Hypoxia-inducible Expression of a Natural cis-Antisense Transcript Inhibits Endothelial Nitric-oxide Synthase

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The destabilization of endothelial nitric-oxide synthase (eNOS) mRNA in hypoxic endothelial cells may be important in the etiology of vascular diseases, such as pulmonary hypertension. Recently, an overlapping antisense transcript to eNOS/NOS3 was implicated in the post-transcriptional regulation of eNOS. We demonstrate here that expression of sONE, also known as eNOS antisense (NOS3AS) or autophagy 9-like 2 (APG9L2), is robustly induced by hypoxia or functional deficiency of von Hippel-Lindau protein. sONE is also up-regulated in the aortas of hypoxic rats. In hypoxic endothelial cells, sONE expression negatively correlates with eNOS expression. Blocking the hypoxic induction of sONE by RNA interference attenuates the fall in both eNOS RNA and protein. We provide evidence that the induction of sONE primarily involves transcript stabilization rather than increased transcriptional activity and is von HippeL-Lindau but not hypoxia-inducible factor 2α-dependent. We also demonstrate that sONE transcripts are enriched in the nucleus of normoxic cells and that hypoxia promotes an increase in the level of cytoplasmic and polyribosome-associated, sONE mRNA. The finding that eNOS expression can be regulated by an overlapping cis-antisense transcript in a stimulus-dependent fashion provides evidence that sense/antisense interactions may play a previously unappreciated role in vascular disease pathogenesis.

Natural cis-sense/antisense (S/AS)3 pairs are highly prevalent in the human genome (1), yet the functional consequences of interactions between sense and antisense mRNA transcripts are poorly understood. Several studies have implicated a role for antisense-mediated regulation of the sense transcript of S/AS pairs (2–4). For example, a natural cis-antisense transcript to hypoxia-inducible factor 1α (HIF1α) has been suggested to negatively regulate HIF1α during prolonged hypoxia (2). Regulation of the sense transcript by antisense RNA can be exerted at the transcriptional, splicing, nuclear export, or translational level. Importantly, antisense transcripts may also regulate the stability of the sense transcript, although the mechanisms implicated have not been defined. Although several orientations between S/AS transcriptional units are possible, tail-to-tail overlaps involving the 3′-UTR predominate (5). Given the prevalence of cis-S/AS interactions, it is vital to understand whether S/AS interactions between 3′-UTR regions are functionally important and to define their mechanisms of regulation. Indeed, trans-S/AS interactions involving microRNAs clearly demonstrate a bias for the 3′-UTR of target mRNAs (6) and have been demonstrated to regulate both mRNA translation and RNA stability, with the former being especially relevant (7). It is possible that functional interactions between S/AS transcripts may result in the generation of short interfering RNA that have the capacity to target either transcript for RNA interference-mediated degradation, although this phenomenon has yet to be directly observed in mammalian cells. A limitation in further understanding these pathways has been the lack of a tractable and biologically relevant model of 3′-UTR cis-S/AS interactions.

We recently identified a natural cis-antisense transcriptional unit to eNOS/NOS3 (8). sONE, also known as NOS3AS/ APG9L2, is oriented tail-to-tail to NOS3, and sONE mRNA is complementary to the eNOS mRNA for 662 nucleotides. This overlap includes portions of the 3′-UTR of the eNOS mRNA. sONE and eNOS mRNA were reciprocally expressed in cultured cells. Relative to endothelial cells, levels of sONE RNA were found to be elevated in nonendothelial cell types, such as vascular smooth muscle cells (VSMCs), which express very low basal levels of eNOS RNA (8). Overexpression of sONE in endothelial cells, which normally express high levels of eNOS but not sONE, resulted in reduced expression of eNOS protein, suggesting that sONE can negatively regulate the expression of eNOS. In contrast, RNA interference-mediated knockdown of sONE in vascular smooth muscle cells resulted in increased expression of eNOS RNA (8).

A number of groups, including our own, have observed down-regulation of eNOS expression following exposure of
endothelial cells to hypoxia (9–11). eNOS mRNA is constitutively expressed and is highly stable in endothelial cells, with half-life measurements following actinomycin D transcriptional arrest averaging 24–48 h (reviewed in Ref. 12). This is much longer than typical mRNAs (13). Hypoxic stimulation of endothelial cells markedly decreases the stability of eNOS mRNAs (9, 11). The molecular mechanisms responsible for the long half-life of the eNOS transcript in normoxic endothelial cells are not fully understood but are mediated, at least in part, by protein binding to the 3’-UTR of the eNOS mRNA (14–16). The mechanisms responsible for alteration of eNOS RNA stability by hypoxia are not known.

In light of the potential for sONE to regulate eNOS post-transcriptionally, and considering that eNOS RNA is destabilized by chronic hypoxia, it was of interest to determine if sONE plays a functional role in the hypoxic down-regulation of eNOS. Here we describe robust hypoxic induction of sONE mRNA expression in various cell types in vitro and vascular tissues in vivo. sONE was not induced at the transcriptional level but was up-regulated by post-transcriptional stabilization of the sONE transcript. Additionally, sONE expression was increased in the absence of functional VHL activity, but HIF2α was not involved in sONE induction. Importantly, sONE functionally regulates eNOS expression during hypoxia, since RNA interference-mediated ablation of sONE transcripts resulted in increased levels of eNOS mRNA and protein in the setting of hypoxia. To our knowledge, this is the first report of an antisense RNA playing a functional role in regulating gene expression in response to cellular stimulation.

MATERIALS AND METHODS

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were cultured in M199 (Invitrogen) containing 20% fetal bovine serum (HyClone, Logan, UT), 17 units/ml heparin, and 0.05 mg/ml endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA), as described previously (17). VSMC and human dermal microvascular endothelial cells (MVEC), purchased from Clonetics (Cambrex, East Rutherford, NJ), were cultured as recommended by the supplier. Early passage HUVEC (passage 3–4), VSMC (passage 4–8), and MVEC (passage 4–8) derived from multiple donors were used in these studies. Renal clear cell carcinoma epithelial cell lines containing mutant VHL (786-O) stably transfected with hemagglutinin (HA)-tagged wild-type or mutant (C162F) VHL expression cassettes were maintained in RPMI 1640 with GLUTAMAX-I containing G418 and 10% fetal bovine serum, as previously described (18). 786-O cells stably transfected with empty vector or an shRNA cassette targeting HIF2α (19), were cultured in Dulbecco’s modified Eagle’s medium containing puromycin and 10% fetal bovine serum.

