Hypoxia is a significant perturbation that exacerbates endothelial barrier dysfunction, contributing to the disruption of vascular homeostasis and the development of various diseases such as atherosclerosis and metastasis of tumors. To date, it is not known what strategy might be used to counter the effect of hypoxia on endothelial permeability.

Objective: This study investigated the role of nitrite in regulating vascular integrity under hypoxic conditions.

Methods and Results: We found denitrosylation and the resulting activation of caspase-3 to be critical for hypoxia-induced endothelial permeability. Nitrite treatment led to S-nitrosylation and the inactivation of caspase-3, suppressing the barrier dysfunction of endothelia caused by hypoxia. This process required the conversion of nitrite to bioactive nitric oxide in a nitrite reductase-dependent manner. Using primary human umbilical vein endothelial cells as a model, we showed that in the presence of nitrite, the S-nitrosylated and inactivated form of caspase-3 was unable to cleave β-catenin, a key component in the VE-cadherin complex. Therefore, nitrite treatment led to the maintenance of VE-cadherin-mediated adherens junctions under hypoxic conditions. In in vivo experiments using a zebrafish model, nitrite was found to protect blood vessels from hypoxia-induced vascular leakage.

Conclusions: These results are the first to demonstrate that nitrite plays a critical role in the protection of endothelial barrier function against hypoxic insult. Our findings show that nitrite holds great potential for the treatment of diseases associated with hypoxia-induced disorder of vascular homeostasis. (Circ Res. 2011;109:1375-1386.)

Key Words: nitrite ■ NO ■ caspase-3 ■ hypoxia ■ endothelial barrier dysfunction

A semipermeable barrier lining along the vasculature, the endothelium regulates the exchange of fluids and solutes between the blood and interstitial space. Dysfunction of the endothelial barrier can lead to problems in the regulation of vascular homeostasis and serious pathological conditions. Increased endothelial permeability may lead to development of various diseases, such as capillary leakage syndrome,1 atherosclerosis,2 and tumor metastasis.3 Because endothelial cells form the first layer in contact with blood, perturbations in blood composition are the main cause of dysfunction of vascular barrier. Hypoxia, one such perturbation, leads to increased endothelial permeability,4 contributing to the progression of several diseases involving disrupted vascular homeostasis.5 To date, it remains unclear what governs hypoxia-induced endothelial permeability. It is also not known what strategy might be used to prevent endothelial barrier dysfunction and preserve vascular integrity under hypoxic insult.

The constitutive production of nitric oxide (NO) by the endothelial NO synthase (eNOS) has long been regarded as a key regulator of vascular health.6 Reduced bioavailability of NO contributes to endothelial dysfunction,7 ultimately leading to the development of various cardiovascular diseases.8 Interestingly, in response to hypoxia, which induces an increase of vascular permeability, NO production by the endothelium is significantly diminished.9 One study reported that when a high level of NO donor is artificially delivered to endothelial culture, hypoxia-induced permeability was sup-

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Nitrite, an oxidized form of NO, can be disproportionated or reduced back to NO under conditions of hypoxia and ischemia. Thus, nitrite can function as a natural reservoir of NO in tissues and the circulation. It remains inert when oxygen supplies are normal. However, in response to hypoxia, various endogenous enzymes expressed in different tissues may act as nitrite reductases, converting nitrite back to the bioactive NO. Biochemical analyses have further found xanthine oxidoreductase (XOR), aldehyde oxidase (AO) and eNOS to be functional nitrite reductases during hypoxia. The reduction of nitrite may serve as an alternative way of producing NO and be able to contribute greatly to the protection of tissues against hypoxia- or ischemia-induced damage. We hypothesized that nitrite could be used to deliver more bioavailable NO to the endothelium under hypoxic conditions. If the conversion of nitrite could increase cellular levels of NO, hypoxia-induced permeability might be reduced and endothelial barrier function restored.

This study investigated whether nitrite-induced NO-mediated signal transduction might protect endothelia against hypoxic challenge. Because the switch between denitrosylation and S-nitrosylation of the active-site Cys residue may control the activity of caspase-3, we also investigated what influence nitrite-dependent NO production might have on endogenous caspase-3 in endothelia under hypoxia and whether the inactivation of caspase-3 might enhance the formation of adherens junctions, an important sign of endothelial integrity. We found that through its regulation of caspase-3 activity, nitrite could protect endothelial barrier function under hypoxic conditions. The protective function of nitrite in this context was further supported by in vivo studies using zebrafish as a model. These findings are the first to identify a new role for nitrite.

Results

Hypoxia-Induced Cys Denitrosylation and Activation of Caspase-3 May Promote Endothelial Barrier Dysfunction

We began by examining whether the activation of endogenous caspase-3 would enhance endothelial permeability under hypoxia. For this, mouse pancreatic microvascular endothelial cells (MS-1) were exposed to normoxia or hypoxia for 16 hours in the absence or presence of a caspase-3 specific inhibitor Z-DEVD-fmk and measured for permeability. Hypoxia-induced increases of endothelial permeability were abolished when the activity of caspase-3 was inhibited by Z-DEVD-fmk (Figure 1A and Online Figure I), suggesting that caspase-3 possibly played a key role in this process. The enhanced permeability could be due to multiple events such as disruption of intercellular junctions or apoptosis. We tested the possible function of caspase-3 involved in these events. As shown in Figure 1B, the hypoxic condition caused an overwhelming loss of cell-cell contacts concomitant with a retracted morphology of cells. This change was significantly suppressed by Z-DEVD-fmk (Figure 1B). We also evaluated the influence of apoptosis on the regulation of endothelial permeability. A semiquantitative measurement showed that after exposure to hypoxia only ≈20% MS-1 cells were found to be apoptotic (Online Figure I). Although apoptosis played a minor role in this experimental condition, this caspase-3-dependent event (Online Figure I) might also contribute to barrier dysfunction. We sought to identify the potential mechanism underlying the activation of caspase-3 in endothelia exposed to hypoxia. To do this, we used the time-dependent profile of HIF-1α-Bnip3 signaling as guidance to assess the onset of hypoxic response in MS-1 cells, because the activation of this pathway reflects the hypoxic environment inside cells. As expected, hypoxia-exposed cells had a gradual elevation in the accumulation of HIF-1α (Figure 1C), a transcriptional factor that induces the expression of bnip3 gene. This inducible expression of Bnip3 was evident as early as 6 hours after hypoxia (Figure 1C), suggesting that hypoxia-induced downstream signaling, including the activation of caspase-3, might be already switched on at this time. We hypothesized that the active-site Cys residue of caspase-3 might undergo denitrosylation, possibly leading to an increase in its enzymatic activity. This hypothesis was tested using the biotin-switch method (BSM). Stimulation of cells with hypoxia for 6.5 hours resulted in a decreased level of Cys S-nitrosylation of caspase-3 (Figure 1D), coinciding with a significant increase in its activity (Online Figure I). Taken together, our findings demonstrate that Cys denitrosylation and activation of caspase-3 might play a key role in increasing endothelial...
Figure 1. Denitrosylation and activation of caspase-3 regulates hypoxia-induced endothelial barrier dysfunction. A and B, MS-1 cells were exposed to normoxia (N) or hypoxia (H) supplied with Z-DEVD-fmk (25 μmol/L) for 16 hours. Endothelial permeability (A) and morphological change (B) were determined. C, MS-1 cells were exposed to hypoxia for the indicated times. Aliquots of lysates were subjected to immunoblotting with anti–HIF-1α and anti-Bnip3 antibodies. D, After exposure to hypoxia for 6.5 hours, the S-nitrosylation levels of endogenous caspase-3 in MS-1 cells were analyzed by the BSM (upper panel). Lower panel shows the result of the densitometric analysis of the gel image as the ratio of biotinylated caspase-3 relative to total caspase-3 in lysate. Data shown in A and D are presented as mean ± SEM (n = 3). *P < 0.05 compared with normoxic controls. #P < 0.05 compared with untreated cells.

