Dissection of a Hypoxia-induced, Nitric Oxide–mediated Signaling Cascade

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Befitting oxygen's key role in life's processes, hypoxia engages multiple signaling systems that evoke pervasive adaptations. Using surrogate genetics in a powerful biological model, we dissect a poorly understood hypoxia-sensing and signal transduction system. Hypoxia triggers NO-dependent accumulation of cyclic GMP and translocation of cytoplasmic GFP-Relish (an NFκB/Rel transcription factor) to the nucleus in *Drosophila* S2 cells. An enzyme capable of eliminating NO interrupted signaling specifically when it was targeted to the mitochondria, arguing for a mitochondrial NO signal. Long pretreatment with an inhibitor of nitric oxide synthase (NOS), L-NAME, blocked signaling. However, addition shortly before hypoxia was without effect, suggesting that signaling is supported by the prior action of NOS and is independent of NOS action during hypoxia. We implicated the glutathione adduct, GSNO, as a signaling mediator by showing that overexpression of the cytoplasmic enzyme catalyzing its destruction, GSNOR, blocks signaling, whereas knockdown of this activity caused reporter translocation in the absence of hypoxia. In downstream steps, cGMP accumulated, and calcium-dependent signaling was subsequently activated via cGMP-dependent channels. These findings reveal the use of unconventional steps in an NO pathway involved in sensing hypoxia and initiating signaling.

INTRODUCTION

Oxygen supply can limit the performance of an athlete or the growth of a microorganism. Not surprisingly, evolution has produced remarkable specializations for oxygen delivery and developed adaptive responses to oxygen insufficiency. The limitations of these mechanisms are seen in cardiac infarct and stroke when debilitating damage results from temporary interruption in oxygen supply. Here we explore the activation of a poorly understood hypoxia response pathway that is likely to make important contributions to adaptation to and survival of hypoxic stress.

In mammals, nitric oxide (NO) plays numerous signaling roles contributing to neuronal function, immune defenses and importantly to vascular tone in the circulatory system. Its identification as a vasodilatory molecule (EDRF: endothelial-derived relaxation factor) uncovered the role of NO as a biological regulator, and linked it to physiological activities of tremendous importance to human health (Moncada and Higgs, 2006). Biochemical investigation identified nitric oxide synthase (NOS) and elucidated a signaling pathway in which NO binds to the heme group of the beta subunit of soluble guanylyl cyclase (sGC) to activate cGMP synthesis and subsequently reduce blood vessel tone and blood pressure (Krumenacker *et al.,* 2004). However, this canonical pathway represents only the tip of the iceberg: identification of widespread roles of NO in diverse organisms has led to the realization that it is a general and ancient biological regulator, and multiple pathways of NO genera-

tion and action have been suggested. Much has yet to be learned about these other aspect of NO biology.

In addition to activating sGC through a heme group, NO exhibits other types of interaction that are candidates for mediating aspects of its physiological action. Notably, in a process known as nitrosylation, or nitrosation, NO can modify free sulfydryl (thiol) groups of cysteines in proteins to produce nitrosothiols, SNOs. Transfer of the NO adduct from one sulfydryl to another (transnitrosylation) is likely to play a signal transduction role (reviewed in Stamler *et al.,* 2001). Study of this posttranslational modification, which is proposed to be a widespread mediator of signaling, is a relatively new field, and the list of proteins that are modified through nitrosylation is expanding rapidly. Because NO is highly reactive, transport of an NO signal in tissues can be facilitated through reaction with glutathione and movement of the resulting *S*-nitrosoglutathione (GSNO), which can subsequently signal by modifying thiol groups on target proteins by transnitrosylation (Lipton *et al.,* 2001; Foster *et al.* 2003). The discovery of GSNO reductase (GSNOR), which reduces GSNO to restore GSH and to eliminate the NO adduct as NH_4^+ (Jensen *et al.,* 1998), revealed the importance of the control of this NO metabolite. GSNOR-deficient mice show enhanced sensitivity to both damaging consequences of an excessive NO signal, such as increased sensitivity to endotoxic challenge (Liu *et al.,* 2004), and beneficial effects, such as reduced infarct size after occlusion of a coronary artery (Lima *et al.,* 2009). These findings suggest that GSNO participates in NO signaling and that GSNOR down-regulates signaling. Additionally, stimulation of ventilation by a further metabolite of GSNO (Lipton *et al.,* 2001) and pharmacological activities of S-nitrosothiols (Palmer *et al.,* 2007) revealed the hypoxia-mimetic activity of these compounds and suggested that they have an important role as hypoxia signals.

NO contributes to homeostasis of local oxygen levels in mammals and influences the extent of damage after inter-

ruption in blood supply. Local hypoxia caused by metabolic demand triggers increased blood perfusion to match oxygen delivery with need. This is accomplished by sophisticated adjustments in the involved capillary bed and in the upstream vessels (Segal, 2005). Investigations into this important phenomenon identified multiple signals contributing to this response, including NO, but the signaling mechanisms are imprecisely defined. NO is involved in the shear-dependent vasodilation that appears to contribute to increases in blood flow to hypoxic tissues (Ungvari *et al.*, 2001) and may make additional contributions. However, the means of NO generation during hypoxia remains enigmatic. One enzyme that generates NO, NOS, uses O_2 as a substrate and cannot produce significant NO in severe hypoxia (Leone *et al.*, 1991; Rengasamy and Johns, 1991). In the presence of O_2, NOS activity generates NO, which is unstable and is rapidly oxidized to nitrite (NO_2^- (Ignarro *et al.*, 1993). One intriguing proposal avoids the difficulty of synthesizing NO in low oxygen by suggesting that hemoglobin carries a reservoir of NO in the form of an SNO adduct, generated on the free sulfhydryl group ($-SH$) on a particular cysteine. This NO is released when oxygen tension is low (reviewed in Stamler *et al.*, 2001). However, a recent genetic test failed to identify obvious defects in mice lacking the cysteine residue of hemoglobin that forms the SNO adduct (Isbell *et al.*, 2008). An alternative proposal suggests that nitrite serves as the intravascular storage molecule for NO and that it is converted to NO in hypoxia (Kim-Shapiro *et al.*, 2006; Feelisch *et al.*, 2008). This proposal is in accord with a long-appreciated possibility that NO can be produced by biological reduction of nitrite (Walters and Taylor, 1965; Fulop *et al.*, 1995; Zweier *et al.*, 1995; Millar *et al.*, 1998). Exogenous addition of nitrite contributes to alleviation of hypoxic symptoms (reviewed in Lundberg *et al.*, 2008), but the mechanism by which this occurs remains unclear.