Cell Treatment and RNA Extraction—Cells were grown in atmospheric O2 (21%) or in a temperature- and humidity-controlled hypoxia chamber (ThermoForma, Marietta, OH). Less than 1% O2 was maintained by the use of Ultra High Purity gas in a sealed chamber (5% CO2, 10% H2, 85% N2; Praxair, Mississauga, ON). To mimic hypoxia, cells were treated with a 140 μM concentration of the iron-chelator desferrioxamine (DFO) (Sigma). After treatment, RNA was extracted as described previously (17).

Extraction of Nuclear and Cytoplasmic RNA—Nuclear and cytoplasmic extracts were generated from normoxic and hypoxic HUVEC. Cells were washed with phosphate-buffered saline, pelleted, and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, and 0.2 units/μl RNaseOUT (Invitrogen)) for 5 min on ice. Nuclei were pelleted at 300 × g for 2 min. RNA was extracted from the supernatant (cytoplasmic fraction) and the nuclei pellet using guanidinium-based extraction protocols. Validation of the efficiency of nuclear/cytoplasmic partitioning was performed by quantifying levels of unspliced, pre-mRNAs (see below for primer sequences), which are known to be highly enriched in the nucleus due to their short half-life (20).

In Vivo Expression of sONE in Hypoxic Rats—Male Sprague-Dawley rats (200–250 g) were exposed to 10% O2 for 16 h or 7 days, and RNA was extracted from the descending thoracic aorta, as previously described (21). All experiments were performed in accordance with the standards of the Canadian Council on Animal Care, and ethics approval was granted by the institutional animal care committee of the University of Toronto Faculty of Medicine and St. Michael’s Hospital.

Determination of RNA Half-life—HUVEC were grown in normoxic or hypoxic conditions for 16 h, after which the RNA polymerase inhibitor DRB (60 μM; Sigma) was added prior to RNA extraction at various time points following transcriptional arrest. 1 h post-DRB addition was used as time 0, and RNA half-life was assessed by quantifying eNOS and sONE RNA levels following transcriptional arrest using real-time RT-PCR (see below). Half-life was estimated by calculating the exponential decay rate of sONE and eNOS RNA using a line-of-best-fit algorithm in Microsoft Excel.

Quantitative Real Time RT-PCR—2 μg of total cellular RNA was treated with heparinase (1 unit; Sigma-Aldrich) and DNase I (5 units; Fermentas; Burlington, ON) and used in first-strand cDNA synthesis using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. cDNA was diluted to a final volume of 100 μl. All quantitative RT-PCR analyses were performed in triplicate on 2 μl of cDNA using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) using Taqman® or SYBR® green technology (Applied Biosystems), as previously described (8) (See Table 1 for primer sequences). Normalization to the reference genes cyclophilin A, TATA-binding protein (TBP), or 18S yielded similar results. Serial 10-fold dilutions of plasmids corresponding to target amplicons were used to develop standard curves so that copy numbers could be deduced by absolute quantification. For the determination of eNOS (exon 11/12) and sONE (exon 7/8) copy numbers, the cycle at which the exponential portion of the amplification curve for 1000 copies of target amplicon passed through the cycle threshold (CT) = 0.2) was 35.3 ± 0.4 and 31.4 ± 0.3 cycles, respectively. Additionally, relative quantification was determined using the ΔΔCt method (22) with similar results.
Hypoxic Regulation of sONE

| Gene name          | Species | Forward primer (5'→3') | Reverse primer (5'→3') | Probe           |
|-------------------|---------|------------------------|------------------------|----------------|
| NOS3/eNOS         | Human   | GCC ATC ACC AGG AAG AGG| TCA CTC GCT TGG CCA TCA C| FAM-CCA AGG CGG TGA AGA TCT CGG C-BHQ |
| NOS3as/sONE       | Human   | ACC CTG ATG AGG AGA ACG C| TCT GTG GTC ACC TGA AAC CTT| FAM-TGC CTC TAG CCC CAG ACA ACA GTG G-BHQ |
| Nos3as/sONE       | Rat     | GCT TCA GCT TCC TCC CCA| TCT GGG COT CTG TGG TCT CCT G| |
| CD31              | Human   | CAT ATG CAG ACC TCA GAA| TCTCAA AGC AGA AAC CAA G| TGG |
| CD34              | Human   | CAT CAT CTC CCA CTA AAC| CAC TTC TCT GAT GCC TGA ACA TTT| |
| VEGFA             | Human   | GCA GAC CAA AGA AGA ATA| CAC TCG GGC TGG TCA CAT| |
| VEGF              | Rat     | TCG AGG AAA GGG AAA GGG| TTT GCA GCA ACA TTT ACA COT| TAC |
| CXCR4             | Human   | TCA A| CTT G C| |
| GAPD/GAPDH        | Human   | CAA GGA GCC ACC ACC GCA TCT| | |
| CYP2A/CycloA      | Human   | GAC GGC GAC CCC TGG G| TCT GCT TTT GGG ACC TCG T| |
| 18S rRNA          | Human   | AGG AAT TGA CCG AGG GGC| GGA CAT CTA AGG GCA TCA CA| |
| 18S rRNA          | Rat     | GAC GAT AGA TTA AGG AGT T| TGC ACA ATT AGG GAT GGA GTT ATT A| |
| TBP               | Human   | CCG CAG CCT CCG AGA GTC C| GCA ATG GTC TTT AGG TCA AGT TTA| |
| Tbp               | Rat     | CCC CTA TCA TCA CTC GCA| CAC CAG AGT TGT TCG TGG CTC TCT T| |
| eNOS pre-mRNA     | Human   | AGC TCG CCT GAT GAT GAT| GTC C| |
| sONE pre-mRNA     | Human   | GAC CAC GGA CAC CCT CAG| CCA AAA CTC TCA CCC AAC TCA| |
| CD31 pre-mRNA     | Human   | GTC C| |
| CD34 pre-mRNA     | Human   | GTC C| |

Since two distinct sONE transcripts exist, each originating from a unique transcriptional start site (8), real time RT-PCR was used to quantify the basal and hypoxia-inducible copy number of each transcript in HUVEC. Primers for the long transcript were located in AS exon 4 (5'-CTC TCG ATG CGT GGA TTA CAA TG-3' and 5'-GCA CAC TGG GCT GAG GGT AGG A-3'), whereas the exon 7/8 primer set (Table 1) detected total sONE transcripts (i.e. total of short and long transcripts). The copy number of the short sONE transcript was determined by subtracting the number of copies of the long transcript from the total number of transcripts. Copy number was determined by comparison with a standard curve using plasmids containing the ampiclon of interest. Since the AS exon 4 primer set does not cross an exon-exon junction, reverse transcriptase-negative samples were used to ensure the absence of contaminating genomic DNA.

