Nitric oxide (NO) is a signaling molecule that regulates many functions, such as vascular tone, blood pressure, neurotransmission, immune response, and oxidation-sensitive mechanisms (1–4). NO may act as an autocrine or paracrine messenger, and its production and degradation are cell type dependent. NO synthesis is catalyzed from L-arginine by a family of nitric oxide synthases (NOSs), varying from picomolar/nanomolar range for short periods to micromolar range for protracted periods (3). NO reacts with molecules such as oxygen, superoxide or metals, nucleic acids, and proteins. The major reactions involving NO support its rapid oxidation into nitrate and nitrite, which are now considered not inert end products but actors of a reverse pathway that represents an alternative source of NO when the endogenous L-arginine/NOS pathway is dysfunctional (5,6).

The effect of NO production on cellular processes is also dependent on its concentration and on the presence of other free radicals. Peroxynitrites, generated from reaction with a superoxide, can interact with several cellular components and are implicated in NO signaling mechanisms involving protein modifications (6). Lower concentrations of NO have been suggested to exert a direct effect on processes such as cell proliferation and survival, whereas higher concentrations have an indirect effect through both oxidative and nitrosative stresses (3,5,6). Free radical interactions also influence NO signaling. One of the consequences of reactive oxygen species (ROS) generation is reduced concentrations of NO (1). The resulting reactive nitrogen species can also have biological effects and increase oxidative and nitrosative stress responses. Overall, cellular responses are differentially regulated by specific NO concentrations, with lower NO concentrations (picomolar to nanomolar) generally promoting cell survival and proliferation and higher concentrations (micromolar) favoring cell cycle arrest, apoptosis, and senescence (6) (Fig. 1A). However, observed divergent effects can be explained by cellular context, cell cycle point, and oncogenic state (6). The molecular mechanisms underlying the proliferative action of NO at low concentrations are not yet clear, but key molecules and pathways involved in NO-mediated inhibition have been better studied. Some basic distinct concentrations of NO have also been proposed for activity, such as for cyclic guanosine monophosphate (cGMP)-mediated processes (30 nmol/l) and nitrosative stress (1 μmol/l) (6).

Molecular Mechanisms of NO Involved in Progression of the Cell Cycle

NO blocks the progression of the cell cycle primarily at the G1/S transition. Hence, NO-induced G1 arrest has been observed in vascular smooth muscle cells (VSMCs) and
other cell lines (7–9). In other contexts and after treatment with NO donors of the nonsteroidal anti-inflammatory drug family, the entry into the G2/M phase was NO sensitive (8,10,11).

As expected, many cell cycle proteins (p21Cip1/Waf1, p27, cyclins, Cdk2, pRb, and the like) are good candidates for final molecular targets of NO, as revealed by exogenous NO, endothelial nitric oxide synthase (eNOS) transfection, and whole expression studies (7,9,12–18). NO is also able to regulate cell proliferation by targeting important mitogenic receptors and their downstream pathways, such as epidermal growth factor receptor (EGFR) signaling pathway (8,19). Moreover, 26S proteasome and apoptosis factors represent other important NO targets (20,21) (Fig. 1B).

**cGMP-dependent and -independent pathways.** A canonical NO pathway involves the selective activation of soluble guanylate cyclase (GC), the generation of cGMP, and the activation of specific cGMP-dependent protein kinases (1,22). However, further mechanisms have emerged that mainly include the formation of NO-induced post-translational modifications (8,22,23). Overall, these modifications activate pathways that are cGMP independent (Fig. 1B).

The involvement of cGMP in growth inhibition has been described in VSMCs, in which NO activates GC with a subsequent increase in cGMP leading to the phosphorylation of a vasodilator-stimulated phosphoprotein and subsequent inhibition of the epidermal growth factor signaling pathway (24,25). Recent findings indicate that other nuclear effects of NO altering the cell cycle can occur. A mechanism involved in the regulation of the cell cycle is the direct interaction of NO-sensitive GC with chromosomes during mitosis (26). More recently, NO has been shown to modulate chromatin folding in human endothelial cells through class II histone deacetylases (27).