Despite the usefulness of model organisms for genetic dissection of signaling systems, there has been relatively little analysis of NO signaling in these tractable systems. Such models have much to offer because the myriad of signaling cascades influenced by hypoxia complicate analyses and put a premium on the ability to isolate particular signaling events, as can readily be done in model systems. In *Drosophila*, exposure to hypoxia has been associated with events as diverse as changes in gene expression (Zhou *et al.*, 2008), a behavioral response (Wingrove and O'Farrell, 1999), activation of Hypoxia-inducible Factor (Lavista-Llanos *et al.*, 2002), inhibition of transcription and translation (Teodoro and O'Farrell, 2003), and suspended animation of *Drosophila* embryos (Foe and Alberts, 1985; Wingrove and O'Farrell, 1999; Teodoro and O'Farrell, 2003). Although some of these events involve NO (Wingrove and O'Farrell, 1999; Teodoro and O'Farrell, 2003), the pleiotropy of the in vivo response confounded analysis of regulation of this signal. Therefore, we sought a simpler experimental context in which to explore its mechanisms of action. We uncovered a role for NO in the signaling process that activates Relish-dependent innate immune responses in *Drosophila* (Foley and O'Farrell, 2003). Because aspects of the immune response can be induced in *Drosophila* S2 cells (Foley and O'Farrell, 2004) and these cells have been successfully used to genetically dissect the inhibition of translation that occurs in hypoxia, using RNA interference (RNAi; Lee *et al.*, 2008), we explored use of these cells to examine the role of NO in hypoxia.

Drosophila S2 cells expressing green fluorescent protein (GFP)-tagged Relish (GFP-Rel) responded to NO donors in culture by translocating this tagged Rel/nuclear factor κB (NF-κB)-type transcription factor from the cytoplasm to the nucleus (Dijkers and O'Farrell, 2007). This NO-induced translocation depends on calcium mobilization and subsequent activation of a calcium-dependent phosphatase, calcineurin. We showed that this pathway also operates in vivo: inhibition of calcineurin in *Drosophila* larvae interfered with Rel-dependent antimicrobial peptide induction in response to either infection or exposure to an NO donor. This previous work showed that this cell culture offers an assay suited to the functional identification of players downstream of NO. The system also allows convenient use of RNAi, pharmacological modulators, and transgenes (Foley and O'Farrell, 2004; Armknecht *et al.*, 2005). Here, we show that hypoxia also induces GFP-Rel translocation to the nucleus, allowing us to use this model to dissect a cascade of signal transduction from oxygen sensing to molecular output.

MATERIALS AND METHODS

Cells, Flies, Reagents, and Antibodies

Drosophila S2 cells were cultured in *Drosophila* Schneider's medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated fetal calf serum, penicillin, and streptomycin. The S2 cell line stably expressing N-terminally GFP-tagged Rel under the control of the copper-inducible metallothionine promoter has been described (Foley and O'Farrell, 2004).

Transient expression in S2 cells by the Invitrogen protocol (Invitrogen, Carlsbad, CA) used pAc5/V5HisB expression vector. The *Fusarium oxysporum* NOR cDNA (N-terminally modified) was a gift from H. Shoun (University of Tokyo, Tokyo, Japan; Nakahara *et al.*, 1993). NOR constructs were amplified and C-terminally hemagglutinin (HA)-tagged. Amplification of a fragment encoding the first 50 aa of *Drosophila* citrate synthase provided a mitochondrial (mt) localization signal when fused to the N-terminus of NOR (mtNOR). Point mutations (S286V/T243V; Okamoto *et al.*, 1997) were introduced in NOR using the following forward primers: T243: gctggcaacgcagtcatggtaaacatgattgct; S286: tgtcgctaccataccgcggttgcactag. GSNOR (*Fdh, Formaldehyde dehydrogenase*) was amplified from cDNA with corresponding primers without the stop codon and cloned with a C-terminal HA tag. All constructs were verified by sequencing.

We used COXIV mAb from Abcam (Cambridge, MA), rabbit anti-cGMP has been described (de Vente *et al.*, 1987), HA mAb from BabCo (Richmond, CA).

RNAi Experiments

RNAi treatments of gene-specific regions were carried out according to (Clemens *et al.*, 2000) but without serum-starving the cells, using the *Drosophila* RNAi library described in Foley and O'Farrell (2004). For generation of double-strand RNA (dsRNA), a Promega T7 Ribomax kit was used (Madison, WI). Primer pairs that were used for the generation of dsRNA: *NOS*: GATCATGTGGGCATCTT-TCC/CCAGGACCAATCAGAATAATGG; *GSNOR*: AGTGGTGTTGGGACAT-GAGG/ATGCATTCGAAGGTGTAATCG; cGMP-dependent channels: *CG17922*: CAAAGAGGAGGAGGAGGAGGAGG/GTGTTCTCCTTGGTTTGAAAGG; *Cng*: AGCAGAACACACTGTAAGTCGC/TGCCCATTTCAATTATTTCAGC; *CG42260*: catagatggtgatgcagatgc/cacttgggctgaagtaccacc; *cngl*: GATACAAG-GACTCCGTTATGGG/GAGGGTAGTCAAACCTCAAAGC; *lh*: CATATC-CAAGCAGAACTCATCG/GGTGTTGATGGAACTCGACG. For the analysis of efficient knockdown of transcripts, total RNA was isolated using TRIzol (Invitrogen).