For quantification of unspliced pre-mRNA, total cellular RNA was reverse transcribed as above. Reactions were also performed in the absence of reverse transcriptase to ensure the absence of genomic DNA contamination. Genomic contamination was routinely <5% of the total signal. Primer sequences are located in Table 1.

For quantification of rat sONE expression in hypoxic rat tissues, 2 μg of RNA was used for first-strand synthesis using random hexamers, and cDNA was diluted to 100 μl. Primers for real time RT-PCR analysis were designed to a conserved region of rat sONE (Table 1). In addition, rat VEGF-A, 18S, and TBP levels were determined (Table 1). The ΔΔCt method (22) was used to determine the change in sONE and VEGF expression, relative to 18S and TBP.

RNA Polymerase II (Pol II) Chromatin Immunoprecipitation (ChIP) Assays—ChIP was performed as described previously (23). 5 μg of anti-Pol II antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added per immunoprecipitation, and control immunoprecipitations containing no antibody were performed in parallel. Pol II and control immunoprecipitated DNA was suspended in 40 μl of water. An input sample (18 μl of 1800 μl total) was removed prior to immunoprecipitation. The cross-links were reversed, and the input sample was diluted 1:10. Primers were directed to sONE exon 3 to detect transcription of both the long and short sONE transcripts (Table 1, sONE pre-mRNA primers). Primers used to amplify the hypoxia response element (HRE) of the VEGF-A promoter (−934 to −785) were 5’-CTA ACC CCG GAA CCA CAC AGC-3’ and 5’-CTG GCC TGC AGA CAT CAA AGT G-3’. The number of copies of the sONE exon 3 amplicon or the VEGF HRE amplicon were determined in 2 μl of bound chromatin, 2 μl of a no antibody control, and 2 μl of a diluted input sample using serial dilutions of genomic DNA. Immunoprecipitated (IP) DNA was calculated by subtracting the number of copies in the no antibody control from the bound chromatin and dividing by input.

sONE RNA Interference—Three Stealth™ small interfering RNAs (siRNA) duplexes were designed to recognize a region in common with the long and short sONE transcripts (Invitrogen). sONE 414 (sense, GGG ACA UCU GUU CUU UUG CCC...
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sONE mRNA Is Hypoxia-inducible and Is Reciprocally Expressed with eNOS mRNA in Endothelial Cells—sONE and eNOS were previously found to exhibit reciprocal expression patterns in endothelial and nonendothelial cells (8). We suggested that this was due, in part, to the post-transcriptional down-regulation of eNOS by sONE in nonendothelial cells. We were interested in assessing whether sONE could be up-regulated in endothelial cells by stimuli that are known to decrease the stability of eNOS mRNA. Therefore, we measured steady-state levels of sONE RNA in hypoxic endothelial cells, since hypoxic stimulation of HUVEC decreases eNOS RNA levels, in part by post-transcriptional mechanisms (9). Hypoxia can be mimicked by treating cells with DFO (26). Endothelial cells (HUVEC) were treated with DFO for various periods of time, and expression of eNOS and sONE was monitored by real-time RT-PCR (Fig. 1A). Basal levels of eNOS and sONE mRNA are shown in Table 2. VEGF mRNA, which is known to be up-regulated by DFO and is a highly hypoxia-inducible transcript (27), was measured in parallel as a control (Fig. 1A). We found that eNOS steady-state mRNA levels modestly increased with short term exposure to DFO (1–4 h) but then dramatically decreased after 24 h of DFO treatment. eNOS levels declined to ~30 and 20% of the mRNA levels of control HUVEC following 24- and 48-h treatment with DFO, respectively. The observed kinetics of eNOS expression was consistent with previously reported findings (28). Importantly, we observed that sONE gene expression was reciprocal in nature to the expression of eNOS. sONE gene expression began to increase after 2 h of DFO treatment and reached a maximum following 24 h of treatment, when mRNA levels increased more than 15-fold with respect to levels in control HUVEC. We have shown previously that treatment of endothelial cells with the histone deacetylase inhibitor, trichostatin A, up-regulates sONE RNA and that this is followed by a decrease in the levels of eNOS RNA (8). Similar to our previous observation that SONE RNA levels were increased by trichostatin A prior to a decrease in eNOS RNA (8), DFO also resulted in an increase in sONE prior to the fall in eNOS RNA levels. The expression of VEGF confirmed that DFO was successful at mimicking hypoxia (Fig. 1A).

Real time RT-PCR was also used to measure the expression of eNOS, sONE, and VEGF in HUVEC that had either been grown in atmospheric O2 (control HUVEC) or grown in a <1% O2 normobaric hypoxia chamber (Fig. 1B). Similar to DFO treatment, sONE was rapidly induced by 4–6 h of hypoxia treatment, and SONE levels continued to increase ~50-fold by 24 h (Fig. 1B). Although eNOS levels were relatively unchanged during short durations of hypoxia (1–4 h), eNOS levels signifi-
cantly decreased following 6 and 24 h. eNOS RNA levels decreased by more than 65% in cells exposed to hypoxia for 24 h. VEGF was induced by hypoxic conditions similarly to sONE, confirming that HUVEC were indeed hypoxic (Fig. 1B).

