelin JM, Howell K, Nichol A, Costello CM, Preston RJ, McLoughlin P. Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. *Circ Res* 2005;97:185–191.
- Nagaoka T, Morio Y, Casanova N, Bauer N, Gebb S, McMurtry I, Oka M. Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* 2004;287:665–672.
- Abe K, Tawara S, Oi K, Hizume T, Uwatoku T, Fukumoto Y, Kaibuchi K, Shimokawa H. Long-term inhibition of Rho-kinase ameliorates hypoxia-induced pulmonary hypertension in mice. *J Cardiovasc Pharmacol* 2006;48:280–285.
- Hyvelin JM, O'Connor C, McLoughlin P. Effect of changes in pH on wall tension in isolated rat pulmonary artery: role of the RhoA/Rho-kinase pathway. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L673–L684.
- Aaronson PI, Robertson TP, Ward JP. Endothelium-derived mediators and hypoxic pulmonary vasoconstriction. *Respir Physiol Neurobiol* 2002;132:107–120.
- Carmeliet P. Cardiovascular biology: creating unique blood vessels. *Nature* 2001;412:868–869.
- Christofidou-Solomidou M, Kennel S, Scherpereel A, Wiewrodt R, Solomides CC, Pietra GG, Murciano JC, Shah SA, Ischiropoulos H, Albelda SM, *et al.* Vascular immunotargeting of glucose oxidase to the endothelial antigens induces distinct forms of oxidant acute lung injury: targeting to thrombomodulin, but not to PECAM-1, causes pulmonary thrombosis and neutrophil transmigration. *Am J Pathol* 2002;160:1155–1169.
- Cleaver O, Melton DA. Endothelial signaling during development. *Nat Med* 2003;9:661–668.
- Howell K, Preston RJ, McLoughlin P. Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. *J Physiol* 2003;547:133–145.
- Tsai AG, Friesenecker B, Mazzoni MC, Kerger H, Buerk DG, Johnson PC, Intaglietta M. Microvascular and tissue oxygen gradients in the rat mesentery. *Proc Natl Acad Sci USA* 1998;95:6590–6595.
- Leong CL, Anderson WP, O'Connor PM, Evans RG. Evidence that renal arterial-venous oxygen shunting contributes to dynamic regulation of renal oxygenation. *Am J Physiol Renal Physiol* 2007;292:1726–1733.
- Archer SL, Wu XC, Thébaud B, Nsair A, Bonnet S, Tyrrell B, McMurtry MS, Hashimoto K, Harry G, Michelakis ED. Preferential expression and function of voltage-gated, O₂-sensitive K⁺ channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction: ionic diversity in smooth muscle cells. *Circ Res* 2004;95:308–318.
- Ho Sui SJ, Mortimer JR, Arenillas DJ, Brumm J, Walsh CJ, Kennedy BP, Wasserman WW. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. *Nucleic Acids Res* 2005;33:3154–3164.
- Jeffery IB, Madden SF, McGettigan PA, Perrière G, Culhane AC, Higgins DG. Integrating transcription factor binding site information with gene expression datasets. *Bioinformatics* 2007;23:298–305.
- Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature* 2005;434:338–345.
- Wang F, Shi S, Zhang R, Hankinson O. Identifying target genes of the aryl hydrocarbon receptor nuclear translocator (Arnt) using DNA microarray analysis. *Biol Chem* 2006;387:1215–1218.
- Yamashita K, Discher DJ, Hu J, Bishopric NH, Webster KA. Molecular regulation of the endothelin-1 gene by hypoxia: contributions of hypoxia-inducible factor-1, activator protein-1, GATA-2, and p300/CBP. *J Biol Chem* 2001;276:12645–12653.
- Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes: Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* 1997;272:23659–23667.
- Ameri K, Hammond EM, Culmsee C, Raida M, Katschinski DM, Wenger RH, Wagner E, Davis RJ, Hai T, Denko N, *et al.* Induction of activating transcription factor 3 by anoxia is independent of p53 and the hypoxic HIF signalling pathway. *Oncogene* 2007;26:284–289.
- Winters SJ, Ghooray D, Fujii Y, Moore JP Jr, Nevitt JR, Kakar SS. Transcriptional regulation of follistatin expression by GnRH in mouse gonadotroph cell lines: evidence for a role for cAMP signaling. *Mol Cell Endocrinol* 2007;271:45–54.
- Taylor CT, Fueki N, Agah A, Hershsberg RM, Colgan SP. Critical role of cAMP response element binding protein expression in hypoxia-elicited induction of epithelial tumor necrosis factor- α . *J Biol Chem* 1999;274:19447–19454.
- Taylor CT, Furuta GT, Synnestvedt K, Colgan SP. Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. *Proc Natl Acad Sci USA* 2000;97:12091–12096.
- Nagy N, Shiroto K, Malik G, Huang CK, Gaestel M, Abdellatif M, Tosaki A, Maulik N, Das DK. Ischemic preconditioning involves dual cardio-protective axes with p38MAPK as upstream target. *J Mol Cell Cardiol* 2007;42:981–990.