GFP-Rel Translocation Experiments and Immunofluorescence

Copper sulfate (0.1 mM) was added to normoxic cells for 6 h to induce GFP-Rel. SNAP (Calbiochem, La Jolla, CA) or 8-Br-cGMP (Sigma, St. Louis, MO) were added to normoxic GFP-Rel cells. After appropriate incubations (e.g., hypoxia), cells were allowed to adhere to Superfrost Plus Gold slides (Fisher Scientific, Hampton, NH) for 2 min, before fixation with 2% formaldehyde, and the percentage of cells displaying nuclear GFP fluorescence was counted using a Leica microscope (Wetzler, Germany). Samples were randomized before counting. The minimum number of cells counted in each individual experiment was 800. Data represent the average of at least three independent experiments. For hypoxic treatments, a 1% O_2 atmosphere was created by continuously perfusing a box with nitrogen under the control of an O_2 sensor (BioSpherics, Redfield, NY), and cells were analyzed after overnight (o/n) incubation. Ca^{2+} scavenger BAPTA-AM, NO scavenger PTIO (2-phenyl-4,4,5,5,-tetramethylinidazoline-1-oxyl-3-oxide), IBMX (3-isobutyl-1-methylxanthine; Sigma), or sGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) were added for 30 min before subjecting the cells to 1% O_2. NOS inhibitor nitro-L-arginine methyl ester (L-NAME) or the inactive isomer *N*-nitro-D-arginine-methylester (D-NAME) was added either 30 min or 48 h
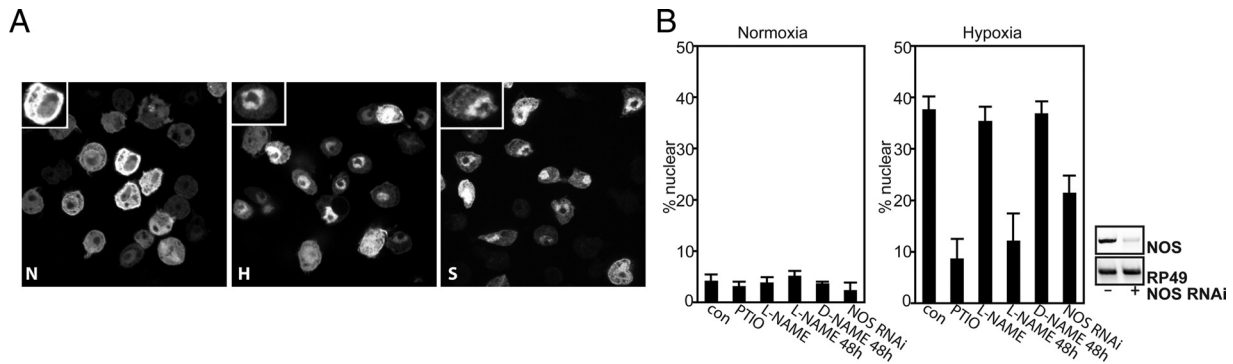
Figure 1. NO is involved in transducing responses to hypoxia. (A) S2 cells expressing GFP-Rel were either left untreated (normoxia, N) or cultured in 1% O_2 for 8 h (hypoxia, H) or in the presence of SNAP (2 mM, S) for 3 h before fixation and analysis for GFP localization. For illustration, insets represent a higher magnification of a cell in the image. (B) Left, GFP-Rel cells were either left untreated (con), treated with PTIO (0.2 mM, 30 min), L-NAME (1 mM, 30 min), L-NAME (1 mM, 48 h), D-NAME (1 mM, 48 h), or with dsRNAi against NOS (5 d) before and during o/n incubation under normoxic circumstances (normoxia), or hypoxia (1% O_2). In this and subsequent figures, the bars are averages of at least three independent experiments; error bars, ±SEM. Right, RNAi against *NOS* knocks down *NOS* mRNA. Cells were treated without or with *NOS* dsRNA, and expression of *NOS* was analyzed by RT-PCR. *RP49* was used as a control for equal input.

before subjecting cells to hypoxia. For antibody stainings, S2 cells adhered to concanavalin A–coated coverslips, fixed in PBS containing 4% formaldehyde (Sigma) for 10 min. Mitochondria were stained using mitotrackerRed (Molecular Probes, Eugene, OR; 50 nM for 30 min) before fixation. DNA was visualized using Hoechst 33258 (Molecular Probes). Images were taken using an Olympus (Tokyo, Japan) microscope driven with Deltavision software (Applied Precision, Issaquah, WA). Images were processed using Adobe Photoshop 7.0 (San Jose, CA).

Western Blotting
Western blotting procedure was previously described in Dijkers and O'Farrell (2007).

RESULTS

NO Is Involved in Transducing Responses to Hypoxia
Previous work demonstrated that GFP-Rel expressed in S2 cells translocates to the nucleus in response to NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP; Dijkers and O'Farrell, 2007), and here we show that it also translocates after reduction in O_2 levels (Figure 1A). The fraction of cells

with GFP-Rel predominantly in the nucleus is easily scored (Figure 1, A and B), providing a robust assay for the contributions of different players to the response.