Nitrite Induces S-Nitrosylation and Inactivation of Caspase-3 Under Hypoxia

We next investigated whether nitrite plays a cytoprotective role in the endothelium. We hypothesized that nitrite might be reduced back to the bioactive form of NO under hypoxia, thus promoting Cys S-nitrosylation and inactivation of caspase-3. Electron paramagnetic resonance (EPR) was used to detect nitrite-induced endothelial NO under hypoxic conditions. Briefly, MS-1 cells were exposed to hypoxic conditions for 5 hours before being treated with nitrite. After treatment, they were further exposed to hypoxia for 30 minutes. Nitrite treatment (100 μmol/L) effectively increased the intracellular NO levels, as evidenced by the typical triplet spectrum of NO–Fe$^{2+}$-diethyldithiocarbamate (Online Figure II). A similar but much stronger triplet EPR spectrum of NO–Fe$^{2+}$–N-methyl-d-glucamine diethiocarbamate (MGD) was produced by adding nitrite (100 μmol/L) to a large number of MS-1 cells cultured in suspension (Online Figure III and Figure 2A). The use of Fe$^{2+}$-MGD as a spin trap also allowed us to detect NO produced by endothelia exposed to a low dose (10 μmol/L) of nitrite (Figure 2A). Together these findings suggest that preincubation of cells under hypoxic conditions for 5 hours had activated endogenous nitrite reductases. Following the same experimental design, we examined the effect of nitrite on regulation of caspase-3 by BSM. As shown in Figure 2B, in the presence of 10 μmol/L nitrite, caspase-3 was no longer under hypoxia-mediated denitrosylation and returned back to the S-nitrosylated form, suggesting that nitrite-derived NO might suppress caspase-3 activity through direct NO modification. Moreover, nitrite worked specifically under hypoxic condition but showed no effect on caspase-3 in normoxic cells (Online Figure IV). Surprisingly, when higher doses of nitrite (50 and 100 μmol/L) were applied, the S-nitrosylation level of caspase-3 (Figure 2B) and endothelial permeability (Online Figure V) were not increased proportionally. Based on these results, we hypothesized that a large quantity of nitrite-converted NO might not be beneficial to endothelia under hypoxia. We next examined whether the active-site Cys163 of caspase-3 in cells treated with nitrite would be more susceptible to S-nitrosylation than it would be in untreated cells incubated with hypoxia. As illustrated in the work flow chart (Figure 2C), after treating hypoxic cells with nitrite, which led to a high degree of S-nitrosylation in the susceptible Cys residues, we processed samples following the general BSM procedure until the completion of SDS-PAGE. Pieces of gel containing caspase-3 were excised and treated with the reagent tris (2-carboxyethyl) phosphine, which reduced all disulfide links back to free thiols, including the biotin-HPDP-tagged Cys residues. These newly generated free thiols were then labeled with N-(2,3-dimethylphenyl)-2-iodoacetamide (DPIAM), which assured us of the formation of more stable derivatives of originally S-nitrosylated Cys residues. After being digested by trypsin, the extracted peptides were subjected to mass spectrometry (MS) analyses. Because an intramolecular disulfide linkage does not exist in caspase-3, the MS intensity of a tryptic peptide containing a DPIAM-labeled Cys residue could be used as an indicator of S-nitrosylation level of caspase-3 in cells. As shown in the MS/MS spectrum (Figure 2D), DPIAM clearly tagged the peptide T22 on the active-site Cys residue. Pieces of gel containing caspase-3 were excised and treated with the reagent tris (2-carboxyethyl) phosphine, which reduced all disulfide links back to free thiols, including the biotin-HPDP-tagged Cys residues. These newly generated free thiols were then labeled with N-(2,3-dimethylphenyl)-2-iodoacetamide (DPIAM), which assured us of the formation of more stable derivatives of originally S-nitrosylated Cys residues. After being digested by trypsin, the extracted peptides were subjected to mass spectrometry (MS) analyses. Because an intramolecular disulfide linkage does not exist in caspase-3, the MS intensity of a tryptic peptide containing a DPIAM-labeled Cys residue could be used as an indicator of S-nitrosylation level of caspase-3 in cells. As shown in the MS/MS spectrum (Figure 2D), DPIAM clearly tagged the peptide T22 on the active-site Cys163. In the extraction chromatography of nano-LC-MS, we found a significantly higher level of the tryptic peptide (T22) carrying the Cys163 in the DPIAM form in the nitrite-treated cells than in the control cells (Figure 2E), indicating that nitrite-derived NO drove the active-site Cys163 of caspase-3 back to S-nitrosylated form. Because the catalysis of caspase-3 is determined by nucleophilic attack of substrates, the S-nitrosothiol modification that disrupted permeability and lead to endothelial barrier dysfunction in response to hypoxia.
Figure 2. Nitrite-derived NO targets the active-site Cys163 of endogenous caspase-3 in endothelia under hypoxia. MS-1 cells were exposed to normoxia (N) or hypoxia (H) for the various times as indicated. Nitrite was added at 5 hours after hypoxia. A, Representative EPR spectra for NO-Fe²⁺-(MGD)₂ adducts formed in culture media of cells containing Fe²⁺-(MGD)₂ complex. NO levels were recorded at 5.5 hours after hypoxia in the absence or presence of nitrite (10 and 100 μmol/L) at 77K. EPR measurements were performed using a Bruker ERS80 spectrometer operating at X-band, 100 kHz. B, Effect of nitrite (10, 50, and 100 μmol/L) on S-nitrosylation levels of caspase-3 was determined by the BSM at 6.5 hours after hypoxia. C, Schematic illustration of the work flow to
the nucleophilic character of Cys163 might have led to the inactivation of caspase-3. Indeed, we showed that hypoxia-induced activation of caspase-3 was diminished in MS-1 cells treated with nitrite, as demonstrated by the enzymatic activity assay (Figure 2F) and the level of cleaved isoform (Figure 2G).

