MINIREVIEW

Role of Nitric Oxide in Cardiovascular Adaptation to Intermittent Hypoxia

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Hypoxia is one of the most frequently encountered stresses in health and disease. The duration, frequency, and severity of hypoxic episodes are critical factors determining whether hypoxia is beneficial or harmful. Adaptation to intermittent hypoxia has been demonstrated to confer cardiovascular protection against more severe and sustained hypoxia, and, moreover, to protect against other stresses, including ischemia. Thus, the direct and cross protective effects of adaptation to intermittent hypoxia have been used for treatment and prevention of a variety of diseases and to increase efficiency of exercise training. Evidence is mounting that nitric oxide (NO) plays a central role in these adaptive mechanisms. NO-dependent protective mechanisms activated by intermittent hypoxia include stimulation of NO synthesis as well as restriction of NO overproduction. In addition, alternative, nonenzymic sources of NO and negative feedback of NO synthesis are important factors in optimizing NO concentrations. The adaptive enhancement of NO synthesis and/or availability activates or increases expression of other protective factors, including heat shock proteins, antioxidants and prostaglandins, making the protection more robust and sustained. Understanding the role of NO in mechanisms of adaptation to hypoxia will support development of therapies to prevent and treat hypoxic or ischemic damage to organs and cells and to increase adaptive capabilities of the organism. Exp Biol Med 231:343–365, 2006

Key words: cardioprotection; hemoglobin; ischemia/reperfusion damage; nitric oxide synthase; nitric oxide stores; peroxynitrite

Favorable and Adverse Effects of Intermittent Hypoxia

Intermittent hypoxia may be imposed by physiological challenges, including strenuous exercise or sojourns at high altitude, or by several diseases, including obstructive lung disease, asthma, and sleep apnea. Extensive basic and clinical research has investigated the impact of intermittent hypoxia on cells, organs, and intact organisms. Much of this research has been directed to delineate mechanisms of physiological responses to acute hypoxic challenges, and to define the cardiovascular adaptations to chronic intermittent hypoxia. Powerful protective capabilities of intermittent hypoxia have been demonstrated, and current studies are revealing important details regarding mechanisms of hypoxia-induced cardiovascular protection.

Since conditions of oxygen deficiency have influenced organisms throughout evolution, mechanisms to sense and adjust to hypoxia are well developed (1). For example, breathing a hypoxic atmosphere activates the sympathetic nervous system to enhance cardiac function and constrict the peripheral circulation, thereby maintaining aortic blood pressure and ensuring adequate delivery of oxygen to the brain and myocardium despite reduced arterial oxygen content. Mechanisms for adaptation to intermittent hypoxia may also provide protection against more severe and/or sustained hypoxia, and, interestingly, confer protection against other stresses, including ischemia (2–5). According to the concept of cross adaptation (2), development of resistance to one factor confers resistance also to other factors, depending on the pattern of gene expression evoked by the primary stress factor. This concept applies to intermittent hypoxia, which elicits a spectrum of direct and cross protective effects (2) that have been used for treatment and prevention of a variety of diseases and to increase the efficiency of exercise training.

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Intermittent hypoxia produces myriad favorable effects in the cardiovascular system, brain, and other organ systems. These effects can be grouped into five major categories:

1) *Adaptation of organs and tissues responsible for oxygen uptake and transport*, exemplified by increased alveolar surface for pulmonary gas exchange (6), proliferation and increased density of vascular networks (7–10), enhanced blood oxygen-carrying capacity due to polycythemia (11), increased mitochondrial density in brain, liver, and heart (12, 13), and hypertrophy of brainstem respiratory neurons (2). This growth of structures and tissues responsible for adaptation to hypoxia, comprising the "systemic structural trace" of adaptation, results from selective increases in RNA and protein synthesis in lungs, heart, bone marrow, coronary blood vessels, and cardiac sympathetic neurons (2). Consequently, adaptation to intermittent hypoxia has been used to treat patients with ischemic heart disease (11, 14), with cardiovascular risk factors, such as obesity and smoking (15), and with post–myocardial infarction heart failure (16). Intermittent hypoxia therapy evoked beneficial hemodynamic changes, increased myocardial contractility, and exerted a persistent antiarrhythmic effect in patients with neurocirculatory asthenia associated with ventricular and supraventricular extrasystole (14). In addition, adaptation to hypoxia improved myocardial contractility in patients with ischemic heart disease. This symptomatic improvement allowed reducing drug doses and withdrawing drug therapy in patients with neurocirculatory asthenia (14). After adaptation to hypobaric hypoxia the body mass index decreased by 10%, low-density lipoprotein cholesterol decreased, and high-density lipoprotein cholesterol increased (15). Patients who breathed hypoxic gas mixtures (FIO_2 14%–16%) from Days 7 to 27 post–myocardial infarction had improved recovery of hemodynamic performance and exercise tolerance compared with untreated patients (16).

2) *Anti-stress action in the brain and other target organs*. Intermittent hypoxia has been shown to improve the mental and physical performance of healthy people functioning under stressful conditions, for example, space flight or military operations (17, 18). Hypoxic therapy has also been employed pre- and postoperatively to decrease stress contributing to postsurgical complications (17, 19, 20). In women adapted to interval normobaric hypoxia 15–25 days before surgery for uterine myoma, the relative volume of myometrial blood vessels was larger and the volume of stroma was smaller than in untreated patients. Adaptation to hypoxia increased the capillary index and neovascularization, which improved myometrial blood supply. Patients adapted to hypoxia displayed less severe symptoms of mental stress, irritability, and sleep disorders (19, 20). Moreover, intermittent hypoxia has been successfully used in complex therapies for neuroses (15), paranoid schizophrenia (2), Parkinson's disease (21), idiopathic cardiac arrhythmias (22), and alcohol abuse (15, 23). For example, adaptation to intermittent hypobaric hypoxia decreased the incidence of ventricular and supraventricular extrasystole by more than 75% in patients with neurasthenic syndrome and nonischemic arrhythmias (22). Adaptation to hypoxia exerted antipsychotic, sedative, and anxiolytic effects and improved response to therapy in patients with drug-resistant schizophrenia (2). Men with stage 2 chronic alcoholism were adapted to intermittent hypoxia at a simulated altitude of 3,500 m for 3 hrs daily over 24 days. After the first 7–10 sessions of hypoxia the patients reported a reduced drive for alcohol and improved sleep, mood, and appetite, and they became more communicative, self-reliant, and independent. Adaptation to hypoxia improved biochemical variables, including decreased liver enzyme release, enhanced antioxidant defense, and reduced lipid peroxidation products (15, 23).

3) *Anti-hypertensive effects*. Intermittent hypoxia modifies water and salt metabolism (2), alters myogenic vascular tone (24), and increases synthesis of vasodilatory factors (25–27). In combination, these actions are antihypertensive, and, indeed, intermittent hypoxia programs have proven efficacious for treating hypertension (17, 28), including preeclampsia in pregnancy (17, 29).

4) *Adaptive changes in the immune system*. Intermittent hypoxia therapy has been effective for treatment of arthritis, bronchial asthma, autoimmune thyroiditis, and allergic dermatitis (30, 31). In children with bronchial asthma, adaptation to hypoxia (3,500 m; 3.5 hrs daily for 20–25 days) increased the attack-free period from 2–4 weeks to 2 months. This effect was associated with decreased circulating immune complexes and increased serum immunoglobulins. Similar beneficial immune responses were observed in adult patients with allergic dermatosis, in whom the affected skin area decreased from 44% to 16%, the continuous lesion area fell from 29% to 8%, and the pruritus score declined from 3.7 to 0.19 (30, 31).

5) *Enhancement of hepatic lipid metabolism*. Adaptation to intermittent hypoxia activates the cytochrome P450 system and 7α-cholesterol hydroxylase in liver (32). By these mechanisms, intermittent hypoxia may provide protection against atherogenic dyslipidemia, an important risk factor for atherosclerosis.

Beneficial Versus Adverse Effects of Intermittent Hypoxia Are Dependent on the Hypoxic Regimen

Chronic or intermittent hypoxia may have serious pathophysiological consequences, including pulmonary and systemic hypertension, myocardial infarction, stroke, and cognitive dysfunction (33–35), depending on the severity and duration of the hypoxia insult. On the other hand, hypoxia may be well tolerated and produce favorable effects if the duration is brief or the reduction in inspired oxygen is more moderate.

Angiogenesis produced by intermittent hypoxia may be protective. Vascular capacity was increased in both left and

right ventricles of rats adapted to intermittent high-altitude hypoxia (36). By increasing vascular endothelial growth factor expression (37, 38), alternating hypoxia and reoxygenation promoted myocardial angiogenesis and thereby decreased the intercapillary diffusion distance for bloodborne fuels and O_2 (39). Coronary flow increased (9) and coronary resistance fell after adaptation to intermittent hypoxia, contributing to cardioprotection from ischemia-reperfusion injury in rats (9, 40). Intermittent hypoxia increased vascularity in skeletal muscles and enhanced exercise performance (8). Similar angiogenic effects of adaptation to hypoxia were observed in rat brain, in which the average intercapillary distance decreased from ~50 to ~40 μM after 3 weeks of hypobaric hypoxia at a pressure of 380 torr (0.5 atm, equivalent to ~10% normobaric oxygen) (41). Increased density of brain vessels could be a mechanism for the antihypertensive effect of adaptation to hypoxia, which prevented the functional rarefaction of arterioles and capillaries in brains of spontaneously hypertensive rats (7, 42).