In addition to decreasing the levels of eNOS RNA, hypoxia also resulted in decreased levels of eNOS protein following a 24-h exposure to hypoxic conditions (Fig. 1C). Together, these studies indicate that sONE is hypoxia-inducible and that the expression of sONE negatively correlates with the expression of eNOS in endothelial cells.

sONE Is Hypoxia-inducible in Multiple Vascular Cell Types—Hypoxia has profoundly different effects on distinct vascular beds in vivo, and cell type-specific changes in the inducibility of hypoxia-regulated genes have been documented (29). To determine whether the hypoxic up-regulation of sONE is specific to large vessel endothelial cells (HUVEC) or is a general response across multiple cell types, two additional human vascular cell types (VSMC and MVEC) were treated with DFO (Fig. S1) or were exposed to hypoxia (Fig. 2). HUVEC and MVEC express high levels of eNOS mRNA and low levels of sONE mRNA. In contrast, VSMC express low basal levels of eNOS mRNA and relatively high levels of sONE mRNA (Table 2). Treatment of VSMC and MVEC with DFO resulted in an increase in sONE transcript levels (Fig. S1). Additionally, hypoxia resulted in a profound induction of sONE mRNA (Fig. 2, A and B). In MVEC, which express high levels of eNOS mRNA, DFO and hypoxia treatment resulted in down-regulation of eNOS mRNA, similar to HUVEC. This can be contrasted with VSMC, which express only trace amounts of eNOS mRNA under normoxic conditions. Neither DFO nor hypoxia treatment affected eNOS RNA levels. The induction of sONE in multiple cell types suggests that hypoxic induction of sONE is not a cell-specific or vascular bed-restricted phenomenon.

sONE Is Hypoxia-inducible in Vivo—To study the regulation of sONE in vivo, rats were exposed to hypoxic conditions (10% oxygen), and sONE expression was analyzed in the aorta. Previous studies have demonstrated that hypoxia causes a decrease in eNOS expression in rat aortas (30). Real time RT-PCR analysis of sONE expression revealed that sONE was induced ~2-fold by 16 h of hypoxia and more than 3-fold after 7 days of hypoxia treatment (Fig. 3). In these same samples, VEGF RNA expression increased ~3-fold following 7 days of hypoxia (Fig. 3). The findings of these experiments suggest that sONE is hypoxia-inducible both in vitro and in vivo. The physiological relevance of sONE induction in vivo will need to be addressed in future studies.

sONE Induction and eNOS Repression Are Reversible upon Reoxygenation of Hypoxic Endothelial Cells—The reversibility of the hypoxic induction of sONE was determined by

![FIGURE 1. sONE is hypoxia-inducible in endothelial cells.](image-url)
returning cells grown in hypoxic conditions (24 h), to a normoxic environment for 24 h (Fig. 4). Following reoxygenation of hypoxic cells, levels of sONE RNA returned to the levels found in normoxic cells (Fig. 4). Similarly, VEGF RNA levels decreased upon reoxygenation of hypoxic cells. Importantly, eNOS RNA levels also returned to baseline levels (Fig. 4), suggesting that repression of eNOS by hypoxia is reversible and that eNOS re-expression is correlated with sONE down-regulation.

**Hypoxia Induces the Expression of Both the Short and Long Variants of sONE**—Northern blotting of sONE expression in multiple human tissues revealed two primary transcripts for sONE (8). Characterization of the two transcripts revealed that the major transcript expressed in human tissues is 2.9 kb in length, consisting of 12 exons (Fig. 5A). The longer variant initiates from a distinct upstream transcriptional start site and contains five additional alternatively spliced exons. Whereas the shorter variant contains an ORF of 363 amino acids, the longer transcript contains an ORF of 924 amino acids, with a high degree of similarity to a yeast autophagy gene, yApg9 (8). Using a primer set that is specific for the longer transcript (AS4) or common to both transcripts (exon 7/8), the abundance of the two transcripts and their hypoxia-inducibility were quantified by real time RT-PCR (Fig. 5B). The amount of the long sONE transcript represented 55% of total sONE mRNA at baseline, suggesting that the long and short variants are similarly abundant in HUVEC (Fig. 5B). Comparison of the hypoxic induction of the short and long sONE transcripts revealed that both transcripts were hypoxia-inducible. However, the shorter variant increased more than 70-fold with longer durations (24 h) of hypoxia, whereas the longer variant increased 18-fold (Fig. 5B). This suggests that hypoxia regulates both the short and long sONE transcripts but that long term hypoxia has a more potent effect on the shorter transcript.

**sONE RNA Is Stabilized by Hypoxia, whereas Transcription of sONE Is Not Induced by Hypoxia**—Several hypoxia-inducible genes are up-regulated by both transcriptional and post-transcriptional mechanisms (reviewed in Ref. 31). To determine whether sONE was transcriptionally induced by hypoxia in HUVEC, the loading of RNA Pol II within the sONE transcriptional unit was monitored by a ChIP assay. Exon 3 of sONE, a genomic region that is common to both the short and the long transcriptional units, was assessed. In normoxic cells, signals generated for Pol II ChIPs were considerably lower than background (Fig. S2), although very little steady-state sONE mRNA is detectable in this cell type. Hypoxia did not significantly change the transcription of the gene throughout the duration of hypoxia (Fig. 6A). In contrast, RNA Pol II was recruited to the
HRE of the VEGF gene during exposure of HUVEC to hypoxia (Fig. 6A). Levels of Pol II at the VEGF HRE were significantly above background in normoxic cells (Fig. S2) but were much lower than levels found at exon 3 of sONE. This is consistent with the low transcriptional activity of the VEGF gene in normoxic cells.

Others have recently utilized real-time RT-PCR quantification of pre-mRNAs as a measure of transcriptional activity of a genomic locus (32). We therefore quantified the levels of unspliced sONE pre-mRNA (exon 3/intron 3) as an additional measure of sONE transcriptional activity. sONE pre-mRNAs were not detectable in the absence of reverse transcriptase (data not shown) and were highly localized to the nucleus of HUVEC (see Fig. 8A). We have previously reported that the sONE gene is transcriptionally active in HUVEC under basal conditions, despite the low steady-state levels of sONE mRNA (8). Consistent with prior findings, we noted some degree of base-line Pol II loading (Fig. S2) and pre-mRNA production (Fig. 6B). Levels of sONE pre-mRNA, which represented 7.8% of total sONE mRNA levels in normoxic cells, were only modestly increased after 6 h of hypoxia, and levels actually decreased following 24 h of hypoxia (Fig. 6B). This can be contrasted with the increase in spliced sONE mRNA, which increased 4- and 11-fold after 6 and 24 h of hypoxia, respectively, in these same samples (Fig. 6B). Taken together, we interpret these data to indicate that the transcriptional activity of the sONE gene does not increase during hypoxic induction of sONE RNA.