Nitrite Suppresses Barrier Dysfunction in Endothelial Response to Hypoxia
Because the activity of caspase-3 played a key role in hypoxia-induced endothelial barrier dysfunction (Figure 1), we hypothesized that nitrite might be able to prevent the dysfunction through S-nitrosylation and inactivation of caspase-3. To test this hypothesis, we incubated MS-1 cells exposed to hypoxia for 16 hours in the absence or presence of nitrite. According to our semiquantitative measurements, nitrite suppressed endothelial permeability, which was otherwise robustly increased in the untreated cells (Figure 3A). These results were concomitant with the assessment of the effect of nitrite on cell morphology. Nitrite abolished hypoxia-induced retraction of cell shape and loss of cell-cell contacts (Figure 3B). Considered together, these findings suggest that nitrite plays a cytoprotective role, which might make possible the maintenance of normal endothelial barrier function even when cells are exposed to hypoxia for sustained periods of time.

Nitrite Inhibits Hypoxia-Induced Barrier Dysfunction Through XOR and eNOS-Dependent NO Production
Having demonstrated the effects of nitrite, we further examined the role of nitrite reductases. Of the several candidates being tested, XOR, eNOS, and AO were constitutively expressed in MS-1 cells (Online Figure VI). We preexposed cells with specific inhibitors against these enzymes before treating them with nitrite (100 μmol/L) and measuring them for NO by EPR. Allopurinol (Figure 4A) and oxypurinol (Online Figure VII), both inhibitors of XOR, attenuated nitrite-derived NO in cells exposed to hypoxia. An inhibitor of eNOS, L-NAME also suppressed hypoxia-induced nitrite reduction (Figure 4A). The AO inhibitor raloxifene had no effect on the NO level (Online Figure VIII), presumably due to a decreased expression of AO in cells under hypoxia (Online Figure VI). Combining allopurinol and L-NAME had a clear effect on the suppression of NO production (Figure 4B), providing us a suitable condition for more detailed analyses. We examined the change of S-nitrosylation levels of caspase-3 when nitrite-derived NO production was attenuated. In the presence of allopurinol and L-NAME, nitrite (10 μmol/L) no longer enhanced the S-nitrosylation of caspase-3 (Figure 4C). Likewise, when we added these inhibitors, nitrite’s effect on the suppression of the activity and cleavage of caspase-3 was lost (Figure 4D). These findings suggest that, when a low dose (10 μmol/L) of nitrite was added, XOR and eNOS functioned effectively as nitrite reductases to provide sufficient NO for cytoprotection. This hypothesis was further tested by additional experiments using allopurinol and L-NAME. As shown in Figure 4E, nitrite was unable to suppress hypoxia-promoted permeability in MS-1 cells when XOR and eNOS were inactivated. Likewise, the rescue effect of nitrite on hypoxia-induced cell retraction and loss of cell-cell contacts were abolished in the presence of allopurinol and L-NAME (Figure 4F). Taken together, our findings demonstrate that XOR and eNOS-dependent NO production play a key role in nitrite-mediated cytoprotection of endothelia against hypoxic injury.

Nitrite Protects Bovine Aortic Endothelial Cells Under Hypoxia Through S-Nitrosylation of Caspase-3
To explore the beneficial effect of nitrite, we performed experiments in primary bovine aortic endothelial cells (BAECs). Our results showed that activated caspase-3 played an essential role in hypoxia-induced disruption of vascular
integrity in BAECs (Online Figure IX). We then examined whether treatment of nitrite would prevent the activation of caspase-3 through S-nitrosylation. The results of BSM showed that endogenous caspase-3 underwent denitrosylation in BAECs exposed to hypoxia (Figure 5A), concomitant with increased activity of caspase-3, as judged by the presence of cleaved caspase-3 (Figure 5A). Importantly, when hypoxic cells were treated with nitrite, the level of S-nitrosylated caspase-3 was reduced, indicating a protective effect of nitrite on endothelial barrier function.

Figure 4. Nitrite reductase activity is required for nitrite-mediated suppression of endothelial barrier dysfunction under hypoxia.

A and B. Representative EPR spectra for NO-Fe²⁺-(MGD)₂ adducts formed in culture media of MS-1 cells under hypoxia. Cells were treated with allopurinol (1 mmol/L) and L-NAME (1 mmol/L) individually (A) or in combination (B) at 4.5 hours after hypoxia. Nitrite (100 μmol/L) was added at 5 hours after hypoxia. NO levels were measured by EPR at 5.5 hours after hypoxia. C through F, MS-1 cells were treated with allopurinol (100 μmol/L), L-NAME (100 μmol/L), and nitrite (10 μmol/L) as described in A. C. The S-nitrosylation levels of caspase-3 were detected by the BSM at 6.5 hours after hypoxia. The activation of caspase-3 (D), endothelial permeability (E), and morphological change (F) were determined at 16 hours after hypoxia. Data shown in D and E are presented as mean±SEM (n=3). *P<0.05 compared with normoxic control. #P<0.05 compared with untreated cells. †P<0.05 compared with nitrite-treated cells.
caspase-3 increased dramatically (Figure 5A), leading to a decrease of its enzymatic activity (Figure 5A). We then tested whether the effect of nitrite on caspase-3 activity might contribute to the regulation of barrier function in BAECs exposed to hypoxia. Our results showed that nitrite significantly suppressed hypoxia-induced increases of permeability (Figure 5B). Based on these findings, we propose that the role of nitrite in endothelial cells is to protect the integrity of barrier function under hypoxia.

