Hypoxia induces a hedgehog response mediated by HIF-1α

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Abstract

Recently, it has become clear that the developmental hedgehog pathway is activated in ischaemic adult tissue where it aids in salvaging damaged tissue. The exact driving force for the initial hedgehog response is unclear and as most physiological and cellular processes are disturbed in ischaemic tissue, hedgehog-activating signals are hard to dissect. Here, we demonstrate that hypoxia per se is able to induce a rapid systemic hedgehog response in adult mice, as evident from expression of the pathway ligand, Sonic hedgehog, as well as the pathway activity marker Patched1 in various organs. Using in vitro models of hypoxia, we showed that the hedgehog response was transient and preceded by the accumulation of HIF-1α, which we hypothesized to communicate between hypoxia and hedgehog expression. Indeed, pharmacological inhibition, knockdown or genetic ablation of HIF-1α abolished hedgehog pathway activation. In conclusion, we have established that hypoxia is translated into a hedgehog response through HIF-1α and this mechanism is likely to be responsible for the hedgehog response observed in various ischaemia models.

Keywords: fibroblasts • hedgehog • HIF-1α • hypoxia

Introduction

The hedgehog (Hh) family of proteins [1] is important in many patterning events in the developing embryo, for instance in determining digit position [2] and the development of the nervous system [3]. Hh signal transduction is highly unusual and some of its pathway components act in a rather unique manner [1]. As shown in Fig. 1A, the Hh pathway contains two receptors, i.e. the 12-pass transmembrane receptor Patched 1 (Ptch1) [4] and the 7-pass transmembrane receptor Smoothened (Smo) [5]. When the pathway is active, due to binding of Shh to Ptch1 (thereby alleviating its inhibitory action on Smo), target gene transcription is initiated through the Gli family of transcription factors [6]. One of these target genes is Ptc1, creating a negative feedback loop [7], and increased expression of Ptch1 is thus indicative of pathway activity. Assaying Ptch1 levels thus enables to analyse tissue responses to generated Shh.

Recently, it has become clear that the Hh pathway is not only active in the developing embryo, but also in the adult organism. Intestinal tract morphostasis is critically dependent on proper Hh signalling [8] and Hh functions in a range of beneficial processes to salvage damaged tissue in different ischaemia models [9–13]. In a kidney ischaemia-reperfusion model, up-regulation by Shh was shown in reperfusion model, and a link with HIF-1α/H9251 was suggested but not mechanistically established [11]. Conversely, in a developmental model, a role for HIF-1α in inducing Shh expression was established by the use of a pharmacological inhibitor, however the implications of this for adult physiology were not addressed [14].

Recent work by Kusano et al. has shown the involvement of Hh in myocardial ischaemia in the adult organism, and the use of Shh cDNA enabled the authors to reduce apoptosis in the afflicted area, to recruit progenitor cells, and to enhance tissue vascularization [10]. The salvage experiments using Shh protein or DNA in the abovementioned papers look like promising therapeutic options but although the authors provide an in-depth description of the involvement of Shh in their experiments, the exact mechanism driving Shh expression during ischaemia remains unknown. In this study, we set out to examine whether hypoxia per se could induce Hh up-regulation and to elucidate the mechanism behind this response.

The response to hypoxic conditions is usually mediated through the hypoxia-inducible factors (HIFs, reviewed in [15]). Under normal oxygen conditions, the HIF-1α transcription factor is
constitutively synthesized, but also subject to degradation thereby keeping HIF-1α protein levels low. Under low oxygen conditions however, the degradation is impaired and HIF-1α levels rise. After binding to the constitutively present HIF-1β subunit, the HIF-1αβ complex binds to consensus sequences present in the promoter region of hypoxia responsive genes to initiate their transcription. HIF target genes are generally involved in processes like angiogenesis and cell survival, and as previously mentioned, possibly Shh as well [11, 14]. In this study, we elucidate the mechanism by which one of the hallmark features of ischaemia, hypoxia, induces an Hh response and shows the requirement for HIF-1α in this Hh response by pharmacological and genetic methods.