**FIGURE 4.** sONE induction and eNOS repression are reversible upon reoxygenation. HUVEC were exposed to hypoxic conditions for 24 h and were then transferred to a normoxic environment for an additional 24 h. sONE, eNOS, and VEGF-A RNA levels were quantified by real-time RT-PCR.

**FIGURE 5.** Both the short and long variants of sONE are hypoxia-inducible. A, schematic of the sONE/NOS3AS locus indicating two major transcripts and the locations of primers used for real-time RT-PCR. Untranscribed regions are indicated as shaded boxes, whereas the open reading frames are indicated by black boxes. B, quantitative real-time RT-PCR was used to determine the relative abundance of the long sONE transcript (primers in AS4) versus the short sONE mRNA (total sONE mRNA (exon 7/8) minus long sONE mRNA (AS4)) in endothelial cells exposed to hypoxic conditions. Although it is below the resolution of the figure, long sONE transcripts represented ~55% of the total sONE transcripts present in normoxic HUVEC. Shown is the mean of three independent experiments.
We previously suggested that the sONE transcript might be unstable in endothelial cells (8). To directly measure the stability of the sONE transcript and to determine whether changes in stability mediate the increase in sONE RNA levels following hypoxia, the half-life of sONE was measured following transcriptional arrest in normoxic and hypoxic (1% O₂, 16 h) HUVEC. sONE RNA had a moderately short half-life of 2.3 h (2.27 ± 0.97 h) in normoxic HUVEC, and the half-life increased nearly 2-fold (1.81 ± 0.16, p < 0.05) following hypoxia, to a half-life of 4.1 h (4.12 ± 1.94 h). A representative experiment is shown in Fig. 6C. As a control, the stability of eNOS RNA was also assessed. As we have previously published (9), hypoxia resulted in a decrease in the half-life of eNOS RNA (half-life of eNOS in hypoxia/normoxia = 0.31 ± 0.19; p < 0.05). These results suggest that sONE transcript stabilization, rather than transcriptional induction, occurs during hypoxia.

The Induction of sONE by Hypoxia is VHL-dependent—The VHL protein regulates gene transcription by ubiquitylating the α subunit of HIFs and targeting them for proteosomal degrada-

tion in an oxygen-dependent manner (33, 34). Consequently, cells that are deficient in VHL functional activity constitutively express hypoxia-inducible genes (35). VHL also regulates genes (36) and cellular function (37) in a HIF-independent manner. To elucidate the role of VHL in regulating sONE RNA levels, we utilized renal epithelial cells (786-O) that are deficient in functional VHL protein (18). These cells were then stably trans-

fected with an expression cassette that constitutively expresses either wild-type or mutant HA-tagged VHL. The mutant VHL protein (C162F) expressed in these cells cannot bind to HIFα proteins, and HIFα is therefore constitutively present in these cells under normoxic conditions (38). Western blot-

ting revealed similar expression of wild-type and mutant HA-VHL (Fig. 7A). sONE was expressed at 3.5-fold higher levels in those cell lines lacking functional VHL activity (Fig. 7B). 786-O cells reconstituted with wild-type HA-VHL were also exposed to hypoxic conditions. Hypoxia resulted in up-regu-

lation of both sONE and VEGF transcripts in these cells (Fig. 7B). It has been previously demonstrated that 786-O cells (VHL-negative) express high levels of HIF2α protein but negligible levels of HIF1α protein (39). To determine whether elevated levels of HIF2α were responsible for sONE induction in the absence of functional VHL, 786-O cells stably expressing an shRNA cassette targeting the HIF2α tran-

script (19) were monitored for sONE expression. These cells

FIGURE 6. Hypoxia increases the half-life of sONE rather than increasing sONE transcription. A, ChIP using anti-RNA Pol II antibodies was performed in HUVEC exposed to various durations of hypoxia. Loading of Pol II at exon 3 of sONE, which is common to both the short and long transcript variants of sONE, and the VEGF HRE, was determined using real-time PCR (see “Materials and Methods” for details). B, unspliced sONE pre-mRNA levels (exon 3/intron 3) were quantified by real time RT-PCR as a measure of the transcriptional activity of the NOS3AS gene. Levels of processed sONE mRNA levels were also quantified. Levels of sONE pre-mRNA did not significantly increase during hypoxia, in contrast to the dramatic increase in the levels of sONE mRNA. C, changes in the half-life of sONE after a 16-h treatment of HUVEC with hypoxia were determined by monitoring the decrease in sONE RNA levels using real time RT-PCR following DRB-mediated transcriptional arrest. 1 h post-DRB addition was used as time 0. A representative experiment is shown (n = 4). The average half-life of sONE in normoxic cells was 2.3 h (2.27 ± 0.97), and the half-life increased nearly 2-fold (1.81 ± 0.16, p < 0.05) following hypoxia, to a half-life of −4.1 h (4.12 ± 1.94).
express greatly reduced levels of HIF2α protein compared with cells transfected with empty vector (Fig. 7C). However, sONE expression was not altered in HIF2α knockdown cells (Fig. 7D). As a control, the expression of VEGF and CXCR4, two well characterized HIF-dependent transcripts (40, 41), were also monitored in these cells (Fig. 7D). VEGF and CXCR4 transcript levels were greatly diminished in two independent clones expressing HIF2α shRNAs. This suggests that HIF2α is not involved in sONE induction in the absence of functional VHL activity. To determine whether transcription of the sONE gene was altered by a lack of VHL activity, RNA Pol II ChIPs were performed in 786-O cells expressing wild-type or mutant VHL. A lack of functional VHL did not result in an increase in sONE transcription (Fig. 7E), suggesting that transcriptional induction was not responsible for the increased sONE levels observed in these cells. In contrast, we observed an increase in the amount of Pol II bound to exon 3 of the sONE gene and the HRE of the VEGF promoter. Shown is the mean ± S.D. of a representative experiment. *, a significant difference between wild-type and mutant VHL cells.