Nitrite Inhibits Hypoxia-Induced Disruption of Adherens Junctions in Human Umbilical Vein Endothelial Cells by Preventing β-Catenin Cleavage

To further explore the role of nitrite, we used BSM to measure the nitrosylation level of caspase-3 in primary human umbilical vein endothelial cells (HUVECs) exposed to hypoxic conditions. Treatment with nitrite suppressed hypoxia-induced denitrosylation (Figure 6A). This effect appeared to be XOR and eNOS-dependent, because the addition of their respective inhibitors, allopurinol and L-NAME, prevented nitrite-mediated enhancement of S-nitrosylation of caspase-3 (Figure 6B). Nitrite suppressed hypoxia-induced activation (Figure 6C) of caspase-3 and its cleavage (Figure 6D). These findings further supported that nitrite controlled S-nitrosylation levels of caspase-3 (Figure 6A and 6B). Because adherens junctions play a critical role in the regulation of vascular permeability,23 we wanted to assess the effect of nitrite on hypoxia-induced interruption of adherens junction in HUVECs. We stained VE-cadherin, which is the principal component controlling the formation of adherens junctions,23 to visualize cell-cell contacts. As shown in Figure 6E, the fully confluent monolayer of HUVECs displayed a typical subcellular localization of VE-cadherin, suggesting the development of well-organized adherens junctions at the cell periphery under the normoxic condition. In response to hypoxia, the number of cells with interrupted junctional VE-cadherin staining increased, which was indicated by the gap formation between neighboring HUVECs (Figure 6E). We observed that overall cell density was unchanged (Online Figure X), suggesting that the effect of hypoxia on junctional disruption was not due to cell loss. In HUVECs exposed to Z-DEVD-fmk, the typical VE-cadherin staining at cell junctions was once more observed despite a low oxygen concentration in the culture (Figure 6E). This result highlights the key role of caspase-3 in hypoxia-induced junctional interruption. Furthermore, nitrite treatment significantly suppressed the effect of hypoxia on adherens junctions. When nitrite was supplied, there was no longer a formation of hypoxia-induced gap between HUVECs in the monolayer (Figure 6E). Taken together, our data suggest that nitrite-mediated inactivation of caspase-3 might play a key role in the maintenance of endothelial junctions, which were otherwise interrupted under hypoxia in a caspase-3-dependent manner.

Because there was no significant change of expression level nor cleavage of VE-cadherin in HUVECs exposed to hypoxia (Online Figure X), we then asked whether other components in the cadherin complex might be targeted by caspase-3. Among them, β-catenin was our primary focus, since this protein functions as a bridge between cadherin and actin cytoskeleton via α-catenin.23 We examined whether the hypoxia-activated caspase-3 could cleave β-catenin in HUVECs. Interestingly, a number of proteolytic forms of β-catenin were detected in response to hypoxia (Figure 6F). Two of them right above the 70-kDa marker in a SDS gel were significantly decreased when cells were exposed to Z-DEVD-fmk, indicating a caspase-3-dependent cleavage of β-catenin in hypoxic cells. An analysis was performed to test the effect of nitrite in the context of hypoxic signaling. As shown in Figure 6G, the level of cleaved β-catenin isoforms was significantly reduced in HUVECs treated with nitrite. Thus, our data introduce a new role for nitrite, the prevention of hypoxia-induced cleavage of β-catenin. We propose that by suppressing proteolysis of β-catenin, nitrite is capable of maintaining the formation of cadherin complex and adherens junctions under hypoxia.

Nitrite Rescues Hypoxia-Induced Vascular Leakage in Zebrafish

We used a zebrafish model to study the effect of nitrite on intact blood vessels. Zebrafish embryos develop quickly. By 24 hours after fertilization (hpf), the embryos’ organ systems, including heart, are usually already formed.24 Thus, blood flow is being driven through the major axial vessels. We used a transgenic line (fli1:EGFP) of zebrafish embryos,25 which is transparent except for green-fluorescently labeled endothelia, allowing us to directly observe possible hypoxia-induced endothelial barrier dysfunction. To evaluate the degree of vascular leakage, we tracked the localization of red-fluorescent microspheres, which were
microinjected into the zebrafish blood vessels at 30 hpf. As shown in Figure 7A, living embryos exposed to hypoxia for 2 hours had significantly higher endothelial permeability than the control group. This led to microsphere extravasations along trunk vessels, particularly pronounced in intersegmental vessels and dorsal longitudinal anastomotic vessels. To investigate how nitrite might regulate vascular permeability, we treated microsphere-bearing embryos with nitrite (10 μmol/L) under hypoxic conditions. Intriguingly, nitrite effectively suppressed hypoxia-induced endothelial barrier dysfunction, as evidenced by a clear decrease in microsphere extravasations in intersegmental vessels and dorsal longitudinal anastomotic vessels (Figure 7A). The number of vascular leakages (Figure 7B) and the maximal distance of microsphere extravasations (Figure 7C) in zebrafish embryos exposed to hypoxia were much greater than those in normoxic controls. On the other hand, in the presence of nitrite, hypoxia had no effect on vascular leakage (Figure 7B and 7C). Thus, results obtained from this zebrafish model provide compelling evidence that nitrite prevents hypoxia-induced endothelial barrier dysfunction in vivo.

Discussion

Recent investigations have suggested that the nitrite treatment can potently inhibit hypoxic pulmonary arterial hypertension and suppress ischemic myocardial damage. However, to date it is not known how broad nitrite acts to inhibit hypoxic or ischemic injury in various tissues across the cardiovascular system. In the present study, we...
discovered a new and beneficial role for nitrite. This study has demonstrated that nitrite-derived NO prevented hypoxia-induced endothelial barrier dysfunction and has revealed the molecular basis for nitrite-mediated protection of hypoxic endothelia. The nitrite effect on intact blood vessels was further elaborated by a zebrafish model. To the best of our knowledge, this is the first report delineating the role of nitrite in regulation of vascular integrity in vivo by inhibiting endothelial permeability under the hypoxic condition.

The most important finding of this study is that nitrite-derived NO production in hypoxic endothelia promotes S-nitrosylation and the inactivation of caspase-3, leading to a sustained barrier function through better maintenance of cadherin junctions (Figure 7D). We showed that endogenous caspase-3 is constitutively S-nitrosylated in endothelia growing in a normal environment. Such modification may be important to ensure that the signaling pathways downstream of caspase-3 are in “switch-off.” Exposure of endothelia to the hypoxic condition in turn...
activates the machinery responsible for denitrosylation of caspase-3. Although the exact mechanism remains elusive, some recent studies have suggested thiolredoxin-mediated denitrosylation of caspase-3 plays a possible regulatory role in this redox signaling pathway. The denitrosylated form of caspase-3 is activated, resulting in cleavage of β-catenin, disruption of adherens junctions and ultimately increased permeability. In the presence of nitrite under hypoxia, the active-site Cys of caspase-3 returns back to the S-nitrosylated form via nitrite reductases-mediated NO production. Our data have demonstrated the critical role of XOR and eNOS in hypoxia-induced nitrite-NO conversion. However, additional nitrite reductases, such as cytochrome c or cytochrome c oxidase, might act synergistically together with XOR and eNOS in this context. Interestingly, in the hypoxic condition used in the current study, eNOS functioned exclusively as a nitrite reductase but it did not produce steady-state NO (Online Figure XI), suggesting a necessary role for nitrite, one in which it is used to deactivate caspase-3 in endothelia exposed to hypoxia. This inactivated form of caspase-3 can no longer cleave β-catenin. Consequently, the cadherin integrity remains intact, preventing endothelia from hypoxia-induced barrier dysfunction.