The antiproliferative effect of NO does not necessarily require cGMP as an intermediate effector because it was also shown in a fibroblast cell line lacking soluble GC (19) or with GC inhibitors (16,28,29). Inhibition of the G1/S transition has been shown to be mostly a cGMP-independent process (7,9), although dual mechanisms were found to mediate the antimitogenic effects in VSMCs (30).

A mechanism for cGMP-independent proliferative arrest is S-nitrosylation, which can reversibly inhibit the catalytic activity of the 26S proteasome and enhance Ras guanine nucleotide exchange under nitrative stress (31,32). A recent study has also demonstrated that NO-mediated Ras nitrosylation in different subcellular compartments regulates downstream pathways stimulating cell proliferation (33). In addition, the EGFR trans(auto)phosphorylation inhibition was found to be independent of cGMP and directly inhibited by NO (19). However, an increase in NO-mediated cGMP enables the disruption of the downstream mitogenic signal through Raf1 phosphorylation (25).

eNOS S-glutathionylation is a pivotal redox regulator of endothelial function and vascular tone. Indeed, in endothelial cells, this modification reversibly decreases eNOS activity with increased production of superoxide anions. Moreover, in hypertensive vessels, eNOS S-glutathionylation is increased with impaired vasodilation (34). The impact of another mechanism, tyrosine nitration, was demonstrated in a recent study that identified several nitrated proteins involved in the different checkpoints toward the cell cycle (35).

Another cGMP-independent mechanism involves mitochondria that influence several pathways modulating cell proliferation, cell cycle arrest, and apoptosis through oxidative and nitrative reactions that are mediated by the NOS mitochondrial isoform (23,36). Moreover, peroxynitrite can also change calcium homeostasis, allowing the opening of the mitochondrial permeability transition pore that promotes mitochondrial signaling of cell death (37). In this context, NO instead exerts a protective action by directly inhibiting the opening of the mitochondrial permeability transition pore. Furthermore, cytochrome c oxidase is one of the most important targets for NO signaling, leading to inhibition of mitochondrial oxidative phosphorylation, the control of apoptosis, and ROS generation (37,38).

**Cell Cycle NO-Dependent Effects in the Cardiovascular System**

**Cell proliferation, inhibition, and apoptosis.** NO is able to inhibit cell growth and proliferation and to induce apoptosis in a dose-dependent manner (7,12,20,28,29,39,40) (Table 1). Indeed, an adequate concentration of NO is required to induce inhibition of cell proliferation. There is a lack of correlation between increased NOS expression and inhibition of cell proliferation in some cellular systems, probably due to the short life of NO that is transformed into inactive compounds (6,8,28).

NO donors and drugs affecting the NO pathway through different mechanisms have been used to inhibit cell proliferation of several cell types. Indeed, it is known that endogenously produced NO can negatively regulate cell proliferation and/or the proliferation of neighboring cells. The increase of endogenous NO depends on the availability of L-arginine and/or inducible NOS expression mediated by several cytokines (41–43).

A proliferative arrest induced by endogenous NO production has been found in different cell types, including VSMCs (8). Proliferation of these cells has been accepted as a common event in the pathophysiology of many vascular diseases. Delivery of L-arginine, pharmacological NO
donors, NO gas, or overexpression of NOS proteins can inhibit proliferation of VSMCs and reduce the injury responses within the blood vessel wall. Preclinical models have been essentially used to establish the potential of NO in the inhibition of VSMC proliferation and induction of cell cycle arrest in models of neointimal hyperplasia and restenosis (11,16,44). A recent study also suggested that NO can inhibit neointimal hyperplasia in a sex- and hormone-dependent manner (44).

Consistent with these observations, VSMCs transfected with eNOS showed inhibition of cell proliferation and of key cell cycle regulatory molecules (45). Moreover, apolipoprotein E is able to increase expression of inducible NOS in VSMCs, thereby inhibiting their proliferation (46).