FIGURE 7. sONE expression is increased in the absence of VHL functional activity, but sONE is not regulated by HIF2. A, epithelial cells (786-O; VHL mutant) were reconstituted with wild-type or mutant (C162F) HA-tagged VHL. Western blotting was performed using antibodies that recognize HA or α-tubulin. Shown is a representative blot. B, expression of sONE and VEGF-A mRNA in cells containing wild-type (wt) or mutant (mut) HA-VHL was determined by real time RT-PCR. 786-O cells reconstituted with wild-type VHL were also exposed to hypoxia for 24 h, and sONE and VEGF-A were quantified. Cyclophilin A mRNA was used for normalization. *, a significant difference (p < 0.05) between groups. C, HIF2α expression was assessed by immunoblot in cells stably transfected with empty vector or a shRNA expression cassette targeting HIF2α. β-Actin was used as a loading control. Shown is a representative blot. D, levels of sONE, VEGF-A, and CXCR4 RNA were quantified in 786-O cells stably transfected with empty vector or in two stable clones containing a HIF2α shRNA expression cassette. sONE RNA levels were not altered in HIF2α knockdown cells. *, a significant difference (p < 0.05) between vector and HIF2α shRNA-transfected cells. E, ChIP analysis was performed in cells reconstituted with wild-type or mutant HA-VHL to assess the relative amount of Pol II bound to exon 3 of the sONE gene and the HRE of the VEGF promoter. Shown is the mean ± S.D. of a representative experiment. *, a significant difference between wild-type and mutant VHL cells.
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As an additional measure of the presence of sONE mRNA in the cytoplasm, and to determine whether sONE mRNA might be translated, polyribosomes were isolated from normoxic and hypoxic (24 h) HUVEC (Fig. 9). Measurement of the absorbance at 254 nm revealed that the 80S peak was eluted in Fraction 6, whereas the polyribosomes were present in Fractions 7–15 (Fig. 9A). Total sONE mRNA (short and long variant) (top left) was not associated with polyribosomes in normoxic cells, but hypoxia resulted in an increased abundance of sONE on polyribosomes (Fig. 9A). However, the longer variant of sONE (AS4) (top right) did not accumulate on polyribosomes under hypoxic conditions. This suggests that the short variant of sONE becomes engaged with polyribosomes, whereas the long variant does not. This is consistent with the massive induction of short sONE transcripts during hypoxia (Fig. 5B). The polysome profile of cyclophilin A (CYP A) mRNA (middle right) was also determined in these same fractions. Hypoxia resulted in a decrease in the presence of cyclophilin A transcripts on polyribosomes. Assessment of the distribution of the eNOS mRNA (middle left) revealed that eNOS was primarily associated with polyribosomes (Fractions 9–12) in normoxic cells. In contrast, eNOS mRNA did not demonstrate nuclear enrichment. As a further control, pre-mRNA and mRNA levels of CD31 and CD34 were also monitored in these fractions, confirming that pre-mRNAs are generally enriched in the nucleus, whereas mRNAs are not. These results suggest that sONE mRNA is atypical in that it is enriched in the nucleus under basal conditions. To determine whether changes in whole cell sONE mRNA levels were attributed to an increase in nuclear or cytoplasmic levels of sONE, sONE mRNA levels were quantified in both of these fractions in normoxic and hypoxic cells (Fig. 8B). sONE mRNA levels increased only modestly in the nucleus, whereas cytoplasmic levels increased more than 30-fold (Fig. 8B). In contrast, the cellular distribution of eNOS mRNA was not altered, since eNOS mRNA levels decreased in both the cytoplasm and nucleus (Fig. 8B).

sONE mRNA Is Enriched in the Nucleus Basally, but Cytoplasmic Accumulation Is Induced by Hypoxia—It is currently not known whether sONE mRNAs are translated in endothelial cells or whether sONE plays a primarily noncoding regulatory role. Interestingly, the sONE transcript is a potential candidate for nonsense-mediated decay due to the presence of a translation stop codon in exon 8 of a 12-exon gene. Nonsense-mediated decay-regulated transcripts are often degraded before entering the cytoplasm (42) or remain associated with their transcriptional unit (43). Additionally, sense/antisense pairs have a propensity to be enriched in the nucleus (44), and RNA editing of double-stranded RNA also results in nuclear accumulation (45). It was therefore of interest to define the subcellular distribution of sONE mRNAs. RNA was isolated from nuclear and cytoplasmic subcellular fractions, and sONE and eNOS pre-mRNA and mRNA were quantified using real time RT-PCR (Fig. 8A). Although pre-mRNA levels for both sONE and eNOS were highly enriched in the nucleus, as expected, levels of sONE mRNA were also enriched in the nuclear fraction. In contrast, eNOS mRNA did not demonstrate nuclear enrichment. As a further control, pre-mRNA and mRNA levels of CD31 and CD34 were also monitored in these fractions, confirming that pre-mRNAs are generally enriched in the nucleus, whereas mRNAs are not. These results suggest that sONE mRNA is atypical in that it is enriched in the nucleus under basal conditions. To determine whether changes in whole cell sONE mRNA levels were attributed to an increase in nuclear or cytoplasmic levels of sONE, sONE mRNA levels were quantified in both of these fractions in normoxic and hypoxic cells (Fig. 8B). sONE mRNA levels increased only modestly in the nucleus, whereas cytoplasmic levels increased more than 30-fold (Fig. 8B). In contrast, the cellular distribution of eNOS mRNA was not altered, since eNOS mRNA levels decreased in both the cytoplasm and nucleus (Fig. 8B). As an additional measure of the presence of sONE mRNA in the cytoplasm, and to determine whether sONE mRNA might be translated, polyribosomes were isolated from normoxic and hypoxic (24 h) HUVEC (Fig. 9). Measurement of the absorbance at 254 nm revealed that the 80S peak was eluted in Fraction 6, whereas the polyribosomes were present in Fractions 7–15 (Fig. 9A). Total sONE mRNA (short and long variant) (top left) was not associated with polyribosomes in normoxic cells, but hypoxia resulted in an increased abundance of sONE on polyribosomes (Fig. 9A). However, the longer variant of sONE (AS4) (top right) did not accumulate on polyribosomes under hypoxic conditions. This suggests that the short variant of sONE becomes engaged with polyribosomes, whereas the long variant does not. This is consistent with the massive induction of short sONE transcripts during hypoxia (Fig. 5B). The polysome profile of cyclophilin A (CYP A) mRNA (middle right) was also determined in these same fractions. Hypoxia resulted in a decrease in the presence of cyclophilin A transcripts on polyribosomes. Assessment of the distribution of the eNOS mRNA (middle left) revealed that eNOS was primarily associated with polyribosomes (Fractions 9–12) in normoxic cells. The association of eNOS mRNA with polyribosomes was attenuated in hypoxic cells.