Biochemical and genetic evidences have shown that β-catenin, which bridges VE-cadherin and actin cytoskeleton, plays a critical role in regulating vascular permeability and integrity. On the basis of such observations, we postulated that signaling processes leading to an abnormal function of VE-cadherin in endothelium may impair the formation of adherens junctions. We further proposed that such a process might be the caspase-3-mediated signaling pathway, especially because β-catenin has been identified as a substrate of caspase-3. In the current study, we showed a hypoxia-dependent cleavage of β-catenin in HUVECs (Figure 6F and 6G). The appearance of 2 cleaved forms, which were 15=20 kDa smaller than the full-length β-catenin, required caspase-3 activity (Figure 6F). Interestingly, the sequence analysis revealed 2 potential caspase-3-targeting sites on the N-terminal region of β-catenin (Online Figure XII). Based on predictions, caspase-3-mediated cleavage on these sites would generate fragments of β-catenin with 15=20 kDa less in their sizes (Online Figure XII), which is what our data has shown (Figure 6F). If this is the case, the N-terminally-truncated form of β-catenin would lose its binding motif specifically for α-catenin (Online Figure XII). As a result, such cleaved β-catenin would no longer link to actin cytoskeleton due to the departure of α-catenin. Consequently, the disruption of cadherin complex may lead to a loss of barrier function as seen in endothelia exposed to hypoxia.

Our identification of nitrite as a potent agent that inhibits hypoxia-induced cleavage of β-catenin is interesting and important. This finding reveals the molecular basis for nitrite-mediated protection of endothelial barrier function against the hypoxic insult. As illustrated in Figure 7D, NO-dependent S-nitrosylation of caspase-3 is a key step to maintain the integrity of VE-cadherin complex because it contributes to the decreased cleavage of β-catenin by nitrite. In this regard, nitrite may function similarly as the caspase-3 specific inhibitor Z-DEVD-fmk. Our study showed that both agents inhibited the cleavage of β-catenin in HUVECs exposed to hypoxia (Figure 6F and 6G). However, taking a close look at our results, we observed that treatment with nitrite significantly diminished the level of all hypoxia-stimulated cleaved forms of β-catenin ranging from 70–85 kDa (Figure 6G), while the incubation of cells with Z-DEVD-fmk mainly affected the formation of two specific fragments of β-catenin (Figure 6F). These findings suggest that, in addition to inactivating caspase-3, nitrite may have a broader effect on protection of β-catenin integrity. In an attempt to elucidate possible functions of nitrite, we turned our attention to its role in the HIF-1α-Bnip3 pathway, which, on activation, may switch on caspase-3-independent signaling for regulation of various cellular events including apoptosis and autophagy. Our preliminary data showed that the stability of HIF-1α and the inducible expression of Bnip3 were significantly suppressed by nitrite in endothelia under hypoxia (Online Figure XIII). Interestingly, this process is likely to be NO-dependent (Online Figure XIII). Thus, we propose that in addition to the direct S-nitrosylation of caspase-3, nitrite-derived NO also acts to downregulate signaling events controlled by HIF-1α. More studies are required to clarify whether proteases that cleave β-catenin in a caspase-3-independent manner could be activated by the HIF-1α pathway in endothelia response to hypoxia.

In addition to a loss of adherens junctions and increased permeability as discussed above, apoptosis is also an important cellular event that promotes endothelial barrier dysfunction. Although the apoptotic signaling only affected a small fraction of cells in the experimental conditions applied in our study (Online Figure I), this event may contribute to barrier dysfunction in endothelia exposed to hypoxia. Indeed, using a sensitive measurement to detect apoptotic events, we demonstrated that nitrite treatment suppressed hypoxia-induced apoptosis effectively in endothelia (Online Figure XIV). In the presence of inhibitors against nitrite reductases, nitrite’s effect on regulation of apoptosis was lost (Online Figure XIV), suggesting a critical role of nitrite-derived NO in this signaling context. Additional investigations are needed to confirm the involvement of NO because the commonly used NO scavenger cPTIO was not functioning well under the hypoxic condition in our experimental setting (Online Figure XV). This effect of nitrite on the inhibition of hypoxia-induced apoptosis may explain in part previous observations that chronic nitrite therapy augmented angiogenic activity in a murine hind limb ischemia model. Perhaps due to reduced apoptosis in the presence of nitrite, endothelia could survive and even proliferate under long-term ischemia, consequently being remodeled for vascular formation. It is anticipated that a diverse array of hypoxia-governed signaling modules, which act synergistically to interrupt the normal barrier function of endothelia, may be targeted and down-regulated by nitrite-derived NO.
In conclusion, our findings open a new avenue for understanding the role of nitrite in preserving the vascular integrity against the hypoxic insult and an understanding of the underlying mechanism. Based on our results, it is tempting to hypothesize about the therapeutic potential of nitrite for the treatment of diseases involving hypoxia-associated endothelial barrier disorder such as atherosclerosis, stroke, and tumor metastasis. We propose that, as illustrated in the living zebrafish embryos (Figure 7A), by reducing pathological vessel leakiness, nitrite may strengthen the endothelial function, further reducing the harmful consequence of these pathological conditions. Due to nitrite’s unique ability to only act in hypoxic tissues but stay inert in healthy tissues, this agent may achieve a high specificity, a critical prerequisite for being considered in clinical use. More experiments are required to explore the therapeutic effect of nitrite on hypoxic or ischemic tissues.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**
- Hypoxia is a common perturbation that promotes endothelial barrier dysfunction, leading to the development of various diseases involving disrupted vascular homeostasis.
- Decreased bioavailability of nitric oxide (NO) contributes to endothelial barrier dysfunction, whereas exogenous treatment with NO donors protects the endothelium from hypoxia-induced barrier dysfunction.
- Nitrite can be reduced back to the bioactive form of NO by endogenous enzymes under conditions of hypoxia and ischemia.

**What New Information Does This Article Contribute?**
- Nitrite-derived NO can prevent endothelial barrier dysfunction under the hypoxic condition.
- The protective effect of nitrite on endothelial integrity is explained by the finding that caspase-3 is targeted and inactivated by nitrite-derived NO under the hypoxic condition.
- Nitrite protects intact blood vessels through suppression of hypoxia-induced vascular leakage in a zebrafish model.