We tested whether NO is involved in hypoxia-induced translocation (HIT) of GFP-Rel. Exposure of S2 cells to the NO scavenger PTIO inhibited HIT (Figure 1B), suggesting a requirement for NO. Because NOS requires O_2 to generate NO, a role for NOS in hypoxia was in doubt. Indeed, the NOS inhibitor L-NAME had no effect when added immediately before hypoxia or even when added 24 h earlier (not shown). However, after prolonged pretreatment (48 h), L-NAME, but not its inactive isomer D-NAME, inhibited HIT (Figure 1B). In addition, RNAi, a technique routinely and effectively exploited in *Drosophila* S2 cells (Clemens *et al.*, 2000), was used to effectively knock down *NOS* levels (Figure 1B, right). A 5-d pretreatment with *NOS* dsRNA reduced HIT (Figure 1B, left). In support of the specificity of this effect, we also obtained similar reduction of HIT when a nonoverlapping region of *NOS* was used for RNAi (not
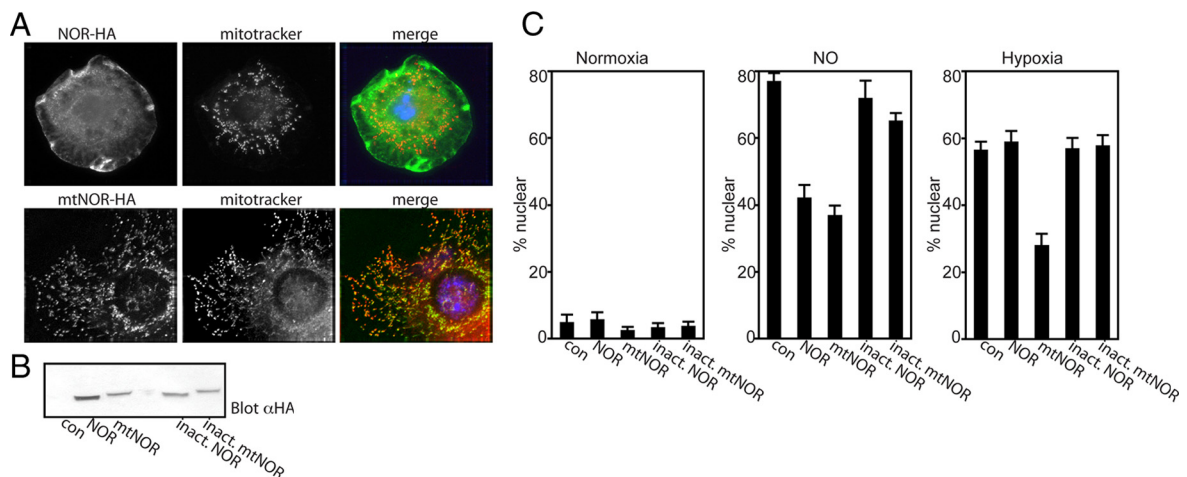

Figure 2. (A) Scavenging of nitric oxide in the mitochondria attenuates responses to hypoxia. Localization of NOR constructs. S2 cells were transfected with NOR-HA or mtNOR-HA construct and stained with mitotracker-Red (50 nM) for 30 min before fixation and staining with anti-HA (green) and DNA staining (Hoechst, blue). (B) S2 cells were cotransfected with GFP-Rel and either empty vector (con), NOR-HA, mtNOR-HA, inactive NOR-HA or inactive mtNOR-HA and analyzed for HA expression by Western blot. (C) Analysis of samples in B for GFP-Rel localization in normoxia or after NO treatment (SNAP, 2 mM, 3 h) or hypoxia (8 h, 1% O_2).

shown). Thus, NOS is required, but the requirement for its prolonged inactivation to have an effect in hypoxia suggests that NOS acts before hypoxia. These results and additional findings reported below suggest that HIT is mediated by NO and provides an experimental setting to examine signal generation and transduction.

Scavenging of NO in the Mitochondria Attenuates Responses to Hypoxia

Because the majority of oxygen use occurs in the mitochondria, it seemed likely that this organelle would participate in the response to reductions in oxygen. To explore this possibility, we wanted to locate the site of NO generation or signaling within the cell during hypoxia. To target NO, we developed a novel tool that uses a fungal NOR (Nakahara *et al.*, 1993) to eliminate NO. Neither *Drosophila* nor mammals possess this enzyme. To analyze NO function in the cytoplasm and mitochondria, we expressed cytoplasmic NOR and mitochondrially targeted NOR (mtNOR) transgenes (Figure 2A). Expression of either of these constructs, but not their catalytically inactive forms (Figure 2B), attenuated nuclear translocation of GFP-Rel in response to SNAP (Figure 2C), demonstrating that fungal NOR is active in *Drosophila* cells. The failure to completely block the NO response might be quantitative, where NOR activity was not sufficient, or some of the NO might have been in an inaccessible form (e.g., SNO; see below). We then examined the influence of NOR on the response to hypoxia. Strikingly, mtNOR attenuated HIT, whereas cytoplasmic NOR and the inactive NOR controls did not. The selective inhibition of the hypoxic response by mtNOR suggests that NO production or signaling occurs in or in association with the mitochondria. This signal needs to be transduced from the mitochondria to the cytoplasm to promote GFP-Rel translocation. Our result suggests that the NO-dependent signal in the cytosol that promotes GFP-Rel translocation is present in a form other than NO itself because it is not sensitive to cytoplasmic NOR.

GSNO Modulates the Response to Hypoxia

One possible means of transporting the highly reactive NO in tissues is in association with glutathione. Nitrosylation of glutathione produces GSNO, which can diffuse and subsequently modify thiol groups on proteins by transnitrosylation or can be broken down to again produce NO (reviewed in Lipton, 2001). GSNOR (also known as glutathione-dependent formaldehyde dehydrogenase) was found to metabolically reduce GSNO (Steffen *et al.*, 2001). In yeast, deletion of *GSNOR* is associated with an increase of nitrosylated proteins in the cell (Liu *et al.*, 2001).