Persistent hypertension is a common maladaption to severe, sustained, or intermittent hypoxia. Frequent alternation of breathing hypoxic gas (6 secs of 2%–3% O_2 every 30 secs) and atmospheric air for several hours per day over 5 to 7 weeks produced a sustained increase in arterial blood pressure in canine (43) and rat (44, 45) models of obstructive sleep apnea. This hypertension resulted primarily from increased sympathetic activity and from oxidative stress (46, 47). It should also be noted that hypoxia induces hyperventilation, which decreases arterial PCO_2, unless ventilation is restricted as in obstructive lung disease, asthma, and sleep apnea. Thus, in addition to a shorter hypoxia stress, sleep apnea differs from other chronic or intermittent hypoxia protocols by elevating arterial PCO_2. This effect may, in part, be responsible for disparate findings of sleep apnea studies, since Neckář *et al.* (48) found that hypercapnia blunted the cardioprotective effects of chronic hypoxia.

The response of endothelium-dependent relaxation to intermittent hypoxia strikingly depends on the hypoxic regimen. Thus, 14 days of intermittent hypoxia (10% FIO_2 for 1 min at 4-min intervals, 12 hrs/day) reduced the bioavailability of nitric oxide (NO) in the cerebral and skeletal muscle circulations and severely impaired endothelium-dependent vasodilation in rats (49). In another rat study, brief (6-sec) exposures to 2%–3% FIO_2 at 30-sec intervals for 7 hrs per day over 35 days produced a sustained elevation of blood pressure (45). In contrast, adaptation to a more moderate intermittent hypoxia regimen (simulated altitude 4,000–5,000 m [equivalent to 12%–10% FIO_2], 4 hrs/day for 40 days) stimulated NO production, preventing development of endothelial dysfunction in spontaneously hypertensive rats (50). In normotensive rats, the same intermittent hypobaric hypoxia regimen did not affect endothelium-dependent relaxation of the aorta, but augmented endothelium-dependent relaxation to acetylcholine and inhibited norepinephrine-induced contractions of smaller arteries (51, 52).

Impaired electron flux through the mitochondrial respiratory chain is an important cause of oxidative stress during intermittent hypoxia (34). Decreased availability of oxygen, the terminal electron acceptor of the respiratory chain, causes ubisemiquinone, a partially reduced free radical form of coenzyme Q, to accumulate. Ubisemiquinone readily transfers its unpaired electron to residual oxygen, generating the superoxide radical anion ($\cdot O_2^-$). Although $\cdot O_2^-$ itself is not especially harmful, it is a precursor of the highly reactive, cytotoxic hydroxyl radical ($\cdot OH$) and, by irreversible condensation with nitric oxide, peroxynitrite ($ONOO^-$) (53). Persistent oxidative and nitrosative stress likely contributes to the morbidity associated with chronic, brief intermittent hypoxia caused by recurrent apneas (34). Accordingly, Joyeux-Faure *et al.* (54) demonstrated that repetitive cycles of 40 seconds of hypoxia (FIO_2 5%) for 35 days increased ischemia-induced infarction of rat hearts. In contrast, Zong *et al.* (3) reported that a 20-day adaptation program using 5- to 10-min periods of hypoxia (FIO_2 9.5%–10%) followed by 4 mins of normoxia repeated five to eight times daily was markedly protective against ischemia-induced infarction of canine hearts.

In contrast to the hypertensive effects of frequent, brief, intermittent hypoxia, as occurs in sleep apnea, adaptation to hypobaric hypoxia at simulated altitudes of 4,000–5,000 m (equivalent to 12%–10% inspired O_2 at sea level) for 4 to 5 hrs per day over 40 days exerted a pronounced depressor effect in spontaneously hypertensive rats (30, 50, 55). Similar antihypertensive effects resulted from normobaric breathing of 9%–14% O_2 for 3 to 8 mins with 3-min normoxic intervals for 40–60 mins per day over 20–30 days in rats and patients (28). Moreover, these hypoxia regimens attenuated both basal and stress-induced sympathetic activity in rats (56, 57).

Intermittent hypoxia improves energy producing metabolic processes by increasing formation of mitochondria in brain and liver, activating electron flux through respiratory complex I, and increasing efficiency of oxidative phosphorylation (12, 58, 59). Reintroduction of oxygen to the reductive intracellular environment during intermittent reoxygenations could generate reactive oxygen species, which may function as signaling molecules activating transcription and eventual synthesis of antioxidant enzymes. Thus, the process of reoxygenation, as well as the period of hypoxia, may play an important role in the adaptation to intermittent hypoxia. The ultimate balance between excessive production of free radicals and enhancement of antioxidative processes directly depends on the experimental regimen.

It is evident that duration, frequency, and severity of hypoxic episodes are critical factors determining whether intermittent hypoxia is beneficial or harmful (60), and protocols used in experimental studies of adaptation to hypoxia have varied greatly (33). Inspired O_2 has varied

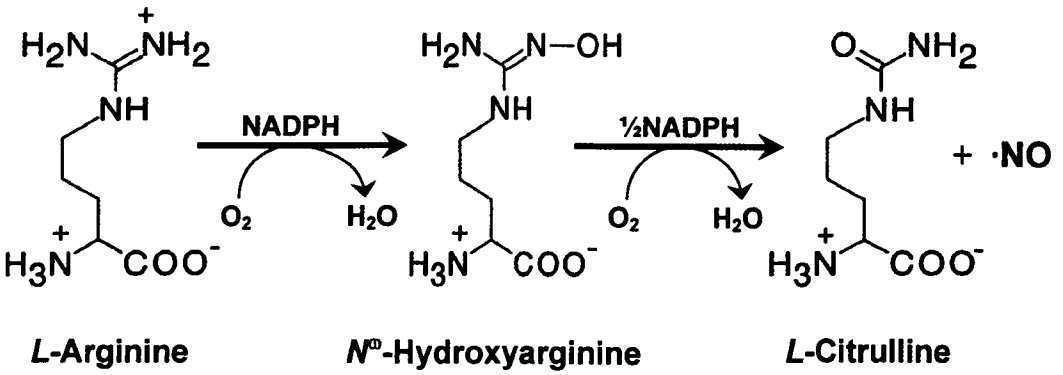

Figure 1. Biosynthesis of nitric oxide from *L*-arginine. NO is produced by five-electron oxidation of the terminal guanidine group of *L*-arginine. The NOS reaction requires flavins and tetrahydrobiopterin as cofactors, while the production of NO itself requires O_2 and NADPH. The reductase domain of NOS transfers reducing equivalents from NADPH to the heme domain, where oxidation of *L*-arginine occurs by a two-step reaction. First, the NOS flavin accepts an electron from NADPH and transfers it to the heme iron, transforming $NOSFe^{3+}$ to $NOSFe^{2+}$. In this step, *L*-arginine is hydroxylated to form an enzyme-bound intermediate, N^{ω}-hydroxyl-*L*-arginine. Next, the heme iron binds O_2 and the enzyme consumes NADPH to oxidize N^{ω}-hydroxyl-*L*-arginine to citrulline and NO (66–68).

from as low as 2%–3% O_2 (43–45) to as high as 14%–16% O_2 (16). The duration of exposure to experimental hypoxia has ranged from seconds to hours, and when the duration of hypoxia was short, hypoxia exposures were often repeated multiple times per day. Intermittent hypoxia programs have varied from as short as 1 day to as long as 90 days. Variable protocols have certainly contributed to current confusion regarding the benefits of intermittent hypoxia.

Adaptation to intermittent hypoxia may be beneficial by increasing efficiency of vascular oxygen transport and energy supply (2, 61), inducing protective antioxidant enzymes such as catalase and superoxide dismutase (62) and heat shock proteins (63), stabilizing cellular membranes (64), and restricting apoptosis (65). There is increasing evidence that NO plays a pivotal role in adaptation to intermittent hypoxia, and the remainder of this review will specifically examine this issue.

NO Formation and Biochemistry

NOS Isoforms, Expression, and Activation. NO is produced by five-electron oxidation of the terminal guanidine group of *L*-arginine. This complex reaction (Fig. 1) is catalyzed by the enzyme NO synthase (NOS), a hemoprotein containing both oxidative and reductive domains. The NOS reaction requires flavins and tetrahydrobiopterin as cofactors, while the production of NO itself requires O_2 and NADPH. The reductase domain of NOS transfers reducing equivalents from NADPH to the heme domain, where *L*-arginine is oxidized by a two-step reaction. First, the NOS flavin accepts an electron from NADPH and transfers it to the heme iron, reducing $NOSFe^{3+}$ to $NOSFe^{2+}$. In this step, *L*-arginine is hydroxylated to form an enzyme-bound intermediate, N^{ω}-hydroxyl-*L*-arginine. Next, the heme iron binds O_2 and the enzyme consumes NADPH to oxidize N^{ω}-hydroxyl-*L*-arginine to citrulline and NO (66–68).

The NOS family consists of three major isoforms: neuronal NOS (nNOS or NOS-I), inducible NOS (iNOS or NOS-II), and endothelial NOS (eNOS or NOS-III). These NOS isoforms were originally named according to the tissue where they were first identified, although later they were found elsewhere. Thus eNOS, a membrane-bound isoform, was first discovered in the vascular endothelium and has been later identified not only in vascular endothelial cells but also in platelets, myocardium, and endocardium. Neuronal NOS is cytosolic, and is present in skeletal muscle as well as in the brain, spinal cord, and peripheral nervous system. The cytosolic inducible NOS isoform is present in immune cells, vascular smooth muscle, astrocytes, fibroblasts, and hepatocytes (for review see Ref. 66). At present the classification of NOS isoforms as constitutive and inducible seems imprecise (for review see Ref. 69), since the constitutive eNOS can be induced in certain situations, such as during chronic exercise (70) or during pregnancy (71), whereas iNOS appears to be present constitutively in some tissues, including human bronchial epithelium (72), rat kidney (73), and some fetal tissues (74).

The constitutively expressed nNOS and eNOS synthesize NO in response to Ca^{2+}-dependent calmodulin binding. Thus, stimuli that increase intracellular Ca^{2+}, such as acetylcholine, bradykinin, ATP, serotonin, and thrombin, elicit NO synthesis by nNOS or eNOS within a few seconds. iNOS is primarily regulated by transcriptional mechanisms and binds calmodulin irrespective of Ca^{2+} concentration.