NO has been implicated in both apoptotic and necrotic cell death, depending on several factors including cellular redox state. Additionally, it can be either an antiapoptotic or a proapoptotic regulator, depending on the pathway and mechanism involved. For instance, at high concentrations of NO, programmed cell death was observed after inhibition of cell proliferation in cardiomyocytes (47). Caspase activation and mitochondrial changes are also involved in the apoptosis initiated after treatment with GEA3162 in murine bone marrow cells (BMCs) (48). NO can also confer protection...
from apoptosis and promote survival of embryonic stem cells, thus delaying their differentiation (21). NO donors were found to cause a dramatic and concentration-dependent induction of apoptosis in a caspase-dependent manner in human neutrophils and murine BMCs (8,48).

**Effects on cell proliferation.** NO can also promote cell proliferation under certain conditions, although the mechanisms responsible for this effect have remained poorly understood (2,6). Interestingly, a recent in vivo study (49) showed that eNOS deficiency may cause collateral vessel rarefaction, thus impairing the activation of a cell cycle gene network during arteriogenesis; this finding has suggested a novel role for eNOS in maintaining native collateral density during growth to adulthood and in collateral remodeling in obstructive disease through regulation of cell proliferation (49). Similarly, NO derived from eNOS has been shown to play an important role in cardiomyocyte proliferation and maturation during early neonatal heart development (50). Still, NO donor treatment determines cell proliferation in several contexts (Table 1) (8,21,51). Interestingly, an NO donor at low concentrations (10 to 50 μmol/l) protected murine bone marrow stromal cells against spontaneous apoptosis (51). Similarly, exposure of embryonic stem cells to low concentrations of NO (2 to 20 μmol/l) can regulate their differentiation by arresting the loss of self-renewal markers and promoting cell survival through inhibition of apoptosis (21). Overall, these studies support the concept that the final effect of NO on the cell cycle is dependent on both concentration and the surrounding context (Fig. 1A).

The existence of multipotent resident cardiac stem cells and adult BMCs that participate in homeostasis of the heart and regeneration of the injured tissue make these cells a valuable resource for the treatment of cardiovascular disease (52,53). The involvement of NO signaling in stem cell cardiovascular biology was demonstrated in the differentiation of embryonic stem cells into myocardial cells (54). Furthermore, a recent study in a porcine model showed that activation of the NO pathway directs BMCs to a preferential cardiomyogenic phenotype and also stimulates cell proliferation (55). However, the precise role of NO in cardiac progenitors and stem cells remains poorly understood.

NO scavenger treatment can increase the proliferation of bone marrow–derived endothelial progenitor cells (EPCs), a subpopulation of adult stem cells that are recruited from bone marrow to the injured vessel to promote endothelial regeneration and neovascularization (53). Data from several studies indicate that the NO pathway may improve the paracrine and angiogenesis efficiency of BMCs in experimental hind limb ischemia (56–58) and in patients with chronic critical limb ischemia (59,60).

### The NO Pathway and Cell Proliferation in Type 2 Diabetes

Hyperglycemia, associated with endothelial dysfunction and reduced new blood vessel growth, is a primary cause of vascular complications in diabetes. Moreover, the ability of patients with type 2 diabetes mellitus (T2DM) to develop coronary collateral vessels is diminished due to a diabetes-associated impairment of EPC count and mobilization (61). The EPC count of patients with T2DM and peripheral arterial disease is substantially lower than that of healthy subjects, nondiabetic patients with vascular disease, and patients with T2DM who do not have vascular disease (53,60,62). Moreover, EPCs isolated from patients with T2DM displayed impaired proliferation and function (63). Additionally, proliferation of EPCs was inversely correlated with plasma glycated hemoglobin levels of these patients, suggesting a relationship between glycemic control and EPC number and proliferation (63).