We tested a putative involvement of GSNOR in hypoxia. First we investigated *Drosophila GSNOR* (*Fdh*) RNAi, which should elevate GSNO. Five days of *GSNOR* dsRNA treatment knocked down *GSNOR* RNA, and, upon GFP-Rel induction, was sufficient to drive GFP-Rel into the nucleus in the absence of hypoxia (Figure 3A, top). Similar results were found when another RNAi was used against a different, nonoverlapping region of *GSNOR*. Next, we checked whether this response depended on NO. Indeed, the response to GSNOR knockdown was suppressed by treatments that down-regulate an NO signal, either L-NAME pretreatment (48 h) or PTIO treatment (Figure 3B, □). These findings suggest that in the absence of hypoxia there is a low level of NO that would be adequate to mediate signaling except that GSNOR-mediated degradation of GSNO normally increases the threshold of NO required for signaling. Perhaps a higher level of GSNOR might down-regulate signaling in hypoxia.
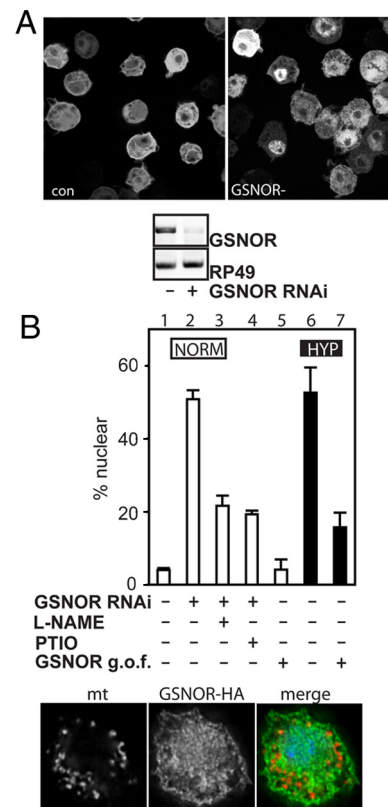

Figure 3. GSNO modulates the response to hypoxia. (A) RNAi against GSNOR promotes translocation of GFP-Rel. (A) Top, GFP-Rel cells were cultured without (con) or with dsRNA against *GSNOR* (GSNOR−) for 5 d before induction of GFP-Rel expression and fixed, and GFP was visualized by fluorescence. Bottom, cells treated as in the top panel were analyzed for *GSNOR* expression by RT-PCR. *RP49* was used as a control for equal input. (B) Top, expression of GFP-Rel was induced (lane 1) or was induced after a 5-d pretreatment with *GSNOR* dsRNA (lanes 2–4). L-NAME was added for the last 48 h of the preincubation (lane 3); PTIO (0.2 mM) was added at the time of GFP-Rel induction (lane 4). Cells were cotransfected with GFP-Rel and GSNOR (lanes 5 and 7) or empty vector (lane 6), without (lane 5) or with subsequent o/n hypoxia incubation (lanes 6 and 7). Cells were fixed, and the percentage of cells displaying nuclear GFP fluorescence was counted. □, normoxic cells; ■, hypoxic cells. Bottom, localization of GSNOR. Cells were transfected with HA-tagged GSNOR, stained for hemagglutinin (HA, green) and mitochondria (mt, red), using a mitochondria-specific antibody, COXIV, and DNA (Hoechst, blue).

To see whether GSNO breakdown can interfere with HIT, GSNOR was overexpressed and was found to block HIT (Figure 3B, ■). To see where GSNO breakdown takes place, we determined the localization of GSNOR and examined the expression of a C-terminally HA-tagged GSNOR transgene (GSNOR-HA). The HA staining was diffusely distributed, without obvious colocalization with a mitochondrial protein (COXIV). We conclude that GSNOR is extra-mitochondrial and hence that the resulting GSNO breakdown by overexpression of GSNOR does not occur in the mitochondria. These observations suggest an involvement of GSNO in hypoxia-induced signal transduction and that GSNO production or its effect depends on NO.

Involvement of cGMP

Because soluble guanylyl cyclases (sGCs) are well-recognized targets of NO and are shown to be activated and to
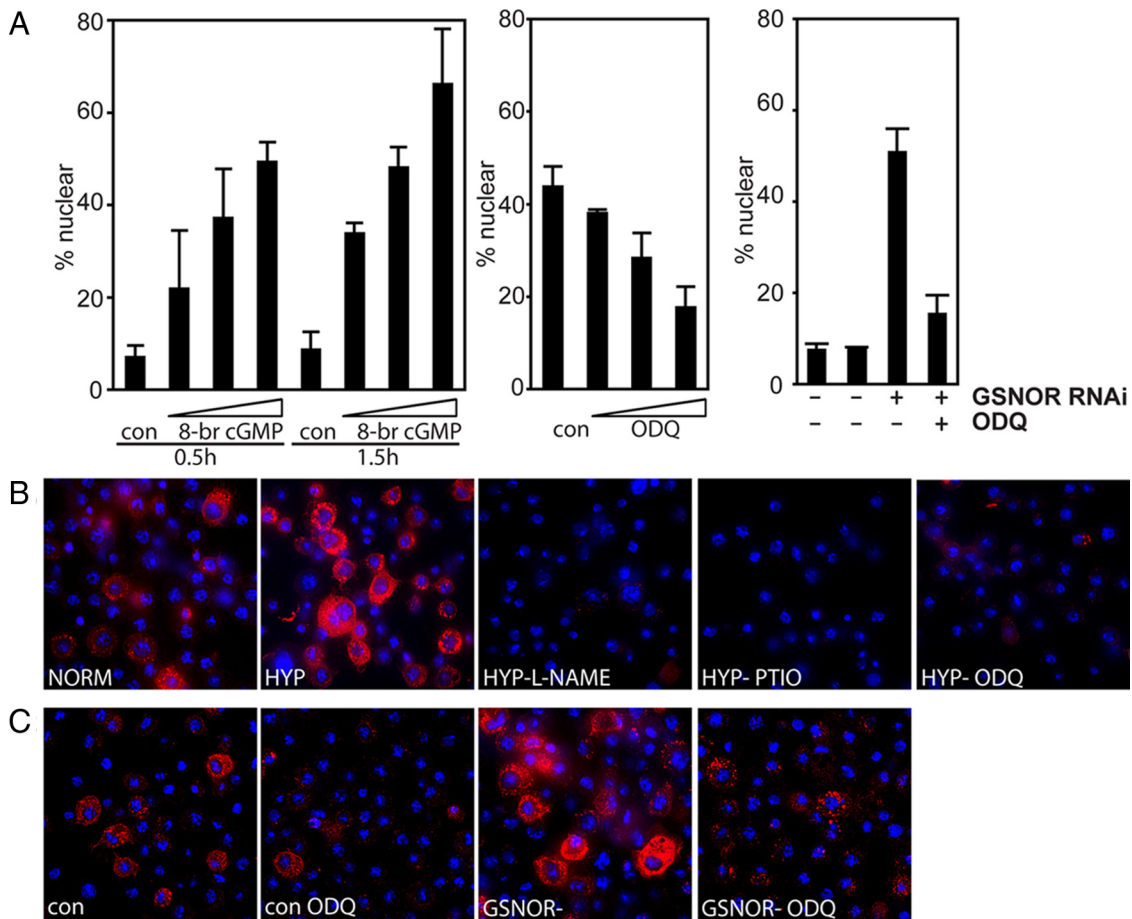
Figure 4. Hypoxia increases cGMP, a necessary and sufficient signal for GFP-Rel translocation. (A) Left, GFP-Rel cells were either left untreated or treated with 8Br-cGMP (0.1, 0.5, or 1 mM) for the times indicated, fixed and analyzed for GFP localization. Middle, GFP-Rel cells were either left untreated or exposed to sGC inhibitor ODQ (50, 10 or 5 μM) for 30 min before exposure to o/n hypoxia, fixation and analysis of GFP localization. Right, control cells (CON) or cells treated with *GSNOR* dsRNA for 5 d (GSNOR−) were left untreated o/n or cultured o/n in the presence of ODQ (50 μM) (con ODQ; GSNOR− ODQ, respectively), and GFP-Rel was localized. (B) S2 cells were cultured under normoxia (NORM) or hypoxia (HYP) in the presence of IBMX (0.2 mM) for 2 h before fixation and staining of cGMP (red) and DNA (blue). The effect of L-NAME (48-h pretreatment HYP-L-NAME), PTIO (HYP-PTIO), or ODQ (HYP-ODQ) on hypoxia-induced cGMP staining was determined. (C) Effect of GSNOR RNAi on cGMP levels in the presence or absence of ODQ. cGMP staining of the cells as in B.