Methods

Animals and exposure of mice to hypoxia

C57BL/6 wild-type mice were purchased from Charles River (Zeist, The Netherlands). All mice were housed and fed according to routine procedures and were used at 8–10 weeks of age. The experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands. Mice were exposed to normobaric hypoxia using a custom-made hypoxia-chamber containing an oxygen sensor (Marin Assist, Hazerswoude, The Netherlands). Mice were placed in the hypoxia chamber and the oxygen level was lowered to 8% within 1 hr. After 16 hrs of 8% O2, animals were killed by bleeding from the vena cava inferior after being anaesthetized by intraperitoneal injection of FFM ((1:1:2 hypnorm (Janssen Pharmaceutica, Beerse, Belgium), dormicium (Roche, Mijdrecht, The Netherlands), sterile water for injection (Braun Melsungen AG, Melsungen, Germany); 0.1 ml per 10 grams body weight). Immediately before the mice were killed, body temperature (intrarectal) and weight were measured, pO2, pCO2, HCO3−, haemoglobin and haematocrit levels of mice exposed to 8% O2 were analysed. Blood was sampled by heart puncture via a lateral approach and analysed using an ABL 505 blood gas analyser and an OSM3 oxymeter (Radiometer, Copenhagen, Denmark).

Materials

The 5E1 Shh-blocking antibody was from the Developmental Hybridoma Bank (Iowa City, IA, USA). Echinomycin was from Alexis Biochemicals (Lausen, Switzerland). Dimethyloxalylglycine (DMOG) and 2-methoxyestradiol (2-ME2) were purchased from Sigma (St. Louis, MO, USA).

Cell culture and exposure of cells to hypoxia

C3H/10T1/2 mouse mesenchymal fibroblasts (from American Type Culture Collection CCL-226, Manassas, VA, USA) were grown in DMEM (Cambrex, East Rutherford, NJ, USA) containing 10% foetal calf serum (FCS; Cambrex). The Shh-LIGHT II mouse fibroblast cell line (ATCC number CRL-2795) was maintained in DMEM with 10% FCS, 0.4 mg/ml G418 and 0.16 mg/ml Zeocin. Hif1α+/− Hras TAg or Hif1α+/− Hras TAg mouse embryonic fibroblasts were kindly provided by Dr. Camenisch and grown as the C3H/10T1/2 cells. H9c2 rat cardiomyoblasts cells [19], ATCC number
CRL1446, were grown in DMEM with 10% FCS. All media were supplemented with L-glutamine. Cells were exposed to normobaric hypoxia using a custom-made hypoxia-chamber and premixed gas containing 1% O2, 5% CO2 and 94% N2 in a normal cell culture incubator. After incubation, cells were quickly lysed for Western blot analysis or luciferase assays.

**Western blotting**

Cells were lysed in Laemmli buffer and brought onto SDS-PAGE gels. After electrophoresis, protein was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 3% Protifar (Nutricia, Zuerterme, The Netherlands) or 5% BSA (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Membranes were blocked in 3% Protifar (Nutricia, Zuerterme, The Netherlands) or 5% BSA (Sigma-Aldrich) in Tris-buffered saline (TBS) for 1 hr. Goat polyclonal α-Pthc1 G-19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 5E1 α-Shh (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), α-Shh (Cell Signaling, Danvers, MA, USA) or α-HIF-1α (Novus Biologicals, Littleton, CO, USA) antibodies were diluted 1:500 (1:1000 for β-actin) in 1% BSA (Sigma-Aldrich) in TBS and membranes were incubated overnight. After 1-hr incubation in 1:1000 α-goat or α-rabbit HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark), blots were imaged using Lumilight Plus ECL (Roche, Basel, Switzerland) on a LAS 3000 Imager (Fuji Photo Film, Tokyo, Japan). Densitometry was performed using the histogram function in Adobe Photoshop 7.0 (mean intensity determined in selection of constant area; Adobe Systems, San Jose, CA, USA).

**Luciferase assays**

Cells were transfected using Effectene (Qiagen, Hilden, Germany) according to the manufacturer’s protocol in 12-well plates (Greiner Bio-One, Kremsmünster, Austria) using 0.5 μg DNA/well. Transfection efficiency for H9c2s was 85% and 70% for the HIF1-α knockout and wild-type MEFs. After transfection and subsequent treatment, cells were lysed with passive lysis buffer as provided by Promega and luciferase activity was assayed according to the Promega Dual-Glo Luciferase Assay System protocol on a Lumat Berthold LB 9501 Luminometer. Firefly luciferase values were corrected for co-transfected Cytomegalovirus (CMV)-driven Renilla luciferase standard to correct for transfection efficiency or dilution effects. Values were expressed as percentage difference to control conditions.

**Transfections**

Transfections of pre-designed siRNA (control and Silencer HIF-1α siRNA; ID#158954), obtained from Ambion Inc. (Austin, TX, USA), were performed using RNAiFect (Qiagen) according to the manufacturer’s protocol and as described previously [29]. Cells were incubated with transfection complexes for 16 hrs, after which fresh medium was added for another 6 hrs preceding further experimentation.