EPCs require NO-cGMP signaling for proper function, including migration, and decreased bioavailability of NO has been proposed as one of the determinants of vascular damage in diabetes (61). Patients with T2DM have a lower overall systemic fraction of L-arginine that is converted to NO compared with healthy individuals (64). Interestingly, eNOS uncoupling in diabetic EPCs resulted in excessive superoxide anion production and reduced bioavailability of NO, implying an intimate relationship between oxidative

### Table 1 Effects of Exogenous NO on the Proliferation of Cardiovascular-Related Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NO Donors</th>
<th>NO Donor Action</th>
<th>First Author (Ref. #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic VSMC</td>
<td>DETA/NO</td>
<td>100 μmol/l to 1 mmol/l</td>
<td>Tanner et al. (7) and Kapadia et al. (20)</td>
</tr>
<tr>
<td>Umbilical arteries VSMC</td>
<td>SNAP</td>
<td>100 μmol/l</td>
<td>Ishida et al. (12,39)</td>
</tr>
<tr>
<td>HUVEC/coronary aortic endothelial cells</td>
<td>SNAP, S-nitrosglutathione, SNP</td>
<td>100 μmol/l</td>
<td>Helle et al. (28)</td>
</tr>
<tr>
<td>Peripheral blood mononuclear cells</td>
<td>GEA3162, GEA3175, SNAP</td>
<td>1–30 μmol/l GEA: 100–500 μmol/l SNAP</td>
<td>Kosonen et al. (29)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>SNAP, SNP, DETA/NO</td>
<td>100–600 μmol/l</td>
<td>Gooch et al. (40)</td>
</tr>
<tr>
<td>Murine bone marrow cells (Jaws II)</td>
<td>GEA3162</td>
<td>30–100 μmol/l</td>
<td>Taylor et al. (48)</td>
</tr>
<tr>
<td>Embryonic stem cells</td>
<td>DETA/NO</td>
<td>2–20 μmol/l</td>
<td>Tejedo et al. (21)</td>
</tr>
<tr>
<td>Murine bone marrow stromal cells (OP9)</td>
<td>SNAP</td>
<td>10–50 μmol/l</td>
<td>Wong et al. (51)</td>
</tr>
</tbody>
</table>

DETA/NO = N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]propane-1,3-diamine; GEA3162 = 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride; GEA3175 = 1,2,3,4-oxatriazolium,3-(3-chloro-2-methylphenyl)-5-[(4-methylphenyl) sulfonyl]amino], hydroxide inner salt; HUVEC = human umbilical vein endothelial cells; NO, nitric oxide; SNAP = S-nitroso-N-acetylpenicillamine; SNP = sodium nitroprusside; VSMC = vascular smooth muscle cells.
stress and EPC damage (65). Prolonged exposure of early and late EPCs to high glucose concentrations reduces their number and proliferative ability and the extent of phosphorylation of eNOS and some members of the PI3-kinase/Akt signaling pathway (66). This study also showed that impaired NO-related rather than oxidative stress–related mechanisms are involved in the EPC dysfunction induced by high levels of glucose (66). In light of such evidence, pharmacological or genetic interventions affecting the molecular pathway involving eNOS activity have been hypothesized to counteract diabetic EPC dysfunction. Thus, the stimulation of NO production or its signaling cascades may increase the number and function of EPCs and attenuate endothelium damage, independently of the vasodilatory effects of NO. In this regard, a recent study showed that treatment of glucose-stressed EPCs with superoxide dismutase attenuated generation of O$_2^-$, restored production of NO, and partially restored their ability to form colonies, whereas treatment with insulin increased production of NO but did not change generation of O$_2^-$ and their ability to form colonies. However, this ability was fully restored after combined treatment with superoxide dismutase and insulin (61). Additionally, Chen et al. reported that in vitro treatment of EPCs from patients with T2DM with an NO donor drug could also reverse impairment of EPCs induced by high levels of glucose acting on cell proliferation (66).