generate cGMP in hypoxia (reviewed in Postovit *et al.,* 2005), we assessed if cGMP would be sufficient to promote GFP-Rel translocation and if sGC inhibition would interfere with HIT. Addition of 8Br-cGMP, a cell-permeable cGMP analog, promoted GFP-Rel translocation in normoxic cells (Figure 4A, left), suggesting that cGMP is sufficient for signaling. Addition of the heme-oxidizing sGC inhibitor ODQ suppressed HIT (Figure 4A, middle), suggesting that sGCs mediates at least part of the hypoxic response. Preliminary data with RNAi against sGC in hypoxia supports this conclusion (not shown). The ability of *GSNOR* RNAi to induce GFP-Rel translocation (Figure 3A) is also inhibited by ODQ (Figure 4A, right), suggesting that GSNO acts through cGMP.

To visualize cGMP generation during hypoxia, we examined staining with a cGMP-specific antibody that has been extensively characterized (de Vente *et al.,* 1987). To eliminate cGMP degradation by phosphodiesterases, phosphodiesterase inhibitor IBMX was included. The cGMP staining revealed modest and variable staining of normoxic S2 cells, and this staining was dramatically elevated in hypoxic cells (Figure 4B). Inhibition of NO signaling by adding PTIO or by pretreating (48 h) with L-NAME blocked the cGMP in-

duction, as did ODQ, suggesting that cGMP elevation in hypoxia is dependent on NO and sGC, similarly to HIT.

To determine whether GFP-Rel translocation induced by *GSNOR* RNAi (Figure 3A) is accompanied by sGC-dependent cGMP elevation, as suggested by Figure 4A, we tested whether *GSNOR* RNAi enhanced cGMP staining. Indeed, *GSNOR* RNAi promoted cGMP staining, and this enhancement was inhibited by ODQ (Figure 4C). These findings indicate that NO mediates induction of cGMP levels in response to hypoxia, and further suggest that GSNO might act as secondary mediator of this effect.

Responses Downstream of cGMP Include Calcium Mobilization via cGMP-dependent Channels

Recently, we examined GFP-Rel translocation following treatment of S2 cells with an NO donor and demonstrated that calcium acts downstream of NO. Cytoplasmic calcium elevation is also sufficient to promote GFP-Rel translocation (Dijkers and O'Farrell, 2007). We expected that calcium would similarly mediate the NO-dependent response to hypoxia. We tested involvement of calcium in HIT and whether it acted downstream of cGMP generation. To this
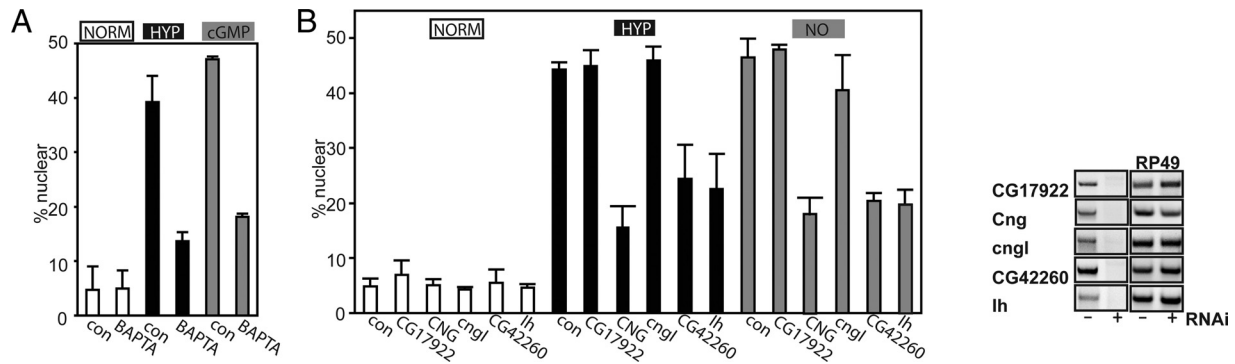
Figure 5. Hypoxia promotes calcium mobilization via cGMP-gated channels. (A) GFP-Rel cells were untreated (con) or treated with Ca²⁺ scavenger BAPTA-AM (25 μM), for 30 min before exposure to hypoxia (o/n, 1% O₂) or addition of 8-Br-cGMP (1 mM, 1.5 h), and GFP localization was determined. (B) Calcium mobilization is mediated via cGMP-gated channels. Left, analysis of RNAi-mediated knockdown of the cGMP-gated channels as in the left panel on GFP-Rel localization in normoxia or hypoxia (1% O₂, o/n). Right, efficacy of dsRNA-mediated knockdown. S2 cells were pretreated with dsRNA against different cGMP-gated channels (*CG17922, CNG, cngl, CG42260*, or *Ih*) for 4 d and mRNA expression was measured by RT PCR. *RP49* was used as a control for equal input. Left, analysis of RNAi-mediated knockdown of the cGMP-gated channels as in the left panel on GFP-Rel localization in normoxia or hypoxia (1% O₂, o/n).