**Cell viability assay**

Cells were seeded in 96-well plates and treated with actinomycin D for 6 hrs, during the last 2 hrs, 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) was added and subsequently cells were lysed in isopropanol and absorbance was read at 560 nm.

**Quantitative RT-PCR**

After hypoxia, H9c2 cells were lysed in Trizol (Invitrogen, Carlsbad, CA, USA) and RNA was isolated according to manufacturer’s protocol. After cDNA synthesis, LightCycler 480 System (Roche Applied Science, Penzberg, Germany) quantitative PCR was performed with the primers described in [14].

**Statistics**

Statistical analyses were conducted using GraphPad Prism version 4.00, GraphPad software (San Diego, CA, USA). Data are expressed as means ± S.E.M. Comparisons between two conditions were analysed using two-tailed unpaired t-tests. *, P < 0.05; **, P < 0.01; ***, P < 0.005.

**Results**

**Hypoxia induces a widespread Shh response in mice**

To study the possible involvement of the Hh pathway in hypoxia, mice were subjected to decreasing concentrations of oxygen. In contrast to previous observations [16–18], the threshold of non-lethal hypoxia was 8% O2 in our hands. The previously reported level of 5 or 6% O2, which was claimed not to interfere with normal mouse behaviour, caused immediate death in our animals. At the observed threshold of 8% O2, the mice became severely hypothermic within 1 hr. Furthermore, the hypoxic mice showed signs of dyspnea and did not eat, drink (resulting in reversible weight loss of 14.43% ± 0.17 [mean ± S.E.M.] after 24 hrs of hypoxia) or move about normally. Within minutes after re-exposure to ambient oxygen levels, body temperature and behaviour normalized (data not shown). Confirmation that lowering ambient oxygen levels to 8% reduces systemic oxygen levels was obtained from blood gas measurements, arterial pO2 decreased 2.6-fold from 90 mmHg to 35 mmHg, as a consequence of lowering ambient O2 levels from 21 to 8%. Venous oxygen levels were 12 mmHg during hypoxia. In addition, pCO2 and HCO3− values were severely reduced, suggesting a metabolic acidosis. Together, these data indicate that the treated mice suffer from quite a severe state of systemic hypoxia.

To evaluate the activation of the Hh pathway in hypoxic tissue, we performed Western blot analysis for Shh on whole organ homogenates from mice exposed to hypoxia for different time periods. Figure 1B shows increased expression of Shh in spleen, liver, brain and to a lesser extent in lung following hypoxia. This is unexpected, as the expression of Shh in the adult organism is anticipated to be rather limited. However, the observed Shh response to hypoxia is reminiscent of the up-regulation seen in ischaemia experiments and might provide a mechanistic explanation for these previous findings.
Quantification of Western blot analysis for Ptch1 (Fig. 1C) shows Ptch1 up-regulation in the analysed organs, and although liver does not show increased Ptch1 expression, Ptch1 is induced in lung, spleen and brain. Thus, these organs not only generate Shh in response to hypoxia, but also respond to it by activation of the Hh pathway, leading to Ptch1 expression.

**Normobaric hypoxia induces a Shh response in an in vitro model for hypoxic cardiac muscle**

The abovementioned data do not explain the exact mechanism by which Shh expression is triggered by hypoxia, and to be better able to assess the mechanism behind this Hh response, we employed an in vitro model system to study Hh pathway activation. Rat cardiomyoblast cells (H9c2 [19]) were subjected to low oxygen levels (1% normobaric) in an airtight thermostated chamber and rapidly lysed to avoid any reperfusion effects.

After hypoxic stimulation, cells were lysed and immunoblotted. As expected, HIF-1α levels rose quickly after exposure to low oxygen levels (Fig. 2A, upper panel). Within 1 hr, maximal levels were reached. Together with the accumulation of HIF-1α, an increase in mature Shh protein levels was observed (lower panel), peaking about 1 hr later than HIF-1α. In response to the expressed Shh, Ptch1 levels were also elevated. After 6 hrs of hypoxia, levels of the analysed proteins had normalized. Densitometric quantification and statistical justification of Western blots as shown in Fig. 2A is shown in Fig. 2B. The kinetics of these results predict that hypoxia leads to rapid HIF-1α accumulation, which initiates Shh transcription and translation, leading to pathway activation, and as a negative feedback, Ptch1 expression. To determine if the rather rapid up-regulation of Shh depends on de novo protein synthesis rather than enhanced protein processing, we analysed the level of Shh transcripts in H9c2 cells following hypoxia. Quantitative real-time RT-PCR revealed a robust transcription of Shh mRNA within an hour of hypoxia that rapidly declined in time (Fig. 2C). This suggests that the observed Shh protein up-regulation observed in Fig. 2B is indeed caused by enhanced protein expression.