NO Biochemistry. The complex biological chemistry of NO and its derivatives, known collectively as reactive nitrogen species, is discussed in several authoritative reviews (75–82). Figure 2 summarizes the chemistry of NO and its derivatives pertinent to cell-physiological conditions and for which empirical evidence exists.

Although it contains an unpaired electron, NO is a relatively nonreactive free radical, and many of its physiological and pathological actions are mediated by its more aggressive derivatives. Irreversible, diffusion-limited condensation of NO and $\cdot O_2^-$ generates $ONOO^-$. Nitrogen dioxide radical ($\cdot NO_2$) is produced by direct reaction of NO

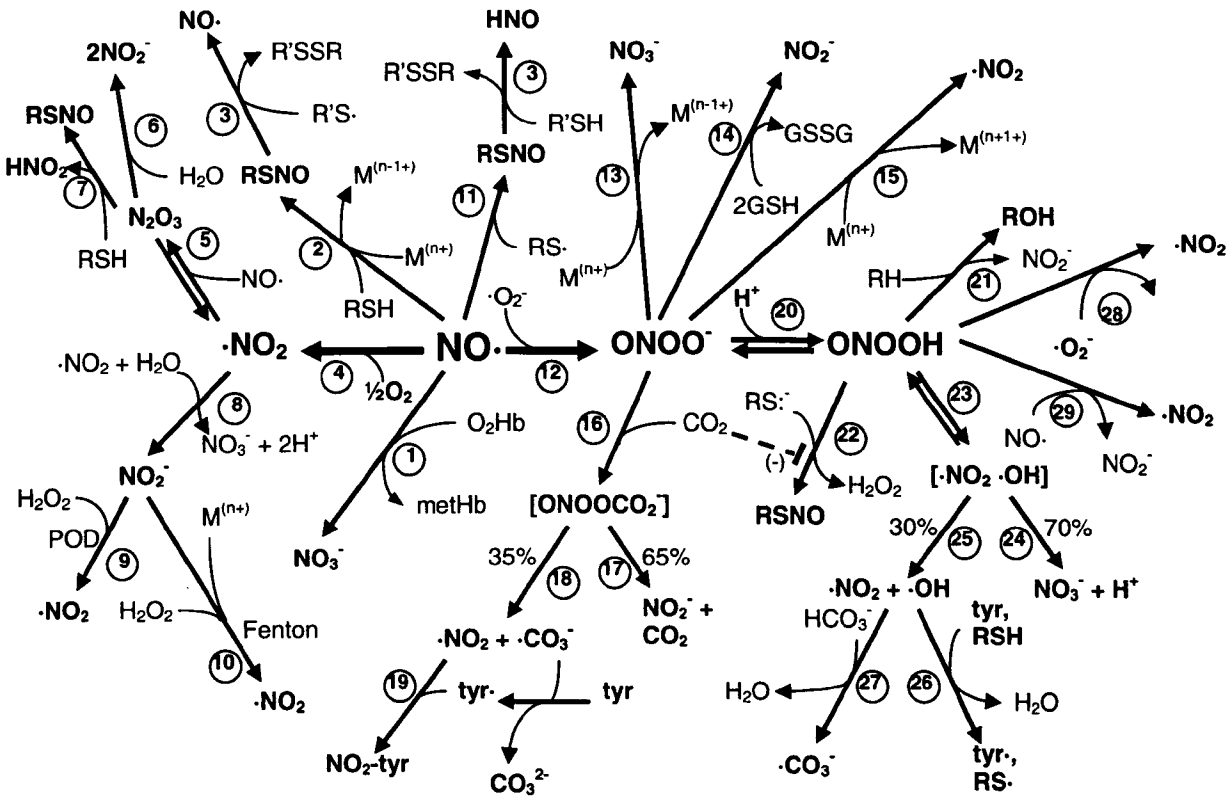

Figure 2. Biological chemistry of nitric oxide and its derivatives. The chemistry summarized here is limited to reactions supported by empirical evidence. Circumscribed numbers indicate the following reactions and pathways: 1. Oxidation of NO· by oxyhemoglobin (O_2Hb), yielding methemoglobin (metHb) and nitrate (NO_3^-) [75, 83, 84]; 2. Transition metal ($M^{(n+1)}$)-catalyzed *S*-nitrosylation of thiols [75, 76]; 3. Liberation of NO· or nitroxyl (HNO) from *S*-nitrosothiols (RSNO) with concomitant formation of mixed disulfides (R'SSR) [85, 86]; 4. Oxidation of NO·, yielding nitrogen dioxide radical ($\cdot NO_2$) [79, 84, 87]; 5. Autoxidation of NO· and $\cdot NO_2$ via formation and hydrolysis of dinitrogen trioxide (N_2O_3) [82–84], yielding (6) nitrite (NO_2^-) [88, 89]; 7. Nitrosation of sulfhydryls by N_2O_3 [88–90]; 8. $\cdot NO_2$ oxidation to NO_2^- and recycling via (9) peroxidase activity (POD) and (10) Fenton chemistry [79]; 11. Direct biradical condensation of NO· and thiyl radicals (RS·), forming RSNO [75, 90, 91]; 12. Biradical condensation of NO· and $\cdot O_2^-$, forming $ONOO^-$ [75, 76, 92]; 13. Transition metal-catalyzed $ONOO^-$ rearrangement to NO_3^- [78, 92]; 14. $ONOO^-$ detoxification by selenoenzymes, with concomitant oxidation of glutathione (GSH) to glutathione disulfide (GSSG) [77, 78, 92]; 15. Transition metal-dependent reduction of $ONOO^-$ to $\cdot NO_2$ [78]; 16. CO_2: $ONOO^-$ condensation and spontaneous decomposition of the $ONOO^-$:CO_2 adduct [78, 92–94] to (17) nonreactive products [78, 79] and (18) free radicals [78, 79, 92]; 19. Nitration of tyrosyl radical by $\cdot NO_2$ after single-electron oxidation of tyrosine by carbonate radical ($\cdot CO_3^-$) [77, 91, 94]; 20. Protonation of $ONOO^-$ to its conjugate acid ONOOH [75, 78]; 21. Oxidation of aliphatic and aromatic hydrocarbons (RH) by ONOOH [78]; 22. Thiol *S*-nitrosylation by ONOOH [95], suppressed by physiological concentrations of CO_2 [90]; 23. ONOOH decomposition via the caged [$\cdot NO_2$ ·OH] intermediate [78] to (24) a nonreactive product, NO_3^- [78, 79, 84], or (25) the radicals $\cdot NO_2$ and ·OH [78, 79]; 26. ·OH oxidation of biomolecules, yielding free radical products [78]; 27. Single-electron oxidation of bicarbonate by ·OH, yielding $\cdot CO_3^-$ [79]; ONOOH decomposition by reaction with (28) $\cdot O_2^-$ [96] or (29) ·NO [96].

with oxygen (Fig. 2) and by decomposition of $ONOO^-$ following its condensation with CO_2 and spontaneous decomposition of the unstable product, $ONOOCO_2^-$. Protonation of $ONOO^-$ generates peroxynitrous acid (ONOOH), which spontaneously decomposes to the radicals $\cdot NO_2$ and ·OH. In addition, $\cdot NO_2$ reacts with bicarbonate, yielding carbonate radical ($\cdot CO_3^-$). These derivatives, and to a limited extent NO itself, chemically modify biomolecules, including proteins, lipids, and DNA. For example, $\cdot NO_2$ is sufficiently reactive to nitrate unsaturated carbon-carbon bonds, for example, in membrane lipids; to abstract hydrogen from C-H bonds of phenols and thiols (79); and to nitrate tyrosyl radicals (81, 91). Peroxynitrite and ONOOH are powerful oxidants and nitrating/nitrosating agents that can initiate lipid peroxidation cascades (95); modify and mutate DNA bases (95); produce single- and double-stranded breaks in DNA (80, 81); deplete cellular thiols (81); activate enzymes, including matrix metalloproteinases (97, 98), poly-ADP ribosyl synthase and polymerase (80, 81), and sarcoplasmic reticular Ca^{2+} ATPase (99); nitrate aromatic compounds, for example, tyrosyl residues (75, 78, 81); and inactivate mitochondrial respiratory complexes (91). Biradical reaction of NO with thiol radicals generates *S*-nitrosothiols, which react with sulfhydryls to liberate nitroxyl (HNO) (86). HNO converts glutathione and other sulfhydryls to sulfanilamide ($RS(O)NH_2$) derivatives (100, 101). Figure 2 presents only a few of these myriad modifications, including *S*-nitrosation and oxidation of protein thiols, mixed disulfide formation, tyrosine nitration, and oxidation of the endogenous antioxidant glutathione. By compromising membrane integrity, damaging structural and contractile proteins, and inactivating enzymes, $ONOO^-$ and its products can inflict lethal cellular injury (Fig. 3). Thus, excessive formation of