FIGURE 8. The sONE transcript is enriched in the nucleus of normoxic cells, but levels increase in the cytoplasm following hypoxia. A, RNA was extracted from nuclear and cytoplasmic fractions of normoxic HUVECs, and levels of sONE and eNOS pre-mRNA and mRNA were quantified by real time RT-PCR. As a control, the nuclear and cytoplasmic levels of CD31 and CD34 pre-mRNA and mRNA were also determined. Shown above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values above is the ratio of nuclear to cytoplasmic abundance. Note that relative quantification was used for determining CD31 and CD34 mRNA levels, so values are in arbitrary units for these two transcripts. All other values.
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Therefore, hypoxia not only results in a decrease in total levels of eNOS mRNA, but the eNOS remaining in the cytoplasm is less likely to be actively engaged on translating polyribosomes. As a control, two mRNAs known to be hypoxia-regulated, VEGF (bottom left) (46) and GAPDH (47) (bottom right), were also assessed. As expected, VEGF and GAPDH mRNAs became more highly associated with polyribosomes in hypoxic cells.

**FIGURE 9.** sONE is not localized to polyribosomes in normoxic cells but becomes engaged with polyribosomes following hypoxia. A, a representative $A_{254}$ trace of isolated ribosome-associated complexes from normoxic cells is shown. The 80S monosome (Fraction 6) is indicated, as are the polyribosomes (Fractions 7–15). B, RNA was extracted from each fraction, and levels of sONE (total and long/AS4 transcripts), eNOS, cyclophilin A (CYPA), GAPDH, and VEGF RNA were quantified by real time RT-PCR. Whereas sONE (short), GAPDH, and VEGF levels increased on the polysome fractions, eNOS and CYPA mRNA levels decreased. Shown is a representative experiment of three.
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The data indicate that sONE negatively regulates eNOS RNA and protein levels during hypoxia. Levels of sONE RNA increase prior to the decrease in steady-state eNOS RNA levels during prolonged exposure to hypoxia (Fig. 1). To provide functional evidence that sONE negatively regulates eNOS RNA levels in endothelial cells during hypoxia, RNA interference was used to knock down expression of sONE during hypoxia, and changes in eNOS RNA and protein levels were assessed (Fig. 10). In endothelial cells transfected with nontargeting, control siRNAs, 24 h of hypoxia resulted in more than a 5-fold increase in sONE expression and a 65% decrease in eNOS mRNA. Transfection of multiple independent siRNAs directed to the sONE transcript prevented the hypoxic induction of sONE (Fig. 10A). In contrast, the hypoxic induction of VEGF was not inhibited by sONE siRNAs. Importantly, sONE siRNAs resulted in an attenuation of the fall in eNOS RNA levels during hypoxia (Fig. 10A). To determine whether eNOS protein levels were similarly affected, Western blotting was used in hypoxic HUVEC transfected with control or sONE siRNAs. The drop in eNOS protein levels following hypoxia was attenuated in cells transfected with sONE siRNAs (Fig. 10B). These results suggest that sONE plays an important functional role in regulating eNOS RNA and protein levels during hypoxia in endothelial cells.

**DISCUSSION**

Functional deficiency of NO contributes importantly to perturbations in vascular homeostasis in the setting of chronic hypoxia. As an example, mice deficient in Nos3 develop more severe pulmonary hypertension and exaggerated pulmonary artery remodeling in the setting of hypoxia (48, 49). Reintroduction of the eNOS gene or the administration of exogenous NO restores vascular function in models of pulmonary hypertension (50, 51). Importantly, although eNOS expression is decreased by hypoxia, the mechanisms responsible are poorly understood. We considered whether sONE, an overlapping cis-antisense transcript to eNOS, might play a functional role in the post-transcriptional down-regulation of eNOS during hypoxia. sONE has previously been reported to contribute to the endothelial cell-specific expression of eNOS via post-transcriptional mechanisms, and sONE induction was associated with the down-regulation of eNOS in endothelial cells treated with histone deacetylase inhibitors (8). Here we demonstrated that sONE and eNOS expression were similarly affected, Western blotting was used as a loading control. Shown is the mean ± S.D. of a representative experiment of four. *p < 0.05 difference between control and sONE siRNA-transfected cells. NS, not significant. B, the effect of sONE knockdown on eNOS protein expression during hypoxia was determined by performing eNOS Western blots. Lamin A/C was used as a loading control. Shown is a representative experiment of four.

**FIGURE 10.** sONE post-transcriptionally regulates eNOS during hypoxia. HUVEC were transfected with multiple independent siRNAs directed to the sONE transcript and after 16 h were exposed to hypoxic conditions for 24 h. A, real time RT-PCR was used to determine the expression of eNOS, sONE, and VEGF-A RNA. 18 S, TBP, and cyclophillin A were used for normalization. Changes in the expression of eNOS, sONE, and VEGF in hypoxic endothelial cells were calculated relative to a GC-matched siRNA control. Shown is the mean ± S.D. of a representative experiment of four. *p < 0.05 difference between control and sONE siRNA-transfected cells. NS, not significant. B, the effect of sONE knockdown on eNOS protein expression during hypoxia was determined by performing eNOS Western blots. Lamin A/C was used as a loading control. Shown is a representative experiment of four.
sONE mRNAs were predominantly nuclear in normoxic cells and that hypoxia resulted in transcript stabilization and an increase in cytoplasmic levels of sONE mRNA. HuR binds to the elongin-binding site of VHL (amino acids 157–184 (53)), which is a hot spot for mutation (55). Indeed, the mutant VHL protein that we used in our studies (C162F) would be expected to interfere with HuR/VHL interactions, allowing HuR to mediate the stabilization of hypoxia-inducible transcripts, such as VEGF. Hypoxia increased the expression of both the short and the long variants of sONE. These transcripts share a common 3’-UTR but originate from two separate transcriptional start sites. This suggests that shared regions of the transcripts, possibly the 3’-UTR, may be involved in the stabilization of sONE during hypoxia.