To formally confirm activation of the Hh pathway transcription factors in the cardiomyoblasts, these cells were transfected with a Gli-reporter construct and exposed to 1% O2 or stimulated with 300 ng/ml Shh for times indicated. After lysis, luciferase activity was assayed and values were expressed as percentage increase relative to t = 0. Mean +/- S.E.M. is shown, n = 3.
Using Western blot analysis of H9c2 lysates together with a concentration range of commercially available N-terminal Shh, the intracellular concentration of Shh in the cells after 2 hrs of hypoxia was estimated to be about 10 pg/10^6 cells.

Normobaric hypoxia induces a Shh response in fibroblasts

To be able to use embryonic fibroblasts from mice knockout for relevant genes, we first needed to confirm that the Hh pathway is also activated in fibroblasts in response to hypoxia as observed in the myocardioblasts. The Hh pathway activity in response to hypoxia was assessed in a murine fibroblast cell line routinely used for Hh research (NIH-3T3 fibroblasts) stably transfected with the abovementioned Gli-reporter construct (Shh-LIGHT II [21]). As shown in Fig. 3A, the fibroblasts respond to hypoxia by activating the Hh pathway, suggesting that these fibroblasts are a good model for studying hypoxia-induced Hh responses.

To confirm that Shh expression and secretion is mediating the Hh pathway response to hypoxia, Hh pathway activation to hypoxia was determined in the abovementioned Shh-LIGHT II cells pre-treated with 1 μg/ml 5E1 Shh-blocking antibody, IgG control, or no antibody and exposed to 1% O2 for 120 min. Antibodies were present in the medium throughout the entire experiment. After lysis, luciferase activity was assayed and expressed as described for Fig. 2C. Mean+/−S.E.M. is shown, n = 6.

HIF-1α mediates the Shh response to hypoxia

Classically, cells sense hypoxic conditions through HIF-1α. HIF-1α accumulates in response to hypoxia and mediates the transcription of various target genes. An *in silico* analysis of the mouse Shh promoter region revealed HIF-1α consensus sequences (5’ tacgtgct 3’) at positions −61 to −53 (taaccgtc)
and −1058 to −1050 (tacctgcc) with a 75% match. To test if HIF-1α accumulation is required for the Shh response to hypoxia, we pre-treated Shh-LIGHT II cells with a panel of inhibitors known to inhibit HIF-1α accumulation, including 2-ME2 [23]. 2-ME2 is a pharmacological inhibitor that depolymerizes microtubules and thereby blocks HIF-1α nuclear accumulation in response to hypoxia [24] and thus paralyses the protein in acting as a transcriptional activator. As can be seen in Fig. 4A, pre-treatment of cells with 30 μM 2-ME2 was able to abrogate Hh pathway activation following hypoxia. Although this inhibitor is widely used as an inhibitor of HIF-1α, it is also known to interfere with other processes dependent on microtubule dynamics [24], and we therefore used another inhibitor known to affect HIF-1α activity in a different way. By using echinomycin, an inhibitor of HIF-1α binding to DNA, we were able to abrogate the activation of the Hh pathway response to hypoxia as well [25]. As both these inhibitors abrogated Hh pathway activity at least some specificity for HIF-1α inhibition could be argued.
By stabilizing HIF-1α under normoxic conditions using the proline hydroxylase inhibitor dimethyloxalylglycine (DMOG [26]), we could observe Hh pathway activation, further confirming the observed induction of Hh pathway activation under hypoxic conditions to be dependent on HIF-1α (Fig. 4A).

A more conclusive model to study the requirement for a specific protein is provided by cells from mice knockout for the gene encoding that protein. To confirm the necessity of HIF-1α in hypoxia-induced expression of Shh, we subjected fibroblasts from mice knockout or wild-type for HIF-1α (Hif1a<sup>-/-</sup> Hras TAg or Hif1α<sup>-/-</sup> Hras TAg mouse embryonic fibroblasts [27]) to hypoxia and determined Shh protein levels. As shown in Fig. 4B, Shh levels were raised by low oxygen conditions in fibroblasts from wild-type mice, whereas fibroblasts from HIF-1α knockout mice were incapable of this response. This confirms that indeed, HIF-1α is required for Shh expression in response to hypoxia.