Increased endothelial permeability has been regarded as a key step contributing to hypoxia-induced vascular barrier dysfunction, which in turn promotes the onset of diseases such as capillary leakage syndrome, atherosclerosis, stroke, and tumor metastasis. To date, it remains unclear how vascular disorders associated with hypoxia could be prevented. Here, we show for the first time that nitrite treatment suppresses the barrier dysfunction caused by hypoxia. Our data support a novel concept that endogenous caspase-3 is denitrosylated and activated in the hypoxic endothelium leading to disruption of adherens junction and increased permeability. Treatment with nitrite, which is reduced back to bioactive NO by endogenous enzymes, results in renitrosylation and inactivation of caspase-3, thus preventing the adverse effect of hypoxic insult on vascular integrity. Using zebrafish as a model, we show that nitrite suppresses hypoxia-induced vessel leakiness; thus strengthening the endothelial barrier function. These findings suggest that nitrite treatment may be useful for the treatment of diseases involving hypoxia-associated vascular disorders.
Nitrite-Mediated S-Nitrosylation of Caspase-3 Prevents Hypoxia-Induced Endothelial Barrier Dysfunction

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Reagents
All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Z-DEVD-fmk was obtained from R&D. Ferrous ammonium sulfate was purchased from J.T.Baker. S-Nitroso-N-acetylpenicillamine (SNAP) was a product of Merck. 100 nm red-fluorescent microspheres was obtained from Thermo Scientific.

Cell Culture, Hypoxia and Immunoblotting
Mouse pancreatic microvascular endothelial cells (MS-1, from ATCC) and bovine aortic endothelial cells (BAECs)\(^1\) were maintained in high glucose DMEM supplemented with 5% and 10% FBS, respectively. Human umbilical vein endothelial cells (HUVECs) were isolated as previously described,\(^2\) and were maintained in M199 medium supplemented with 20% FBS, 30 \(\mu\)g/ml ECGS and 5 \(\mu\)g/ml heparin. For normoxic and hypoxic exposure, endothelial cells were exposed under serum depletion and low glucose conditions. To achieve the hypoxic condition in culture medium, 100 ml of low glucose DMEM (Gibco) in a 250 ml tissue culture flask with 0.2 \(\mu\)M vented seal cap was kept in a MiniMACS anaerobic workstation (Don Whitley Scientific) supplied with 95% N\(_2\) and 5% CO\(_2\) for 3 days before using. Afterwards, the oxygen level in the medium was shifted from 7.6 ppm to 0.4 ppm as measured by a dissolved oxygen meter (G&B instruments). The oxygen level in the MiniMACS anaerobic workstation was adjusted to 1% as monitored by an oxygen sensor at all times. For experiments defined as under hypoxic conditions, endothelial cells (2x10\(^5\) cells per 1 ml oxygen-removed low glucose DMEM in a single well of 6-well culture plate) were placed in the anaerobic workstation for various times as indicated without any perturbation during incubation. For immunoblotting, aliquots of total lysates (25 \(\mu\)g) were subjected to immunoblotting with antibodies recognizing caspase-3 (#9662), Bnip3 (#3769) VE-cadherin (#2158, all three from Cell Signaling); HIF-1\(\alpha\) (#1536, R&D) and \(\beta\)-catenin (#610153, BD Biosciences).

Endothelial Permeability Assay
The procedure was modified from the published protocol.\(^3,4\) Briefly, endothelial cells (3.5x10\(^5\)) were seeded on 0.4% gelatin-coated transwell plates (24 mm diameter; 0.4 \(\mu\)M pore size; Millipore). Cells were cultured in phenol red-free medium before exposed to hypoxia. Prior to the end of hypoxic treatment, FITC-dextran (4 kDa for MS-1 cells; 40 kDa for BAECs; 1 mg/ml) was added to the upper chamber. After incubation for 5 min, an aliquot (100 \(\mu\)l) of medium from the lower compartment was collected for measurement by an ELISA reader equipped with a fluorescence attachment (Infinite F200, Tecan).
Measurement of S-nitrosylation of Endogenous Caspase-3

The S-nitrosylation level of caspase-3 was detected by the biotin-switch method as previously described with the exception of initial alkylation of reduced Cys residues by 50 mM IAM. Biotin-tagged proteins were subjected to immunoblotting with anti-caspase-3 antibody, or SDS-gel for mass spectrometric analysis. As shown in Fig. 2C, gel slices containing caspase-3 were proteolyzed with trypsin and then incubated with 25 mM ammonia bicarbonate supplemented with 5 mM tris (2-carboxyethyl) phosphine (TCEP) and 1 mM N-(2,3-dimethylphenyl)-2-iodoacetamide (DPIAM) at 37°C for 1 h. The extracted peptides were cleaned up by ZipTip (C18, Millipore) followed by liquid LC-MS/MS analysis using a nanoAcquity system (Waters) coupled with an LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Scientific). MS/MS data were processed by Bioworks 3.3.1 and searched against the UniProt database using Mascot 2.2 server. The matched MS/MS were manually validated.

EPR Measurement for NO Formation

Nitrite-derived NO by hypoxic endothelial cells was trapped by either Fe²⁺-(DETC)₂ or Fe²⁺-(MGD)₂ complex as described previously with minor modifications. For measurement of intracellular NO generation with Fe²⁺-(DETC)₂ complex, ferrous sulfate (FeSO₄·7H₂O) and DETC were each dissolved separately in deoxygenated, iced-cold 0.9% NaCl. Fe²⁺-(DETC)₂ complex was then prepared immediately before using by mixing 3 mM ferrous sulfate solution with 3 mM DETC solution. For each treatment, Fe²⁺-(DETC)₂ colloid (500 µl) was added drop-wisely to the surface of monolayer of endothelial cells (in 10-cm dish) followed by incubation for 30 min. Cells then were scraped gently while phenol red-free DMEM (600 µl) were added. The cell suspension was collected and transferred into an EPR tube (Wilmad LabGlass) followed by snap-freezing with liquid nitrogen. For extracellular NO trapping, ferrous ammonium sulfate and MGD were each dissolved separately in deoxygenated distilled water. The Fe²⁺-(MGD)₂ complex was then freshly prepared by mixing 1 mM Fe²⁺ with 5 mM MGD under hypoxia. Cells (5-8x10⁷ cells per treatment) were trypsinized and resuspended in phenol red-free DMEM before transferred into the hypoxic incubator. Prior to the end of each treatment, the Fe²⁺-(MGD)₂ complex was added to the cell suspension and incubated for additional 30 min under hypoxia. Cells were then transferred to an EPR tube and snap-frozen by liquid nitrogen. EPR spectra were performed with Bruker ER580 pulse and CW spectrometer (Bruker) at 77K with 1 mW microwave power, 100 kHz modulation frequency, 5 G modulation amplitude, 5242.88 ms time constant, 327.68 ms conversion time and 4 scans. SNAP was used as a positive control for the NO detection.

Caspase-3 Activity Assay

Caspase-3 catalytic activity was measured using a colorimetric assay kit (Millipore) according to the manufacturer’s instructions. Briefly, whole cell lysates from 2 x 10⁵ cells were prepared with lysis buffer. 70 µg of cell lysate was reacted with substrate reaction buffer containing a colorimetric
substrate of caspase-3, DEVE-p-nitroaniline (Ac-DEVE-pNA). This mixture was incubated for 2.5 h at 37°C. The absorbance was evaluated at 405nm by an ELISA reader (Infinite F200, Tecan).