What is the normal biological role of sONE? The open reading frame of the longer sONE transcript is homologous to a known yeast autophagy gene, yAtg9p (8, 56). Autophagy is the degradation pathway for intracellular components triggered by nutrient deprivation (57) and exogenous stimuli that limit nutrient and energy availability, including hypoxia (58). Overexpression of the ORF of the longer sONE variant has been demonstrated to result in autophagosome formation (56). However, since sONE RNA is expressed at very low levels in normoxic endothelial cells, and considering that sONE mRNA is enriched in the nucleus, there is currently no evidence that the native sONE ORF produces a functional protein in normoxic endothelial cells. In contrast to the longer sONE variant, the shorter variant shares only minimal homology with the ORF of yAtg9p (8). We have demonstrated that the shorter variant is the predominant mRNA induced by hypoxia. Moreover, it was the major mRNA associated with polyribosomes. It is important to note that polyribosome association does not necessitate sONE translation in hypoxic endothelial cells. For example, microRNAs localize to polyribosomes (59, 60) to inhibit the translation of target mRNAs that are already engaged on the ribosome (61). We cannot exclude a model in which sONE localizes to polysomes for the purpose of inhibiting the translation of eNOS mRNAs. The amount of eNOS mRNA actively engaged with polyribosomes was decreased during hypoxia, and total eNOS protein levels were decreased in hypoxic cells. Additionally, sONE knockdown in hypoxic cells resulted in an increase in eNOS protein levels. In regard to the biological role of sONE in human endothelial cell types, we favor a model in which sONE plays an important noncoding biological role, specifically that sONE mRNA regulates eNOS mRNA post-transcriptionally during hypoxia. We posit that interaction between eNOS and sONE mRNAs results in the destabilization of eNOS mRNA and may also interfere with its translation.

We anticipate that the significant overlap (662 nucleotides) between the human eNOS and sONE mRNAs is key to the mechanism by which sONE regulates eNOS expression in endothelial cells. The overlap between eNOS and sONE mRNA (662 nt) is atypical compared with the median of known cis-S/AS pairs (139 nt) (62). Several models could be put forward for the regulation of eNOS RNA levels by sONE. eNOS mRNA is highly stable in endothelial cells, and changes in RNA stability and adenylation of the eNOS transcript have been implicated in the response of endothelial cells to several physiological and pathological stimuli, including shear stress (63), exposure to inflammatory cytokines (15, 64), and hypoxia (9). A multiprotein complex binds to the eNOS 3’-UTR in endothelial cells to actively stabilize eNOS RNA (14, 15). eNOS and sONE may compete for RNA-stabilizing proteins, or sONE/eNOS RNA interactions may prevent the formation of this stability complex on the eNOS 3’-UTR. Competition for stabilizing complexes has previously been observed in the regulation of CHOP RNA by its overlapping transcript, MetRS (65). Since eNOS and sONE mRNA demonstrate reciprocal expression patterns in cells, either eNOS or sONE may be preferentially stabilized in a given cell type. However, hypoxia results in increased sONE expression even in cell types with relatively high levels of sONE RNA, suggesting that hypoxia exerts an additional stabilizing effect. Since sONE mRNA is enriched in the nucleus of normoxic cells, but cytoplasmic levels increase following hypoxia, sONE mRNA could regulate eNOS in either the nucleus or the cytoplasm during hypoxia. We considered whether interactions between eNOS and sONE mRNA might prevent the nuclear export and cytoplasmic stabilization of eNOS mRNA. However, we did not observe a change in the nuclear/cytoplasmic distribution of eNOS mRNAs in hypoxic cells, suggesting that nuclear export of eNOS was not affected.

Although sONE might affect the formation of stabilizing complexes on the eNOS 3’-UTR, an alternative model for eNOS destabilization involves the RNA interference pathway. If long double-stranded mRNAs were to form between these two transcripts, they could be targeted for processing by Dicer to form siRNAs (66). Generation of siRNAs from naturally occurring sense-antisense partners has been observed in plants (67), but no evidence has been found for their existence in mammalian cells (68, 69). However, siRNAs generated from bidirectional transcription of the 5’-UTR of L1 retrotransposons have been identified in human cells that play a role in suppressing retrotransposition (70). Others have argued that the RNA interference pathway is not importantly involved in the regulation of natural S/AS genes (71). This recent work demonstrated that sense and antisense RNAs are often present in different subcellular compartments and do not physically interact in the cytoplasm. Long double-stranded RNA species are known to activate the protein kinase R pathway and elicit global translational inhibition (reviewed in Ref. 72). Compartmentalizing sense and antisense transcripts may avoid the activation of this pathway under physiological conditions. Although we demonstrate that sONE mRNA is basally enriched in the nucleus, sONE levels were dramatically increased in the cytoplasm following hypoxia. This important nuance could allow for the de novo physical interaction between the mRNAs in the cytoplasm. Whether such interaction might activate the protein kinase R pathway is currently not known, but global translational inhibition is known to occur in hypoxic cells (73). Additionally, the functional importance of interactions that occur at or near the site of transcription should not be discounted, especially considering the fact that RNA interference can occur in the nucleus (74, 75). Finally, even following hypoxic stimulation of HUVEC, there remains a 100–1000-fold difference in the copy numbers between eNOS and sONE tran-
scripts. It is especially noteworthy that the RNA-induced silencing complex (RISC) is able to amplify the effect of guide strand-mediated RNA degradation in the RNA interference pathway (76). Determination of whether the RNA interference pathway is involved in eNOS/sONE regulation will require future validation.

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