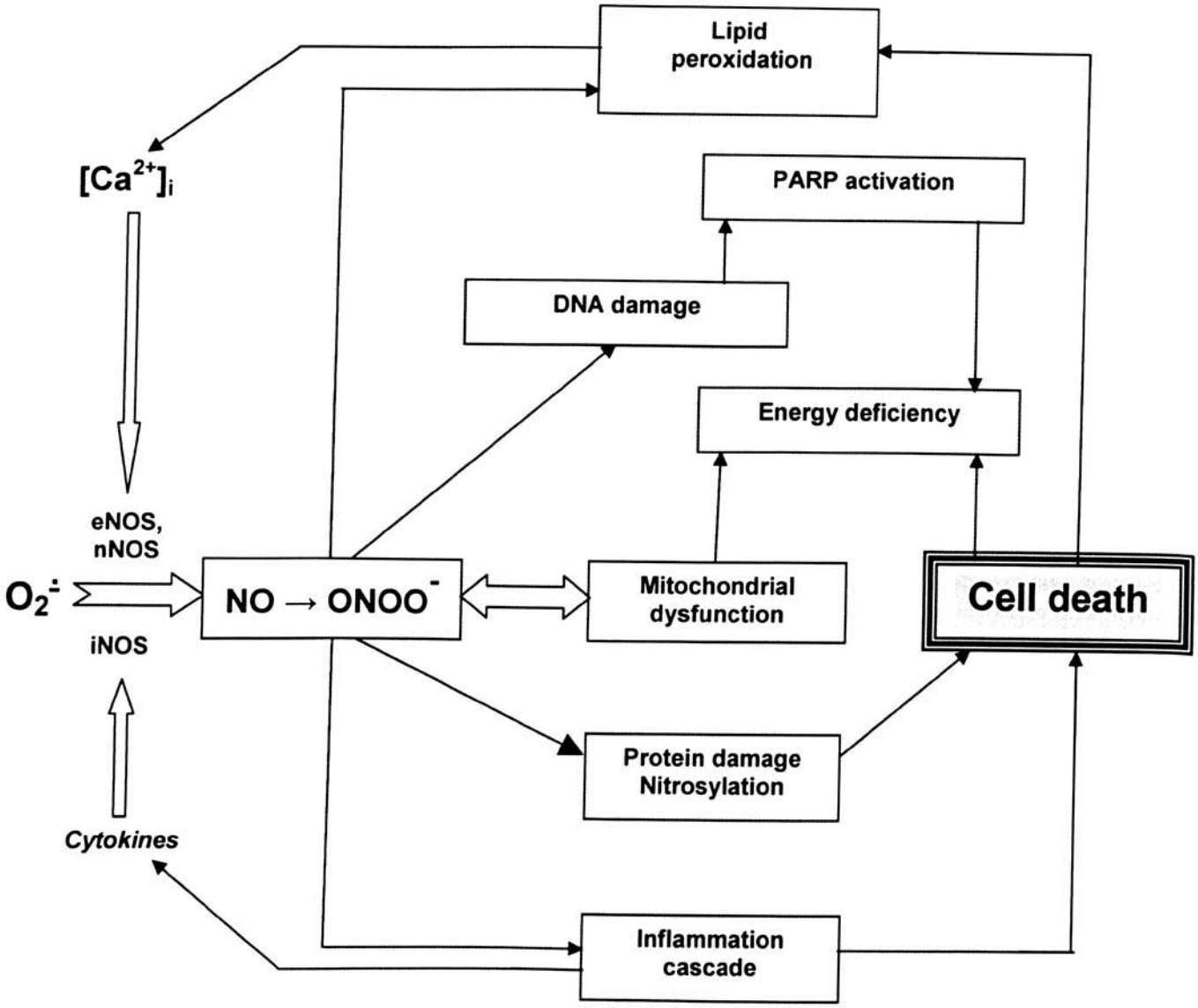

Figure 3. $ONOO^-$-dependent detrimental mechanisms. See text for details. eNOS: endothelial nitric oxide synthase (NOS); nNOS: neuronal NOS; iNOS: inducible NOS; PARP = poly-ADP ribosylpolymerase.

NO, which occurs, for example, upon coronary artery reperfusion, can injure myocardium.

Hypoxia Modulates NOS Activity and NO Bioavailability

Hypoxia may influence NO production, NO tissue concentration, and NOS expression by several mechanisms (Fig. 4): (i) limitation of NO production due to inadequate NOS substrate O_2; (ii) effect of O_2 on NOS feedback inhibition; (iii) modulation of NO bioavailability; (iv) induction of hypoxia inducible factor (HIF)-1 and other NOS transcription factors; (v) changes in intracellular Ca^{2+} concentration and Ca^{2+} influx; and (vi) induction of NOS-regulating heat shock proteins.

Oxygen: a Key NOS Substrate. Studies of the relationships between O_2 concentration and NOS isoform activities in bovine brain, aortic endothelial cells, and macrophages yielded apparent K_m values of 23.2, 7.7, and 6.3 μM O_2 for nNOS, eNOS, and iNOS, respectively (102). Another study estimated the K_m value for nNOS to be as high as 400 μM O_2 (103). Since the K_m values of NOS isoforms are within the normal range of tissue O_2 concentration, any reduction of tissue O_2 would decrease NO production (102, 104). Indeed, acute, profound hypoxia (0.1%–0.2% O_2) applied to cell cultures decreased NO production by all three NOS isoforms by 60%–80% (103, 104). Less severe hypoxia (4.8% O_2) (105) suppresses NO synthesis only moderately, and this effect of hypoxia is blunted by increased Ca^{2+} influx, which activates Ca^{2+}/

calmodulin-dependent NOS isoforms. In severe hypoxia, Ca^{2+} activation of NOS cannot compensate for reduced O_2 availability, and NO deficiency may develop (105, 106).

Oxygen Modulates NO Feedback Inhibition of NOS. NO can bind to $NOSFe^{2+}$, forming a heme-NO complex ($NOSFe^{2+}NO$), which prevents the heme from binding O_2 and thus increases the apparent K_m for O_2 (103, 104). By this mechanism, NO feedback inhibits NOS, so the enzyme usually functions at only a fraction of its catalytic capacity. During steady state NO synthesis, between ~70% and 90% of nNOS exists as a ferrous-NO complex (107).

Feedback inhibition of NOS by NO is modulated by O_2 concentration, since O_2 and NO compete for the heme iron. Also, the rate of decay of the heme iron-NO complex is dependent on O_2 concentration; as O_2 concentration decreases, less NO is displaced by O_2 and NO production declines (103). As the heme-NO complex subsequently dissociates and then binds O_2, the active enzyme ($NOSFe^{3+}$) is regenerated (107, 108). The sensitivity of the ferrous-NO complex to O_2 influences the overall NOS response to O_2. It is primarily by this mechanism, rather than the effect of O_2 as a substrate, that NOS produces NO in proportion to the O_2 concentration across the physiologic range (0–250 μM) (109).

Oxygen Tension Regulates NO Bioavailability. Although O_2 is required for NO synthesis, under some conditions NO concentration has been found to vary inversely with O_2. A large increase in PO_2 can lower NO concentration by oxidizing NO to NO_2^- and NO_3^-. Conversely, NO bioavailability may increase as NO production falls during hypoxia. Heyman *et al.* (110) found that interventions known to intensify hypoxia in renal medulla, such as NOS inhibitors or radiologic contrast media, paradoxically increased tissue NO in renal cortex. Other measures known to ameliorate hypoxia, such as furosemide, *L*-arginine, and hypotension, reduced NO. The authors proposed (110) that NO scavenging by oxygen is reduced or, more likely, NO release from hemoglobin is increased as PO_2 falls, whereas hyperoxemia accelerates NO removal.

Hypoxia Affects NOS Gene Expression. The response to chronic hypoxia also involves altered expression of NOS genes (111, 112). Hypoxia induces transcription factors such as hypoxia-inducible factors (HIF-1 and HIF-2) and nuclear factor kappa B (NF-κB) (112). HIF-1 is a heterodimer consisting of α and β subunits. Constitutively expressed and O_2-independent, HIF-1β can heterodimerize with other proteins that contain bHLH-PAS domains. HIF-1α content is exquisitely controlled by intracellular O_2 concentration. Under normoxic conditions (FIO_2 21%) HIF-1α is tagged for proteosomal degradation by O_2-dependent proline hydroxylation (33, 113). HIF-1α content and HIF-1 transcriptional activity progressively increase as FIO_2 decreases from 21% to 5%, then more sharply as FIO_2 falls below 5%. Within 1 min of reoxygenation, HIF-1 decomposes and the HIF-1α subunit is proteolytically degraded. The rapid dynamics of HIF-1 may be especially important for adaptation to brief periods of intermittent hypoxia.

HIF-1 regulates genes containing the hypoxia-responsive element (HRE), a *cis*-acting transcriptional-regulatory motif that includes one or more binding sites for HIF-1 (112). During hypoxia, HIF-1 activates many target genes, including those involved in NO synthesis, erythropoiesis, angiogenesis, glycolysis, and cell proliferation (33, 112). A HRE has been identified within the iNOS gene (114), suggesting that iNOS expression could be regulated like classic oxygen-regulated genes, for example, erythropoietin (115). nNOS mRNA accumulation has been observed after hypoxia *in vivo* (116, 117). This increase may involve a general cellular stress response, leading to increased nNOS gene transcription, or direct activation of nNOS transcription through binding of HIF-1 (118). HRE motifs have been detected in the nNOS genomic sequence, but their functionality is not yet known (119). Gess *et al.* (120) demonstrated that hypoxia increased eNOS mRNA in all investigated organs and suggested the existence of a HRE in the eNOS gene, which would mediate eNOS gene regulation by hypoxia in a fashion similar to that of the erythropoietin gene. More recently, Coulet *et al.* (121) showed that human eNOS is a hypoxia-inducible gene, whose transcription is stimulated through HIF-2 interaction with two contiguous sites located at −5375 to −5366 of the human eNOS promoter.

A transcription factor for a variety of genes (122, 123), NF-κB may contribute to hypoxic induction of iNOS gene expression. During adaptation to intermittent hypoxia, NF-κB may be activated by reactive oxygen species generated during alternating hypoxia and reoxygenation (123). However, the contribution of NF-κB to the increased NO production during intermittent hypoxia has not been determined.

Although hypoxia causes pulmonary hypertension, it is interesting to note that hypoxia stimulates NOS expression in pulmonary vessels. Toporsian *et al.* (124) observed an increase in pulmonary eNOS gene expression after 12-hr hypoxia in rats. eNOS expression increased above baseline in pulmonary arterioles already expressing the gene, and the fraction of arterioles newly expressing eNOS began to increase after 1 day of hypoxia; eNOS expression continued to increase for several days before stabilizing. Other investigators also reported accumulation of rat lung nNOS and eNOS mRNA after prolonged hypoxia (125–127).