To assess the requirement for HIF-1α in the activation of the Hh pathway transcription factors by hypoxia, we employed Gli-reporter transfection of the abovementioned fibroblasts from mice knockout or wild-type for HIF-1α (Fig. 4C). In the absence of HIF-1α, no activation of the Hh pathway transcription factors could be observed, providing definitive evidence that HIF-1α is essential in mediating the Hh response to hypoxia. The requirement for HIF-1α was confirmed with siRNA knockdown of HIF-1α in Shh-LIGHT II cells (Fig. 4D). Following siRNA knockdown of HIF-1α, no induction of Hh pathway activation by hypoxia could be observed.

Figure 4E (left panel) shows the reduction in HIF-1α protein level after siRNA transfection and subsequent hypoxia (the hypoxia was necessary to induce HIF-1α to measurable levels). Although the reduction in protein is definitely not complete, it is enough to abrogate the Hh response as shown by the data from Fig. 4D. Quantification of several silencing experiments is shown. HIF-1α protein levels in the Hif1α<sup>-/-</sup> Hras TAg or Hif1α<sup>-/-</sup> Hras Tag MEFs are shown in the middle panel, showing complete absence of HIF-1α as expected in the knockout cells. Finally, the effect of 2-ME2 on HIF-1α accumulation under hypoxic conditions was confirmed in the rightmost panel. In conclusion, the presented data show that cells are able to respond to an oxygen-poor environment by expression of Shh, mediated by HIF-1α.

Discussion

In this study, we set out to investigate whether one of the most prominent features of ischaemia, i.e. hypoxia, was able to induce an Hh response both in <em>vivo</em> and in <em>vitro</em>. By exposing mice to low oxygen levels, we were able to show a widespread Hh response. Mechanistic studies in cardiomyoblasts and fibroblasts mimicked the effects observed in <em>vivo</em>, and showed that HIF-1α accumulation was required for the Hh response. These findings show that of the plethora of disturbed processes in ischaemic tissue, hypoxia <em>per se</em> is able to induce an Hh response. Based on these data, we propose the following model for Hh action in hypoxic tissue: an occluded vessel restricts blood flow to tissue, which subsequently becomes hypoxic. HIF-1α levels rise due to these hypoxic conditions leading to de novo expression of Shh subsequently activating its pathway. It is tempting to speculate that as a consequence, transcriptional targets of Shh, like among others, vascular endothelial growth factor (VEGF) and the angiopoietins (Ang 1/2) are expressed, thereby leading to tissue vascularization and the reduction of tissue damage. However, this latter notion needs to be confirmed in future experiments.

The proposed model would not only explain the up-regulation of Shh as seen in ischaemia models, but might also have intriguing implications for other conditions in which individuals are exposed to low oxygen levels, for instance flight or high altitude (sometimes leading to significant arterial hypoxia without symptoms), or situations such as COPD or asphyxia. An interesting preliminary observation in this regard comes from pilot experiments involving non-smoking volunteers. After challenging the volunteers with tobacco smoke, isolated neutrophils were found to have almost two-fold higher levels of Ptch1 mRNA within 6 hrs after first inhalation. It should be noted however that tobacco smoke does not only induce hypoxia, but affects many other physiological processes, making the data from these experiments inconclusive and further studies into this phenomenon are planned.

Some issues should be kept in mind while interpreting our data. The murine experiments depicted in Fig. 1 clearly indicate that systemic hypoxia leads to Shh expression but they also indicate that some discrepancy between Shh levels and the subsequent Hh pathway activity in a given organ exists. Especially the fact that Shh protein levels are easily detected in liver of mice subjected to hypoxia whereas no Hh pathway activity is detected in these livers is puzzling. One could however argue that the amount of Shh produced is not sufficient to block all Ptch1 receptors. Alternatively, the majority of hepatocytes might not have a functional Hh pathway and consequently Shh expression does not lead to pathway activation (see also the data on HepG2 cells in [28]). Whatever the explanation however, it does not conflict with the main message of our manuscript.

Another matter that should be regarded while interpreting our data is the speed with which we kill the mice after taking them out of the hypoxic chamber. Although this was performed as quickly as possible, it was done in atmospheric oxygen levels, making short-term reperfusion not a completely unlikely contributor to our <em>vivo</em> models.

In summary, we sought to determine whether hypoxia triggers the Hh pathway, and how. Our data confirm that hypoxia indeed leads to HIF-1α-dependent Shh expression and consequent Hh pathway activity. We suggest that this activation by hypoxia <em>per se</em> might explain the observed Shh expression in the ischaemia models as described by others.

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