purpose, we preincubated cells with a cell-permeable Ca²⁺ chelator (BAPTA-AM) and subsequently subjected them to either hypoxia or treatment with 8-Br-cGMP. Ca²⁺ scavenging suppressed HIT, as well as 8-Br-cGMP–induced GFP-Rel translocation (Figure 5A). These data show that hypoxia-induced GFP-Rel translocation requires calcium and that this occurs downstream of cGMP.

A study of oxygen-influenced behavior in *Caenorhabditis elegans* identified an sGC, *GCY-35*, and two downstream cGMP-dependent channels, *Tax-2* and *-4*, as components in an oxygen-sensing signal transduction pathway. *Drosophila* has several cGMP-dependent channels homologous to *Tax-2* and *-4*: *CG17922*, *Cng (CG7779)*, *cgnl (CG9176)*, *CG42260*, and *Ih (CG8585)*. We tested putative involvement of these channels in HIT by RNAi. We targeted each of five candidate cGMP-dependent channels with two nonoverlapping dsRNAs that effectively knocked down the levels of the cognate RNA (Figure 5B, right) and tested the effect on GFP-Rel translocation induced by hypoxia or by NO. Knockdown of *Cng (CG7779)*, *CG42260*, or *Ih (CG8585)* attenuated the induced translocation in response to either inducing condition. This suggests that cGMP generation can promote calcium mobilization through the activation of cGMP-dependent channels and that this calcium mobilization occurs downstream of NO signaling.

DISCUSSION

The complexity of an organism's response to hypoxia confounds investigations of NO signal generation and transduction. Here, we describe a cell-based model of hypoxia-induced NO-mediated signaling that is amenable to a surrogate genetic approach using RNAi as well as expression of NO-modulating enzymes. Our data demonstrate the importance for mitochondria for NO signaling during hypoxia and implicate GSNO as a signaling intermediate to promote cGMP generation and subsequent calcium mobilization (Figure 6).

Means of NO Generation and NO Intermediates

NO donors induce GFP-Rel nuclear translocation (Dijkers and O'Farrell, 2007), and induction of translocation by knockdown of GSNOR suggests that GSNO is also an inducer. Reciprocally, the response to hypoxia is blocked by the NO scavenger PTIO, by prolonged treatment of L-NAME, expression of the enzymatic NO scavenger mtNOR, or by scavenging of NO-derived GSNO by GSNOR expression. Thus, NO signaling is sufficient to induce GFP-Rel translo-
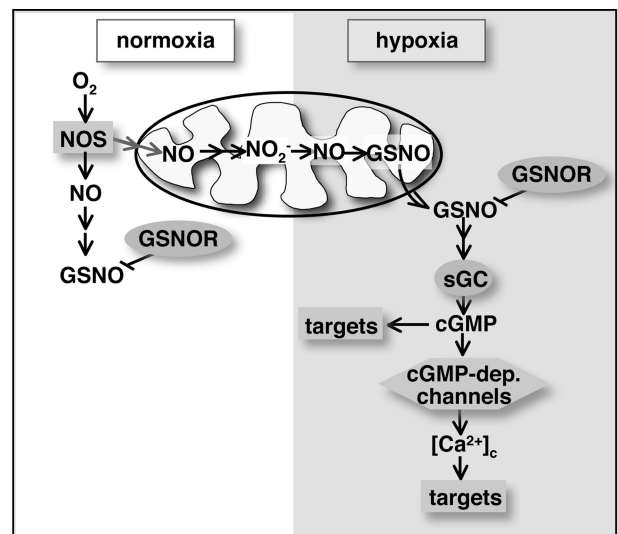

Figure 6. Model for hypoxia-induced signaling. During normoxia (white area), activity of NOS presumably generates a pool of NO, possibly nitrite, which is stored in the mitochondria. During short-falls of oxygen (hypoxia, shaded area), this pool gets converted back to NO. NO is present in the cytoplasm as GSNO. In the cytoplasm, generation of cGMP through sGC subsequently activates cGMP-dependent channels, resulting in mobilization of calcium through these channels. The two most novel steps in this proposed pathway are the production of NO by mitochondrial reduction and the activation of sGC by GSNO. The reductive production of NO by the mitochondrial has been documented in other contexts (Kozlov *et al.*, 1999; Nohl *et al.*, 2000). The activation of sGC by GSNO is suggested by our data and a recently recognized diversity in the mechanisms regulating sGCs leads us to suggest an activating input by transnitrosylation (see *Discussion*). Activation of this signaling pathway may allow adaptation to conditions of hypoxia. The signaling pathway as delineated in this article was defined by our data using S2 cells.

cation and is necessary in the context of the hypoxia response.