The eNOS-inducing effect of hypoxia in lungs and pulmonary circulation may be simulated by metabolic alteration of redox state. Hypoxia led to an increase in the cellular $NAD(P)H/NAD(P)^+$ ratio, which augmented activation of eNOS transcription by AP-1, a redox-sensitive transcription factor. In this process, hypoxia increased eNOS mRNA transcription in a manner inversely proportional to PO_2 (128). This increase in pulmonary eNOS expression may reflect an adaptive response to blunt hypoxia-induced pulmonary vasoconstriction. However,

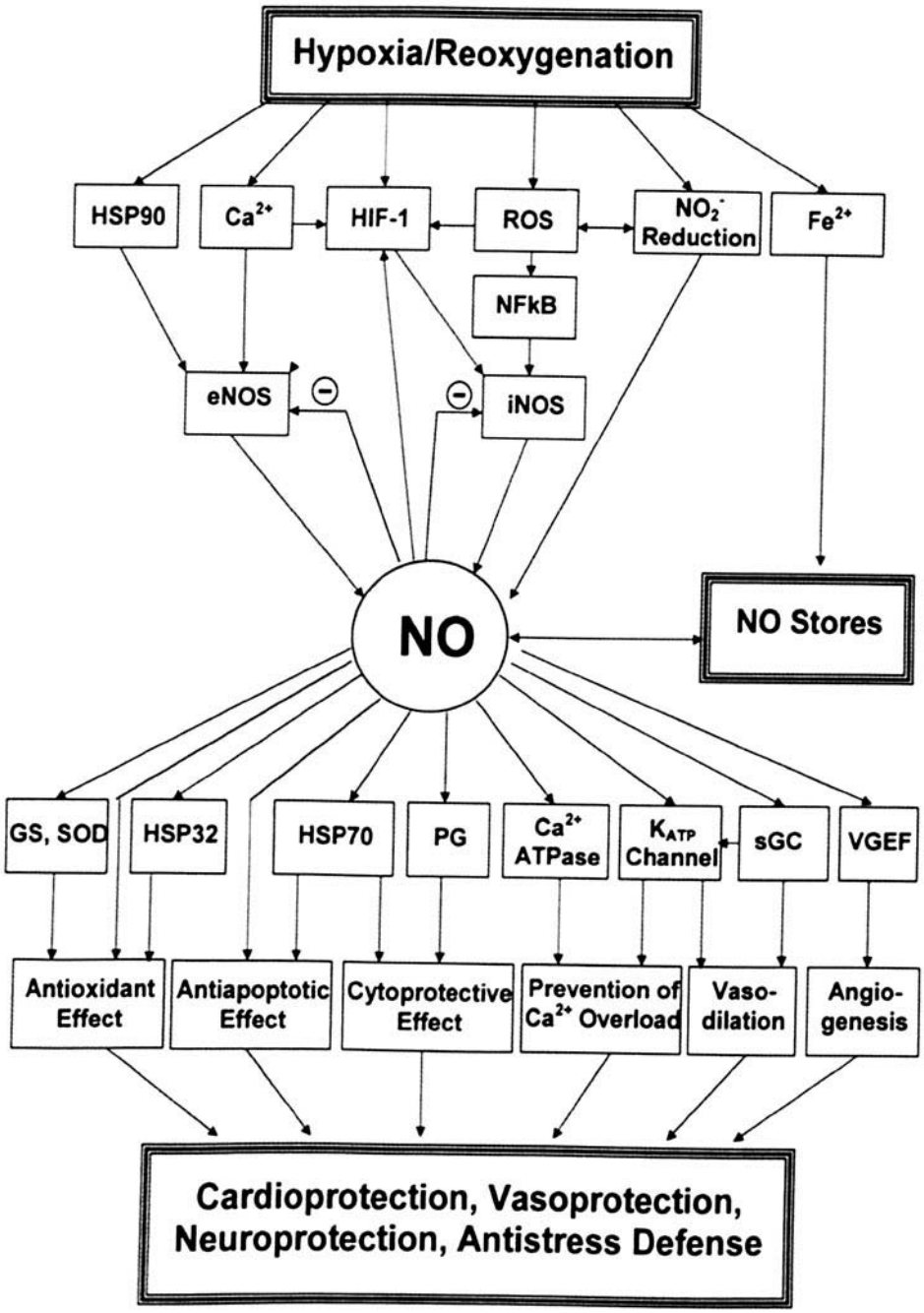

Figure 4. NO-dependent mechanisms in protective effects of adaptation to hypoxia. See text for details. NOS = NO synthase; iNOS = inducible NOS; eNOS = endothelial NOS; SOD = superoxide dismutase; GS = glutathione; HSP = heat shock protein; HIF-1 = hypoxia-inducible factor 1; ROS = reactive oxygen species; PG = prostaglandins; VEGF = vascular endothelium growth factor.

this notion contradicts the decline in pulmonary endothelial NO production observed in the same model by Shaul *et al.* (126) following prolonged hypoxia. Shaul *et al.* (126) suggested that diminished availability of NOS cofactors during hypoxia limited NO production despite increased NOS expression. Other studies demonstrated hypoxic down-regulation of eNOS expression in pulmonary endothelial cells (117). Hypoxia reduced eNOS mRNA and/or protein in human (129), porcine (130), and bovine (131) pulmonary artery endothelial cells. These hypoxia effects were presumably due to both decreased transcription and destabilization of eNOS mRNA (131).

In nonpulmonary endothelial cells, the findings are also controversial. Increased eNOS mRNA and protein contents were observed in bovine aortic endothelial cells incubated with 1% O_2 (132), in cerebral blood vessels during ischemia (133), and in hypoxic human renal proximal tubules (134). Other reports, however, demonstrated reduced eNOS expression in human umbilical vein and bovine aortic endothelial cells exposed to low PO_2 (135, 136). Reduced

eNOS expression resulted from decreased eNOS mRNA stability and eNOS promoter activity (135).

Post-translational regulation of iNOS by prolonged hypoxia was demonstrated in murine macrophages (137), in which 24-hr exposure to hypoxia (PO_2 23 ± 1.4 mm Hg) lowered iNOS activity but did not affect iNOS protein content. In these cells, hypoxia disrupted interactions of iNOS with the cytoskeletal protein α-actinin 4, causing iNOS displacement from the submembranal regions, a location which may be important for normal iNOS activity.

Hypoxia Affects NOS Activity and Expression by Altering Intracellular Ca^{2+}. Luckhoff and Busse (138) found a close positive correlation between increases in intracellular Ca^{2+} and NO formation in endothelial cells exposed to hypoxia. Hampl *et al.* (105) showed that after 10 min moderate hypoxia (4.8% O_2), Ca^{2+} began to enter the cells and activated eNOS. However, more prolonged hypoxia terminated the Ca^{2+} entry and decreased NO synthesis. The Ca^{2+}-dependent mechanism of hypoxia-enhancement of NO production may involve both NOS activation and expression of genes regulated by Ca^{2+} influx through plasma membrane L-type Ca^{2+}-channels (139). In this process, temporal dynamics of Ca^{2+} transients are more important for gene expression than the steady-state Ca^{2+} concentration (140). In PC12 cells exposed to 60 cycles of 30 secs 1.5% O_2/4 mins 20% O_2, HIF-1α protein increased 3-fold. A Ca^{2+}-activated mechanism involving calmodulin-dependent protein kinase appeared to stabilize HIF-1α, which in turn increased HIF-1 transcriptional activation of the iNOS gene (141). Thus, hypoxia-induced Ca^{2+} influx activates eNOS and induces iNOS expression.

Hypoxia Induces NOS-Regulating Heat Shock Proteins. Hypoxia induces a spectrum of heat shock proteins (HSPs), including HSP27 (142), HSP32 (143), HSP70 (63, 142, 144), and HSP90 (145). Association of HSP90 with eNOS activates NO production (146, 147) and, importantly, limits $\cdot O_2^-$ generation by uncoupled eNOS (148, 149). Furthermore, HSP90 activates nNOS as well as eNOS (150). In contrast, induction of HSP70 may suppress NO production by nuclear accumulation of the p65 subunit of NF-κB (151, 152). Thus, hypoxia-induced HSPs can either increase or decrease NO production.

Cardio- and Vasoprotective Functions of NO

The role of NO in cardiovascular injury and cardio- and vasoprotection remains controversial. Both NO donors and NOS inhibitors have been reported to protect against myocardial ischemia/reperfusion (IR) injury (60, 153–170). NO may protect myocardium by increasing coronary flow (171–173), decreasing neutrophil accumulation (174), maintaining endothelial function (175), preserving calcium sensitivity and contractile function without increasing energy demand (176), and reducing myocardial oxygen consumption (177, 178), although the relative importance of these protective mechanisms is unclear (179). Paradoxically, inhibition of NOS ameliorated myocardial ischemia-reperfusion injury by mimicking ischemic preconditioning (155), blunting $ONOO^-$ formation (180), and suppressing nitrosative injury by NO derivatives (153, 180). The NO cardioprotection controversy is exemplified by two recent reports in genetically modified mice. Flogel *et al.* (157) reported that postischemic recoveries of phosphocreatine and left ventricular developed pressure were enhanced in hearts of eNOS-deficient versus wild-type mice. Conversely, Brunner *et al.* (181) reported that myocardium of transgenic mice overexpressing eNOS was more resistant to IR injury than that of wild-type mice.

Bolli's analysis of the literature in 2001 (158) found that of 92 studies which examined the impact of NO on IR injury in nonpreconditioned myocardium, 73% concluded that endogenous or exogenous NO was cardioprotective, and only 12% reported a detrimental effect of NO. The proportion of studies showing a protective effect of NO was similar for *in vivo* and *in vitro* studies. It remains a challenge to explain why some studies found detrimental effects of NO. A reasonable explanation is that NO effects are concentration-dependent. The NOS inhibitor *L*-NMMA, which dose-dependently reduced NO release into coronary venous effluent, revealed NO's dual character in myocardial IR injury; thus, low doses of *L*-NMMA improved postischemic recovery of left ventricular contractile performance and myocardial ATP content and decreased myocardial creatine kinase release during reperfusion (163, 180). In contrast, higher doses of *L*-NMMA, which caused coronary vasoconstriction, had adverse effects. Coadministration of NOS substrate *L*-arginine abolished the diverse effects of *L*-NMMA (155).