**Immunofluorescence Staining**

Endothelial cells (2 x 10^5) were plated on glass coverslips. After treatment, cells were fixed with 4% paraformaldehyde, permeabilized by 0.2% Triton X-100, and then stained with anti-VE-cadherin antibody or DAPI. Samples were visualized using a Zeiss LSM 510 confocal microscope.

**Measurement of Vascular Leakage in Zebrafish Embryos**

The Tg(fli1:EGFP) line of zebrafish were maintained at 28.5°C on a 14 h light/10 h dark cycle. Embryos were collected by natural spawning in 1-phenyl-2-thiourea (PTU)/E3 egg water at 28.5°C. Microinjection of vascular leakage tracers into embryos was carried out following a published protocol with minor modifications. Briefly, 100 nm red-fluorescent microspheres were freshly coated with BSA (4% in PBS) for 2 h at RT. An aliquot of microsphere suspension (4.6 nl) was injected into a tricaine-anesthetized embryo at 30 hpf through the common cardinal vein using a gas-driven microinjector (IM300, Narishige). Only embryos bearing microspheres after injection were used for further analyses. For visualization of vascular leakage, embryos were exposed to either normoxic or hypoxic condition for 2 h at 28.5°C. The hypoxic condition was achieved by incubating embryos (5 embryos per 2 ml PTU/E3 water in a single well of 24-well culture plate) in a hypoxic chamber (Astec, set up at 1.5% O2) supplied with 95% N2 and 5% CO2. Embryos were anesthetized with tricaine and then mounted in 4% methylcellulose before photographed. Leakage of microspheres from ISVs and DLA Vs was recorded by fluorescent microscopy (Imager A1, Zeiss).

**Apoptosis Assay and Annexin V staining**

Apoptosis detection was majorly performed with an in situ cell death detection kit (Roche) according to the manufacture’s directions. In brief, 2 x 10^5 cells were lysed in 200 µl lysis buffer and centrifuged at 200 g for 10 min at room temperature. 10 µl of supernatant was incubated with 80 µl immunoreagent buffer for 2 h at room temperature. The absorbance was evaluated at 405nm by an ELISA reader (Infinite F200, Tecan). Apoptosis distribution was performed using an Annexin V-FITC apoptosis detection kit (BioViosion) by a FACSCalibur flow cytometry (BD Biosciences) according to the manufacturer’s instructions.

**Statistics**

Statistical significance was determined using a two-tailed Student’s t test. P < 0.05 was considered significant.
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Online Figure I

The effect of Z-DEVD-fmk on hypoxia-induced caspase-3 activation and apoptosis. MS-1 cells were exposed to normoxia (N) or hypoxia (H, 1% O₂) in the absence or presence of Z-DEVD-fmk (25 μmol/L) for 16 h. Caspase-3 activity (A) and the levels of cleaved form of caspase-3 (B) were measured. C, The sub-population of cells undergoing apoptosis was determined using Annexin V/PI staining by a FACSCalibur flow cytometry. Note only ~20% MS-1 cells were in the apoptotic phase after exposed to hypoxia. D, Apoptosis was measured by a cell death detection kit. The data shown in A and D are presented as the means±SE (n=3). *P<0.05 when compared with normoxic controls. †P<0.05 when compared with untreated controls.
Online Figure II

EPR spectra of NO-Fe$^{2+}$-(DETC)$_2$ adducts. A, Representative EPR spectra of NO-Fe$^{2+}$-(DETC)$_2$ adducts formed after 30 min incubation of Fe$^{2+}$-(DETC)$_2$ complex with SNAP (100 μmol/L) under hypoxia (a). When SNAP is absent, no EPR spectrum was observed (b). B, Monolayer of MS-1 cells were exposed to hypoxia, during which nitrite (100 μmol/L) was added at 5 h post-hypoxia. After incubation for 30 min, Fe$^{2+}$-(DETC)$_2$ complex were added drop-wisely to the monolayer of cells. The cellular NO levels were then measured by EPR at 6 h post-hypoxia (a). No EPR spectrum was observed after 30 min incubation of nitrite with Fe$^{2+}$-(DETC)$_2$ complex under hypoxia in the absence of MS-1 cells (b), or after 30 min incubation of Fe$^{2+}$-(DETC)$_2$ complex alone under hypoxia (c).
Online Figure III

**EPR spectra of NO-Fe\(^{2+}\)-(MGD)\(_2\) adducts.** A, Representative EPR spectra of NO-Fe\(^{2+}\)-(MGD)\(_2\) adducts obtained by incubation of SNAP (100 μmol/L) with the Fe\(^{2+}\)-(MGD)\(_2\) complex for 30 min under hypoxia. B, MS-1 cells growing in suspension were exposed to hypoxia, during which nitrite (100 μmol/L) and Fe\(^{2+}\)-(MGD)\(_2\) complex were added at 5 h post-hypoxia. The NO levels in culture media were measured by EPR at 5.5 h post-hypoxia. Note that no EPR spectrum was observed after 30 min incubation of nitrite with Fe\(^{2+}\)-(MGD)\(_2\) complex under hypoxia in the absence of MS-1 cells, or after 30 min incubation of Fe\(^{2+}\)-(MGD)\(_2\) in the absence of nitrite with MS-1 cells which were already exposed to hypoxia for 5 h. C, The level of NO generated from nitrite (100 μmol/L) by MS-1 cells under hypoxia was measured by the EPR method as described in B. Note that the EPR spectrum looks similar to that obtained by incubation of SNAP (10 μmol/L) with the Fe\(^{2+}\)-(MGD)\(_2\) complex for 30 min under hypoxia.
Online Figure IV

Nitrite does not affect the S-nitrosylation level of caspase-3 under normoxic condition. MS-1 cells were exposed to normoxia (N) or hypoxia (H), during which nitrite (10 μmol/L) was added at 5 h post-hypoxia. The S-nitrosylation levels of endogenous caspase-3 were determined by the BSM at 6.5 h post-hypoxia.
Online Figure V

Dose effect of nitrite on hypoxia-induced endothelial permeability. MS-1 cells were exposed to normoxia (N) or hypoxia (H), during which various doses of nitrite were added at 5 h post-hypoxia. Endothelial permeability was determined at 16 h post-hypoxia. Data are presented as the means±SE (n=3). #P<0.05 when compared with untreated cells.
Online Figure VI

**Measurement of the mRNA expression levels of XOR, eNOS and AO.** MS-1 cells were exposed to normoxia (N) or hypoxia (H) for 5 h. The mRNA levels of XOR, eNOS and AO were measured by RT-PCR. Representative images from one of two independent experiments are shown.
Online Figure VII