**New Concepts in the Role of NO in the Cell Cycle and Clinical Insights**

The NO pathway is involved in many cardiovascular conditions, and delivery of specific concentrations of exogenous NO is an attractive therapeutic option for these disorders, including those that are cell proliferation based. A well-known example of cell proliferation–based pathology is neointimal hyperplasia, which has been found to decrease with NO-based therapies in animal model studies. A recent study suggested that particular attention should be paid to the patient’s sex and hormone status when developing these therapies (44). A significant protective role of NO is also implicated in therapeutic neoangiogenesis associated with peripheral ischemia (56–60).

Interestingly, vasorelaxant prostanoids used in clinical practice, such as prostacyclin or its derivatives, have been shown to exert protective effects on endothelial cells by mechanisms that partly involve cyclic adenosine monophosphate–mediated formation of NO (67). Indeed, prostacyclin analogues such as Beraprost or Iloprost can increase the number and migration of EPCs in humans and in ischemic tissues of experimental animal models (68,69). Prostacyclin has been found to exert a direct effect on the function of EPCs in an autocrine or a paracrine manner through an NO-dependent mechanism (70). In this regard, NO-dependent vasoprotective agents such as prostacyclin or statins could have a significant therapeutic role in cardiovascular diseases under pathological conditions, such as diabetes, where EPCs are impaired. Moreover, other drugs such as angiotensin-converting enzyme inhibitors also prevent endothelial dysfunction by increasing eNOS protein expression and activity (71–73).

The biology of NO has stimulated the development of pharmacological agents with different actions on cell proliferation; for example, some agents are able to release NO, such as NO-donating nonsteroidal anti-inflammatory drugs and NO-aspirin, and may also be used against vascular damage. Indeed, NCX-4016, an aspirin-like NO donor, has been found to reduce experimental restenosis (11) and the development of atherosclerosis (74), which are linked to proliferation of VSMCs. Similarly, nebivolol, a beta-blocker that releases NO, was able to reduce experimental atherosclerosis (75). Consistently, there are numerous NO-based therapies for pulmonary hypertension (76), another disease linked to proliferation of VSMCs. Preclinical studies revealed a potent effect of inhaled nitrite in the inhibition of experimental pulmonary arterial hypertrophy and proliferation of VSMCs, although further studies are required to better establish doses, potential toxic effects, and mechanisms of their therapeutic action (77). The chemical versatility of NO has led to the synthesis of a wide range of NO donors, each with a different mode and rate of release and action, suitable for different disease targets. However, long-term use of current NO donors is limited by development of tolerance and toxicity issues, suggesting that novel alternatives should be identified. Nitrosyl-cobinamide has been found to be an effective NO-releasing compound in several models, suggesting that it could be a useful drug for treating hypertension and cardiovascular disease (78). Furthermore, a recent study showed the positive effect of infusion of NO-releasing hydrogel/glass hybrid nanoparticles that induce a reduction of blood pressure and an increase in vascular relaxation without any tolerance and immunologic response (79).

Additionally, in vitro and in vivo studies have shown that some mitochondrial antioxidants are able to decrease ROS production, leading to reduced apoptosis and improved cardiac function. However, the use of these molecules in humans require the development of specific and sensitive tools to monitor mitochondrial oxidative stress and the development of orally available compounds (80).

One of the major weaknesses in studying ROS and reactive nitrogen species is the lack of proper tools to monitor their production in vivo. Thus, sensitive and specific detection methods can be useful for elucidation of the effects of NO on cell proliferation in pathophysiological conditions both in vitro and in vivo. To date, fluorescent probes based on small organic molecules provide dynamic information concerning the localization and quantity of biological molecules of interest (81). This approach could be worthwhile to evaluate their functions in the living body by using less invasive techniques, without the need for isolating tissues or cellular constituents. Today, several design strategies for specific reactive nitrogen species fluorescent probes to use in
bioimaging technologies, including photo-induced electron transfer, are well established and have been applied to many probes. The use of this approach should be suitable for real-time analysis of NO-dependent mechanisms (82–84).

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