It appears that there is an optimal concentration of NO for protection: too little or too much may be detrimental. Although many studies with NOS inhibitors demonstrate that NO is essential for cardiovascular protection (163, 181–183), these studies do not exclude the possibility that excessive NO is harmful. NO overproduction may result from increased iNOS in macrophages and/or vascular smooth muscle cells or excessive activation of eNOS in coronary, cerebral, and peripheral vascular endothelium (184–188).

Acute hypotension is an important consequence of NO overproduction in septic (189), anaphylactic (190), heat (191, 192), hemorrhagic (193), and cardiogenic (187, 194, 195) shock. Both iNOS and eNOS have been implicated in this hypotension. Preferential inhibition of iNOS with nonvasoactive low doses of NO inhibitors limited the fall of blood pressure in septic shock (196) and heat stroke (197). Complete inhibition of NO production induced a considerable increase in blood pressure but failed to improve survival of rats in heat stroke (197). Other authors observed increased mortality of rats exposed to septic shock after NOS inhibition (196–199) due to myocardial ischemia, microvascular thrombosis, and disturbed antimicrobial defense (198) and a direct cardiotoxic effect (199–201).

Increased levels of NO were detected in both plasma (202) and aorta (187, 203) of rats subjected to heat stroke or acute myocardial infarction. This NO overproduction was accompanied by excessive endothelium-dependent relaxation of isolated blood vessels (203, 204), which inversely correlated with blood pressure (205). These studies support an important contribution of eNOS to NO- induced acute hypotension.

The relative contributions of iNOS and eNOS to NO overproduction have also been studied in rats subjected to acute heat stress (206). In these experiments, heat stress induced NO overproduction in the heart, liver, kidneys, spleen, brain, and small intestine, detected by electron paramagnetic resonance. Cycloheximide, an inhibitor of protein synthesis, reduced heat stress–induced NO production in liver by 73%. Since iNOS is virtually absent from hepatocytes under normal conditions, this result demonstrated a large contribution of *de novo* synthesized iNOS to NO overproduction in heat stress.

The major mechanism by which excessive NO promotes cell injury likely involves the reaction of NO with $\cdot O_2^-$ to generate cytotoxic $ONOO^-$ (Fig. 2). The potential for $ONOO^-$ toxicity is greatest when NO and $\cdot O_2^-$ are produced in roughly equal amounts in the same location (96). Excess amounts of either precursor can decompose ONOOH by thermodynamically feasible reactions (Fig. 2: reactions 28, 29; Refs. 96, 207). Also, NO can impair the function of essential proteins by nitrosylating iron-sulfur clusters and thiol residues. NO itself is a poor nitrosylating agent, but will readily react with sulfhydryl radicals generated by one-electron thiol oxidations (101) or with sulfhydryls following its conversion to more aggressive reactive nitrogen species (86). The resulting inhibition of key enzymes of the tricarboxylic acid cycle and the mitochondrial respiratory chain, disruption of mitochondrial calcium metabolism, or damage to DNA may result in cell death by apoptosis and/or necrosis through activation of poly(ADP-ribose)polymerase (Fig. 3) and subsequent inhibition of glycolysis and depletion of ATP (208). Pharmacological inhibition of poly(ADP-ribose)polymerase is considered a promising approach in the treatment of pathologies involving NO overproduction (209).

Adaptation to Hypoxia Limits NO Overproduction and Corrects NO Deficiency. NO overproduction in rats contributed to detrimental effects of acute, severe hypobaric hypoxia produced by a simulated altitude of 11,000 m in rats (63). In this study, inhibition of NOS or trapping of NO prolonged survival during subsequent hypoxic exposure. When rats were conditioned by an 8-day program of intermittent hypoxia and then subjected to acute, severe hypoxia, NO overproduction in the brain was prevented, and survival of these adapted rats was markedly increased. This protection appeared to be due to a moderate increase in NO synthesis during adaptation to hypoxia, since inhibition of NOS during adaptation abolished the protection.

Intermittent hypoxia may also protect in conditions of NO deficiency. Endothelial dysfunction often results in increased vascular tone and hypertension (210), and impaired vascular smooth muscle relaxation is associated with NO deficiency, as evident from abnormally low plasma concentrations and urinary excretion of the stable NO metabolites, nitrite and nitrate (50, 211, 212). The NO deficiency may be due to reduced synthesis by eNOS (213) or increased NO oxidation or trapping by free radicals (214). When stroke-prone, spontaneously hypertensive (SHRSP) rats were conditioned by hypobaric hypoxia beginning at the onset of hypertension (at age 5–6 weeks), the development of hypertension was slowed. This antihypertensive effect was associated with stimulation of endothelial NO synthesis, indicated by increased urinary $NO_2^- + NO_3^-$ excretion, and was also evident in isolated blood vessels (50). These antihypertensive and vasoprotective effects of hypoxic adaptation were mimicked by the NOS-stimulating, β-adrenergic antagonist nebivolol (215). Compared to the β_1-selective antagonist metoprolol, Buval'tsev *et al.* (216, 217) found that nebivolol was a more potent antihypertensive agent and also prevented endothelial dysfunction, myocardial hypertrophy, and vascular remodeling in SHRSP rats.

Intermittent Hypoxia Adaptations Prevent Endothelial Dysfunction and IR Injury by NO-Related Mechanisms. Adaptation of rats to intermittent hypobaric hypoxia stimulated NO production as indicated by doubled plasma concentrations of nitrite and nitrate (50). In this study, hypoxic adaptation dampened the acute fall of blood pressure and excessive endothelium-dependent relaxation associated with myocardial infarction. Daily administration of a NOS inhibitor during adaptation to hypoxia abolished this protection (218, 219), whereas the NO donor, dinitrosyl iron complex (DNIC), mimicked the beneficial effects of adaptation to hypoxia (219, 220).

The cardioprotective effects of hypoxia adaptation and ischemic preconditioning are similar, in that in both cases subsequent ischemic injury and ischemia-induced arrhythmias are reduced (5, 168, 221, 222). Thus, both of these cardioprotective interventions may activate some of the same defense mechanisms. Indeed, Neckář *et al.* found that the cardioprotective effects of prolonged, intermittent hypoxia and ischemic preconditioning were not additive (223). This finding indicates that the mechanisms of hypoxia- and preconditioning-induced protection share some common signaling pathways. Such important experiments have not yet been done in animals adapted to intermittent hypoxia.

Nitric oxide appears to contribute to both the acute and delayed phases of cardioprotection induced by ischemic preconditioning (222). Conceivably, hypoxia-evoked NO protects hypoxia-adapted myocardium by mechanisms similar to those activated by ischemic preconditioning. However, important differences between the stresses of ischemic preconditioning and hypoxia adaptation should be

noted. The ischemic preconditioning insult itself may inflict injury to activate constitutive defense mechanisms and expression of protective proteins. In contrast, neither a single session nor a complete course of adaptation to hypoxia is associated with myocardial injury, so the "structural cost" of hypoxia is much lower than for ischemia (2, 3). In addition, adaptation to hypoxia produces more sustained protection than ischemic preconditioning. The beneficial effects of ischemic preconditioning subside after 3–4 days (224, 225), but protection against ischemia-induced myocardial infarction persisted for at least 35 days after adaptation of rats to intermittent high-altitude hypoxia (5).

In studies of adaptation to intermittent hypoxia, the hypoxic stress is usually considered to be the factor responsible for stimulating cardioprotection. However, the reoxygenation phase may be crucial to the adaptative process. Milano *et al.* (226, 227) tested the hypothesis that repeated, brief reoxygenation episodes during prolonged hypoxia would improve myocardial tolerance to a more severe hypoxic insult. Hearts were isolated from rats conditioned by 14 days hypoxia and sequentially perfused with hypoxic, then hyperoxic, media. Contractile performance during hyperoxia was better maintained in hearts isolated from rats that had been reoxygenated for 1 hr/day throughout the adaptation program.

Although NO plays a role in cardioprotection afforded by both continuous and intermittent hypoxia adaptation (221), the effects of these adaptive processes on NO metabolism differ. Continuous hypoxia increased eNOS expression and basal NO production by eNOS, but iNOS remained undetectable (228). Acute inhibition of NOS abolished cardioprotection evoked by continuous hypoxia in neonatal rabbits (25, 229), whereas administration of the NO donor, *S*-nitrosoglutathione, mimicked the protection (25). In contrast, intermittent hypoxia induced by repeated hypobaric exposures increased myocardial iNOS content and reduced expression of eNOS (230–232). Kolář *et al.* (231) reported that inhibition of NOS had an antiarrhythmic effect in nonadapted but not in hypoxia-adapted hearts, suggesting that hypoxic adaptation suppressed arrhythmias by blunting excess NO production during ischemia and reperfusion. Moreover, *S*-nitrosoglutathione completely abolished the antiarrhythmic effect of intermittent hypoxia. Collectively, these results indicate that excessive NO production during ischemia is arrhythmogenic.

Some studies have demonstrated that delayed cardioprotection observed 24 hrs after ischemic preconditioning resulted from upregulation of iNOS (233). Ding *et al.* (168) reported improved postischemic function of hearts from rats adapted to hypobaric, intermittent hypoxia, compared to nonadapted control hearts. Preischemic $NO_2^- + NO_3^-$ content was higher in adapted hearts. The iNOS-selective inhibitor, aminoguanidine, suppressed protective effects of intermittent hypoxia. $NO_2^- + NO_3^-$ content increased after 30 mins ischemia in control but not in hypoxia-adapted hearts. Relative to preischemia, iNOS mRNA increased after reperfusion in nonadapted hearts but decreased in adapted hearts. Thus, adaptation to intermittent hypoxia may have prevented a burst of iNOS activity and cytotoxic NO overproduction during the initial phase of reperfusion.