The XOR inhibitor oxypurinol suppresses NO conversion from nitrite in hypoxic MS-1 cells. EPR spectra for NO-Fe$^{2+}$-(MGD)$_2$ adducts formed in culture media of MS-1 cells under hypoxia. Cells were treated with oxypurinol (500 μmol/L) at 4.5 h post-hypoxia or left untreated. Nitrite (100 μmol/L) and Fe$^{2+}$-(MGD)$_2$ complex were then added at 5 h post-hypoxia. NO levels were measured by the EPR method at 5.5 h post-hypoxia.
The AO inhibitor raloxifene does not affect NO conversion from nitrite in hypoxic MS-1 cells. EPR spectra for NO-Fe$^{2+}$-(MGD)$_2$ adducts formed in culture media of MS-1 cells under hypoxia. Cells were treated with raloxifene (50 µmol/L) at 4.5 h post-hypoxia or left untreated. Nitrite (100 µmol/L) and Fe$^{2+}$-(MGD)$_2$ complex were then added at 5 h post-hypoxia. NO levels were measured by the EPR method at 5.5 h post-hypoxia.
Online Figure IX

The effect of Z-DEVD-fmk on hypoxia-induced endothelial permeability in BAECs. Endothelial permeability was measured in BAECs exposed to normoxia (N) or hypoxia (H) for 16 h in the absence or presence of Z-DEVD-fmk (12.5 μmol/L). The data are presented as the means±SE (n=3). *P<0.05 when compared with normoxic controls. #P<0.05 when compared with untreated controls.
Online Figure X
Measurements of cell density and the level of VE-cadherin in HUVECs response to hypoxia. A, Cell density of HUVEC’s monolayer was unchanged after exposure to hypoxia (H) for 8 h compared with normoxic controls (N), despite the fact that VE-cadherin-mediated adherens junctions were significantly lost under hypoxia. Cells were double stained with VE-cadherin and DAPI. Images were captured by confocal microscopy with the same exposure time. B, HUVECs were exposed to hypoxia for 6.5 h. Nitrite (10 μmol/L) was treated at 4 h post-hypoxia. Aliquots of lysates were subjected to immunoblotting with anti-VE-cadherin and anti-tubulin antibodies. Representative images from one of three independent experiments are shown.
Online Figure XI

Treatment of MS-1 cells with L-NAME does not affect NO generation under hypoxia in the absence of nitrite. EPR spectra for NO-Fe$^{2+}$-(MGD)$_2$ adducts formed in culture media of MS-1 cells under hypoxia. Cells were treated with L-NAME (1 mmol/L) at 4.5 h post-hypoxia or left untreated. NO levels were measured by the EPR method at 5 h post-hypoxia.
Online Figure XII

Sequence analysis of β-catenin revealed several potential caspase-3-targeting sites. Schematic diagram shows potential cleavage sites of caspase-3 (red arrows). Several proteolytic forms of β-catenin ranging from 70 to 80 kDa may appear in response to caspase-3-mediated cleavage, as indicated by “predicted products” shown in the bottom of the diagram. The α-catenin binding region and the epitope of β-catenin antibody are marked. Note that “predicted products” might be recognized by the antibody against β-catenin used in this study.
Online Figure XIII

The effects of nitrite on the stability of HIF-1α and the inducible expression of Bnip3 in endothelial cells response to hypoxia. A, MS-1 cells were exposed to hypoxia (H) for 6.5 h, during which nitrite (10 μmol/L) was added at 5 h post-hypoxia. Changes of HIF-1α and Bnip3 protein levels were determined by immunoblotting. B, MS-1 cells were treated as described in A with the combined treatment of allopurinol (100 μmol/L) and L-NAME (100 μmol/L) at 4.5 h post-hypoxia. Note that the inhibition of nitrite reductases inhibited nitrite effect on the stability of HIF-1α and the inducible expression of Bnip-3 under hypoxia. C, BAECs were exposed to hypoxia for 6.5 h. Nitrite (10 μmol/L) was supplied at 4.5 h post-hypoxia. Consistent with the observations in MS-1 cells, both the stability of HIF-1α and the inducible expression of Bnip3 were suppressed by nitrite in BAECs response to hypoxia. D, HUVECs were exposed to hypoxia for 6.5 h. Nitrite was added at 4 h post-hypoxia. Note that the stability of HIF-1α was suppressed significantly by nitrite in HUVECs under hypoxia. However, we were unable to detect a clear signal of Bnip3 in lysates prepared from HUVECs. A-D, Representative images from one of three independent experiments are shown.
Online Figure XIV

The effect of nitrite on hypoxia-induced apoptosis. A, MS-1 cells were exposed to hypoxia (H) for 16 h. Nitrite (10 μmol/L) was added at 5 h post-hypoxia. Apoptosis was then determined by a cell death detection kit. B, MS-1 cells were treated as described in A with the inclusion of allopurinol (100 μmol/L) and L-NAME (100 μmol/L) at 4.5 h post-hypoxia. Note that in the presence of allopurinol and L-NAME, nitrite supplement could no longer suppress hypoxia-induced apoptosis. C, MS-1 cells were treated as described in A with the exception that nitrite was added at various times as indicated. The anti-apoptotic effect of CSNO was also examined following the same experimental procedure, in which CSNO (10 and 100 μmol/L) was added at 5 h post-hypoxia. The data are presented as the means±SE (n=3). *P<0.05 when compared with normoxic controls. #P<0.05 when compared with untreated controls. †P<0.05 when compared with nitrite-treated cells.
Online Figure XV

Treatment of MS-1 cells with cPTIO does not affect nitrite-mediated inhibition of hypoxia-induced apoptosis. MS-1 cells were exposed to normoxia (N) or hypoxia (H) in the absence or presence of 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 100 μmol/L) for 16 h. Nitrite (10 μmol/L) was added at 5 h post-hypoxia. Apoptosis was determined by a cell death detection kit. Note that cPTIO at 100 μmol/L has been used extensively as a NO scavenger to confirm the effect of NO on regulating a diverse array of signaling events under the normoxic condition. [...]

However, in the experimental setting applied in the current study, cPTIO failed to ablate NO-mediated cytoprotection contributed by the nitrite treatment under the hypoxic condition. Unlike the treatment of allopurinol (100 μmol/L) and L-NAME (100 μmol/L) that inhibited the beneficial effect of nitrite via down-regulation of nitrite-NO conversion (Online Figure XIV), in the absence or presence of cPTIO, nitrite could suppress hypoxia-induced apoptosis indistinguishably. These results suggest that cPTIO might not function as an effective NO scavenger under the hypoxic condition. Additional investigations are needed to confirm the involvement of NO in nitrite-mediated cytoprotection against hypoxia-induced endothelial insults. The data are presented as the means±SE (n=3). *P<0.05 when compared with normoxic controls. #P<0.05 when compared with untreated controls.
SUPPLEMENTAL REFERENCES