The fast-acting NOS inhibitor L-NAME only suppressed HIT if added well in advance (2 days; Figure 1B, not shown) before inducing hypoxia. Thus, NOS, which requires oxygen for activity (Rengasamy and Johns, 1991), acts before hypoxia to satisfy a precondition needed for later response to hypoxia. Because the half-life of NO in the presence of oxygen and sulfhydryls is only seconds, accumulated NO would decay after brief inhibition of NOS. Apparently, NOS activity yields a more stable product. Although this stable product could in theory be due to some unknown activity of NOS, more likely it is an NO derivative such as nitrite (NO_2^-), which results from rapid oxidation of NO (Ignarro *et al.,* 1993), or an SNO. After prolonged L-NAME exposure even these more stable products would decay.

A number of reports suggest that mitochondria can reduce NO_2^- in hypoxia and thereby convert it to NO (Walters and Taylor, 1965; Kozlov *et al.,* 1999; Castello *et al.,* 2006; Feelisch *et al.,* 2008). If this were the means of generating NO in our experimental setting, we would expect the NO to be produced within the mitochondria. Indeed, we find that targeting of the NO-eliminating enzyme NOR to the mitochondria inhibited HIT, whereas there was no effect with cytoplasmic NOR. We conclude that mitochondrial NO contributes to hypoxia signaling. To influence GFP-Rel, the mitochondrial signal needs to be transduced to the cytoplasm.

NO-induced Signaling in the Cytoplasm: Putative Role of S-Nitrosothiols

The failure of cytoplasmic NOR to block HIT suggests that if mitochondrial NO is transferred to the cytoplasm, it must be very rapidly converted to a form that is resistant to NOR. Our findings that *GSNOR* RNAi induced nuclear translocation of GFP-Rel, whereas overexpression of cytoplasmic GSNOR inhibited HIT (Figure 3, A and B) suggest GSNO as an alternative form of the signal in the cytoplasm. This is consistent with nitrosylation of GSH in conjunction with transfer of NO to the cytoplasm. GSNO could transduce the signal by transnitrosylating target proteins (Liu *et al.,* 2001), and GSNOR would down-regulate signaling by removing GSNO (Jensen *et al.,* 1998). This possibility is consistent with recent findings arguing that GSNO is a hypoxia-mimetic signal (Palmer *et al.,* 2007; Lima *et al.,* 2009).

Although the specificity of GSNOR clearly implicates GSNO levels as an important modulator of signaling, it should be noted that GSNO can breakdown to release NO, as well as transfer the NO adduct to other sulfhydryls (Bryan *et al.,* 2004). Accordingly, although the level of cytoplasmic signal might be coupled to GSNO levels, the direct mediator of the signal might not be GSNO itself.

Signaling through cGMP

The most studied target of NO is sGC, and its product cGMP has a well-established role in vasodilation. Our data show that cGMP is induced by hypoxia, that a cGMP analog can induce GFP-Rel translocation, and that a sGC inhibitor (ODQ) inhibits HIT (Figure 4). We conclude that cGMP is an essential signaling intermediate in this hypoxia-signaling pathway.

Interestingly, *GSNOR* RNAi resulted in cGMP generation, which can be blocked by ODQ (Figure 4, A and C). This is the first time that GSNO signaling has been linked to the activation of sGC. We currently do not know if this means that sGC can be activated by transnitrosylation through GSNO or if GSNO is ultimately converted to NO to activate sGC, a route that is not supported by our data with cyto-

plasmic NOR. Activation of sGC through nitrosylation has been reported (Ignarro *et al.,* 1980), although nitrosylation of sGC has also been associated with subsequent inhibition (Sayed *et al.,* 2007).

It is now realized that the family of sGCs exhibit diverse modes of regulation. Recently, atypical soluble guanylyl cyclases in *Drosophila* have been shown to be activated by anoxia, independently of NO, as well as being activated by NO (Morton, 2004; Vermehren *et al.,* 2006). In contrast, *C. elegans*, which does not possess NOS activity, has a member of the sGC family that is activated by O_2 and mediates a behavioral response to hyperoxia (Gray *et al.,* 2004). Our findings that 1) sGC inhibitor ODQ inhibited the effect of either *GSNOR* RNAi (Figure 4A) or SNAP treatment (not shown), and that 2) hypoxia-induced cGMP generation was ablated by either L-NAME pretreatment or PTIO treatment (Figure 4B) suggest NO/GSNO-dependent activation of sGC during hypoxia. However, preliminary evidence using RNAi suggested redundant contributions from the classical and the atypical sGCs to HIT (not shown). Given the diverse regulatory inputs into this family of sGCs it seems plausible that GSNO can act as activator of cGMP production, as suggested by our data.

Previous work in our lab suggested that treatment with an NO donor results in calcium mobilization (Dijkers and O'Farrell, 2007). HIT was blocked by scavenging calcium (Figure 5A). Similarly, the effect with a cGMP analog was blocked, indicating that cGMP can promote calcium mobilization. Calcium mobilization by cGMP can occur through cGMP-gated channels. In *Caenorhabditis elegans*, the cGMP-gated channels *Tax-2* and *-4* are involved in behavioral responses to hyperoxia downstream of sGC (Gray *et al.,* 2004).

Drosophila larvae display a strong behavioral response to hypoxia (Wingrove and O'Farrell, 1999) that is dependent on NO, as well as the cGMP target PKG, but a role for cGMP-dependent channels therein has not been established. Interestingly, we did find a role for several of these channels in HIT (Figure 5B), suggesting that NO generated in hypoxia promotes calcium mobilization through cGMP-dependent channels. The observation that RNAi against several of these channels interfered with HIT may be explained by the fact that they form dimers and thus function together: in *C. elegans*, mutation of either *Tax-2* or *-4* yielded a similar phenotype as did the double mutant (Coburn and Bargmann, 1996). It would be of interest to see whether cGMP-dependent channels are involved in the *Drosophila* behavioral response to hypoxia.

In summary, we provide a system to dissect NO signaling during hypoxia, which originates in the mitochondria and is transduced to the cytoplasm (Figure 6), promoting cGMP generation and subsequent calcium mobilization. The common involvement of NO signaling in responses to hypoxia and findings that GSNO is hypoxia-mimetic suggest that the pathway described here may be used widely.

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