Delayed cardioprotection by NO derived from iNOS can result from ischemic preconditioning, heat stress, cardiac pacing, or administration of exogenous compounds (234). For instance, endotoxin in sublethal doses limited IR arrhythmias and myocardial necrosis (235). A synthetic endotoxin derivative, monophosphoryl lipid A, was similarly cardioprotective (236). Muller *et al.* (185) cited the induction of adaptive mechanisms such as antioxidant enzymes and heat shock proteins in the heart and the formation of releasable NO stores in blood vessels as examples of long-term benefits of iNOS induction.

Although there are numerous reports of hypoxia-induced cardioprotection in rats and other small mammals, Zong *et al.* (3) reported the first evidence of such protection in a large mammal model of IR. In dogs adapted to intermittent normobaric hypoxia, less than 2% of the ischemic myocardium was infarcted following 60 mins coronary artery occlusion and 5 hrs reperfusion, a protocol that infarcted about 35% of the ischemic territory in nonadapted dogs. Also, life-threatening arrhythmias were absent in the adapted dogs, while ventricular tachycardia and/or fibrillation occurred in over half of the nonadapted dogs (3). In similarly adapted dogs, this group found that myocardial eNOS content and NOS activity were reduced significantly (188). The investigators suggested decreased eNOS may protect myocardium of adapted dogs from excessive NO formation upon reperfusion.

It seems evident that adaptation to intermittent hypoxia produces a broad spectrum of cardio- and vasoprotective effects, related to the ability of hypoxia and reoxygenation to modulate NO metabolism (Fig. 4). The salutary mechanisms involving hypoxic modulation of NO metabolism are not yet fully understood. Below we describe some of the possible pathways leading to such protection.

Mechanisms of NO-Dependent Protection Evoked by Adaptation to Intermittent Hypoxia

Alternative Sources of NO in Adaptation to Hypoxia. NO binding by certain proteins buffers excess free NO and generates NO stores, which can gradually release NO and therefore serve as a nonenzymic source of free NO (237). Although often undetectable in basal conditions, NO stores form in response to increased NO concentration, whether from increased NOS activity or from administration of exogenous NO donors (238). *S*-nitrosothiols and DNIC are two major forms of NO storage and transport in mammals (Fig. 5). Exchange of NO between these two classes of compounds depends on the intracellular contents of iron, low molecular weight thiols, and NO. *S*-nitrosothiols and DNICs exist in proteins and in soluble

thiol ligands, such as cysteine or glutathione. Protein-bound complexes are more stable than low molecular weight NO adducts and are regarded as the principal intracellular NO stores (237).

Nitric oxide stores in blood vessels have been detected by electron paramagnetic resonance spectroscopy (239), histochemical staining for bivalent iron (240), immunostaining for *S*-nitrosated cysteine (241), and photorelaxation responses of isolated blood vessels (242). A facile method for detecting NO stores in the vascular wall is the reaction of *N*-acetylcysteine or diethyldithiocarbamate with DNIC and *S*-nitrosothiols, which releases NO and induces vasodilation in proportion to the size of the NO stores (243, 244). NO storage positively correlates with plasma levels of nitrite and nitrate (245), and chronic administration of DNIC expanded NO stores in rats, whereas chronic NOS inhibition decreased stores (246). Efficiency of NO storage or potential capacity to store NO can be assessed by incubation of an isolated blood vessel with excess NO (26).

Adaptation to intermittent hypoxia results in a progressive increase in NO stores. This may be an adaptive mechanism that protects the cardiovascular system from the harmful effects of excessive NO synthesized during repeated exposure to hypoxia. On the other hand, as nonenzymic NO sources, NO stores may compensate for decreased production of NO by endothelial cells, or feedback-inhibit NO overproduction. Smirin *et al.* (218) reported that prevention of NO storage in the vascular wall abolished hypoxia-mediated protection against NO overproduction, while augmentation of NO stores by a NO donor mimicked this protection. Chronic treatment of rats with the antioxidant *N*-acetylcysteine, which depletes NO stores (247, 248), blunts the improvement in myocardial resistance to injury effected by adaptation to hypoxia (249).

Efficiency of NO storage is genetically predetermined and is apparently related to the inherited capacity for NO synthesis. Adaptation to hypoxia increased total NO production to a similar extent in SHRSP and normotensive Wistar-Kyoto rats, but the size of NO stores was much less in SHRSP than in Wistar-Kyoto rats (26). Since more NO remained unbound in SHRSP rats, adaptation to hypoxia had a pronounced depressor effect compared to Wistar-Kyoto rats. However, the lack of compensatory increase in NO storage capacity in SHRSP rats may exacerbate endothelial injury and dysfunction due to NO overproduction by iNOS in macrophages and vascular smooth muscle (250).

Hemoglobin can bind NO and provide additional NO stores. Although NO is rapidly oxidized by oxygenated hemoglobin, NO reacts with deoxygenated heme groups to form $\beta HbFe^{2+}NO$ in the venous circulation and with ^{93}cys residues of the Hb β-chain to form *S*-nitrosohemoglobin (SNO-Hb) (251). SNO-Hb may be an important source of bioactive NO in hypoxic conditions, since as PO_2 falls below 6 mm Hg, NO is released from ^{93}cys residues in sufficient quantity to dilate vessels (252, 253). However, the functional relevance of this mechanism for controlling blood flow has been challenged. At physiological PO_2, SNO-Hb formation is negligible and irreversible NO oxidation prevails (254). Moreover, SNO-Hb is unstable in red cells (255). Several diffusion barriers exist between the sites of NO formation and hemoglobin, including the red cell membrane (256, 257), the unstirred layer surrounding the red cell (258), and an erythrocyte-free zone of plasma along the surface of vascular endothelium (259). These barriers are estimated to decrease the rate of NO scavenging by intraerythrocytic hemoglobin 500- to 1000-fold (256, 260).

Nitrite represents an important circulating and tissue storage form of NO, since NO_2^- can be recycled to bioactive NO by intravascular and tissue nitrite reductases when the partial pressure of oxygen decreases. NO_2^- ions are reduced to NO by electron-donor systems with the participation of NADH, NADPH, flavoproteins, and either cytochrome oxidase in mitochondria, cytochrome P-450 in endoplasmic reticulum, or deoxyhemoglobin in red cells (260–262). Reduction of NO_2^- to NO has been documented in vascular smooth muscle (263), endothelium (264), and ischemic myocardium (265). Nitrite reductase reactions increase in hypoxic conditions (266); indeed, nitrite reduction by hemoglobin is a major source of NO during hypoxia. Because the rate of NO generation from NO_2^- is linearly dependent on reductions in PO_2 and pH, NO_2^- could be reduced to NO and protect ischemic tissue. Accordingly, NO_2^- administered 5 mins before reperfusion reduced cardiac infarct size by 67% in mice subjected to coronary artery occlusion (267). In another recent study, orally administered NO_2^- was transformed to NO and attenuated hypertension in a dose-dependent manner (268). Thus, the NO_2^- reduction pathway can provide an alternative means of NO generation that might be therapeutically useful.

NO-Dependent Vasodilation and Protection Against Ca Overload. Soluble guanylate cyclase is a principal target of NO, which binds to the cyclase's heme moiety to activate the enzyme and increase synthesis of cyclic GMP (cGMP; Ref. 269). cGMP-dependent protein kinases phosphorylate several target regulatory proteins and modulate ion channel function to produce physiological responses (269, 270), including the well-known vasodilatory effects of NO. Moreover, cGMP-dependent pathways have been implicated in protective effects of NO (167, 271). Increased activation of K_{ATP} channels was demonstrated in chronically hypoxic rabbit hearts (272). The authors suggested that hypoxia-induced NO production activated cGMP-dependent protein kinase, which in turn phosphorylated and activated K_{ATP} channels. The resulting potassium efflux and hyperpolarization of the perfused heart decreased Ca^{2+} influx through L-type channels and thereby conferred tolerance to subsequent ischemia (25).

Clinically, the importance of NO deficiency in development and progression of essential hypertension was implied by a strong inverse correlation between NO production and blood pressure (273, 274). Intermittent hypobaric hypoxia

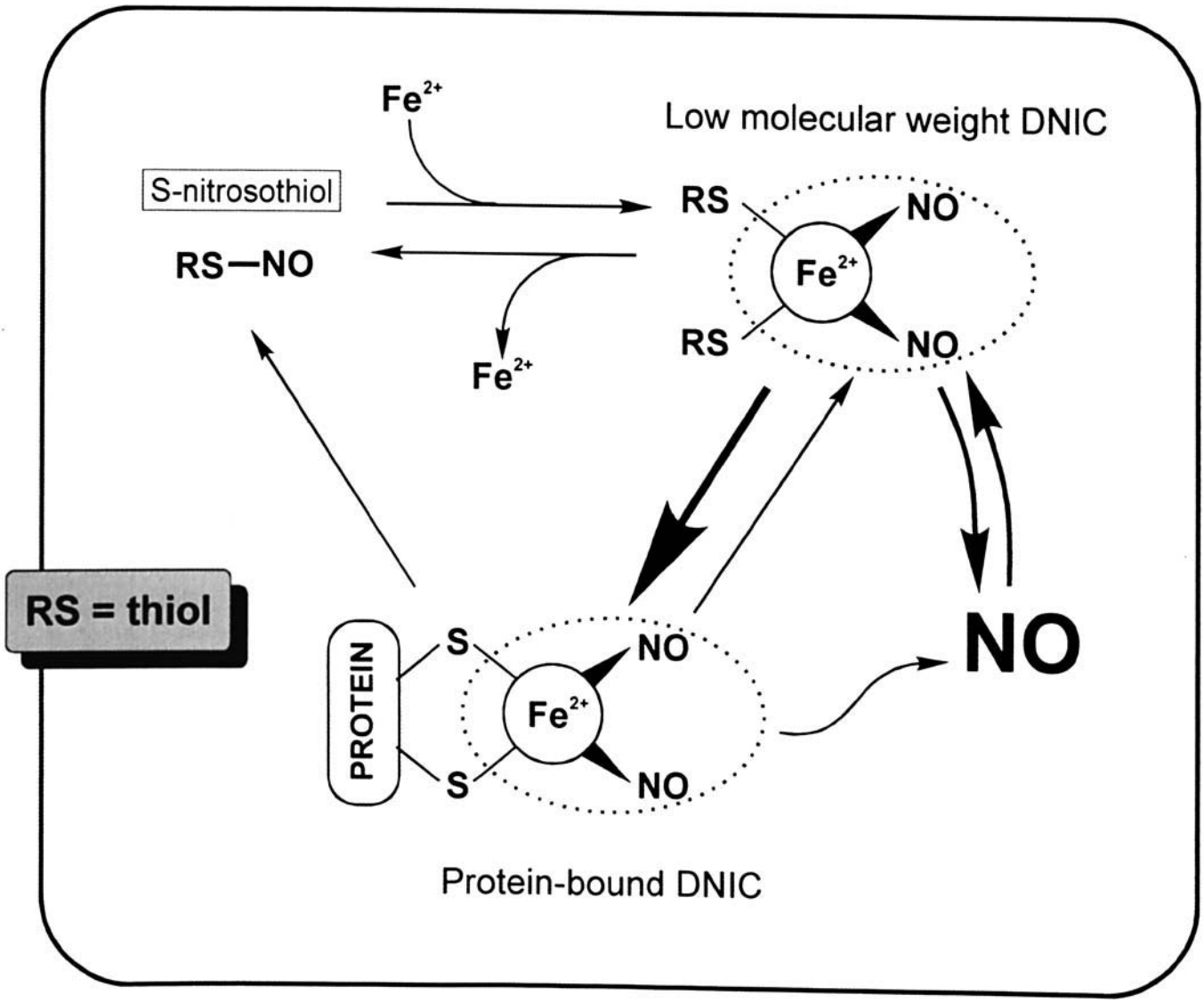

Figure 5. Nitric oxide stores: *S*-nitrosothiols (RS-NO), high molecular weight dinitrosyl iron complexes (DNIC) and low molecular weight DNIC. RS-NO and DNIC exist in two forms: bound to protein sulfhydryl groups (high molecular weight forms), and bound to low molecular weight thiols, particularly cysteine or glutathione. Protein complexes are much more stable than low molecular weight ones and thus are regarded as intracellular NO stores. At low concentrations of low molecular weight thiols, protein-bound DNIC is stable, while at high local concentrations of low molecular weight thiols, protein-bound DNIC can represent a reservoir of biologically active NO. Biological activity of DNIC is attributed to the release of free NO, to the transfer of NO to protein cysteine residues, or to $Fe^{+}(NO^{+})_2$ groups, which possess a high affinity to protein dithiols, to form high molecular weight DNIC. RS-NO and DNIC are interconvertible, depending on intracellular amounts of Fe^{2+}, low molecular weight thiols and NO. Apparently RS-NO performs as the major intercellular transport form of NO. Upon encountering high concentrations of non-heme iron and thiols, RS-NO initiates the formation of DNIC, which degrades to release NO. Thus, NO-containing complexes form a dynamic system of biologically active NO stores, in which NO continuously exchanges between free and bound forms (237, 238).

lowered blood pressure and prevented endothelial dysfunction by increasing NO production in hypertensive patients (273).

Recent studies suggested that the increased NO production resulting from elevated endothelial cell $[Ca^{2+}]$ (275) following prolonged hypoxic exposures blunted subsequent vasoconstriction by decreasing intracellular $[Ca^{2+}]$ (276, 277) and myofilament Ca^{2+} sensitivity in vascular smooth muscle (278, 279). NOS inhibition prevented hypoxic attenuation of vasoconstriction (275).

Adaptive increases in NO synthesis may help prevent calcium overload under pathological conditions by enhancing sarcoplasmic reticular (SR) Ca^{2+} sequestration (280, 281). Adaptation of rats to intermittent hypobaric hypoxia increases SR Ca^{2+} ATPase activity in myocardium by increasing the Ca^{2+} sensitivity of the ATPase and the V_{max} for Ca^{2+} transport; indeed, NO-induced cardioprotection against Ca^{2+} overload in rats paralleled NO induction of SR Ca^{2+} ATPase gene expression (282). Recent evidence indicates that $ONOO^{-}$ mediates NO-activation of the Ca^{2+} pump in vascular smooth muscle and cardiomyocytes by inducing *S*-glutathiolation of a regulatory cysteine residue (99); by enhancing intracellular Ca^{2+} sequestration, this physiological action of $ONOO^{-}$ could protect cells from

Ca^{2+} overload. Moreover, hypoxia adaptation makes the SR Ca^{2+} pump more resistant to oxidative damage (283, 284). Increased resistance of the sarcolemmal Na^+, K^+ ATPase to oxidative stress may also contribute importantly to intermittent hypoxia protection against Ca^{2+} overload (284). Xu *et al.* (285) showed that NO protected this ATPase by scavenging toxic free radicals. Therefore activation of NO-dependent mechanisms by adaptation to hypoxia may protect cells against Ca^{2+} overload by both decreasing Ca^{2+} entry and increasing Ca^{2+} removal and sequestration.

NO-Dependent Induction of Protective Factors. In addition to rapid mechanisms of NO signaling based on post-translational modifications of preexisting proteins, NO can modulate gene expression. This adaptive long-term regulation occurs primarily at the level of mRNA transcription and is controlled by transcription factors (286).

There is some overlap between NO- and hypoxia-induced gene regulation, due at least in part to cross talk between NO and HIF-1α (286). In normoxic conditions NO can induce HIF-1α accumulation by activating its transcription and translation, thereby mimicking hypoxia (287, 288). Transcriptional activation may be initiated by NO-mediated nitrosation of Ras. NO has also been shown to directly enhance HIF activity by nitrosating a cysteine residue in the C-terminal transactivation domain of HIF-1α (289). Other studies suggested that NO upregulated HIF-1 through the PI3K/Akt pathway (290, 291), and this effect was independent of soluble guanylate cyclase activity (290). In hypoxia, NO prevents accumulation of HIF-1α, its association with HIF-1β, and target gene activation (287). The underlying mechanism is NO-dependent activation of prolyl hydroxylase (292) or blockade of electron flow at complex I of the respiratory chain (293). The similar actions of NO and hypoxia on transcriptional activation may help explain why treatment of rats with a NO donor (63, 219, 294) or NOS stimulator (216, 295) mimicked protective effects of adaptation to intermittent hypoxia. Thus, NO administration during hypoxia may be detrimental because it hampers accumulation of HIF-1, whereas during normoxia NO mimics the effect of hypoxia and is protective.

Exogenous NO is reported to either suppress or activate expression of the hypoxia-inducible gene *VEGF*, depending on the redox status of the cell system (286). Endogenous NO induces VEGF synthesis in various cell types, including macrophages, vascular smooth muscle cells (296), and keratinocytes (297). NO enhances VEGF expression by activating Akt kinase, followed by induction of several transcription factors, of which stabilization of hypoxia-inducible factor (HIF-1) is the critical step (298). Enhancement of VEGF expression is another example of NO mimicking hypoxia, the conventional activator of HIF-1 and VEGF synthesis (299).

Another NO-responsive transcription factor is heat shock factor 1, which activates expression of HSP70 and HSP32 (heme oxygenase-1) (300). NO increased expression of both of these protective HSPs (156, 300, 301). HSP70 protects cells from ischemia (302), apoptosis (303, 304), and necrosis (305) and blocks proinflammatory processes (151). HSP32 generates the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin and thus enhances protection against oxidative injury (306). HSP32 promotes cardiac xenograft survival (307), protects against oxidative damage (308), and increases resistance to hyperoxia (309). In addition, NO enhancement of VEGF production and release is also mediated by HSP32 (37).

The hypothesis that HSP70 mediates the protective role of NO in adaptation to intermittent hypoxia was verified by studying the effect of NOS inhibition on HSP70 accumulation and hypoxia-induced protection. A program of mild hypobaric hypoxia (5,000 m; 10–30 mins daily for 8 days) induced pronounced HSP70 accumulation in rat organs and increased the survival time of rats during subsequent, more severe hypoxia (simulated altitude of 11,000 m). The NOS inhibitor *L*-NNA completely abolished both the accumulation of HSP70 and the development of hypoxia resistance. These results suggested that intermittent hypoxia evokes NO-dependent activation of HSP70 synthesis and strengthens resistance against hypoxia stress (63, 219).

In addition to induction of protective HSPs, hypoxia-induced NO production can also mobilize other essential systems of self-defense. NO stimulated synthesis of cytoprotective prostaglandins PGE_2 and PGI_2 through activation of cyclooxygenases (27, 310, 311). NO may contribute to hypoxic enhancement of antioxidative defenses (284), including synthesis of the antioxidants glutathione (306) and superoxide dismutase (312). Finally, NO was proposed to block apoptosis by blunting caspase activation (313) or by activating cytoprotective proteins such as Bcl-xL (314), cAMP response element binding protein (315), or heme oxygenase-1 (316).

In summary, NO-dependent protective mechanisms of adaptation to intermittent hypoxia are based on moderate stimulation of NO synthesis and restriction of NO overproduction directly or through negative feedback by NO originating from NOS and alternative sources. The adaptive enhancement of NO synthesis and/or availability activates or increases expression of other protective factors, which makes the protection more robust and sustained. Understanding mechanisms of adaptation to hypoxia will support development of therapies to prevent and treat hypoxic or ischemic damage to organs and cells and to increase adaptive capabilities of the organism. In this respect strategic modulation of NO metabolism is of specific interest.

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