Review

Nitric oxide and the proliferation of vascular smooth muscle cells

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1. Introduction

Vascular smooth muscle cell (VSMC) proliferation is an important component of vessel wall remodelling in response to injury, for example, after angioplasty or vein grafting, and during atherosclerosis formation. Endothelium-derived nitric oxide (NO) production is both a tonic and an induced regulator of blood vessel tone [1–3]. Its function is impaired by atherosclerosis and, more significantly, by atherogenic risk factors, including hypercholesterolaemia, homocysteinaemia, diabetes, smoking and high blood pressure, even before the appearance of overt atherosclerosis [4–6] Endothelial NO production is dysfunctional after balloon injury and in vein grafts at the time when VSMC proliferation and neointima formation is progressing [5,6]. It has been tempting, therefore, to propose a causal relationship between impaired NO production and increased VSMC proliferation. If so, this might explain, in part, the association between endothelial dysfunction and atherogenesis.

The primary purpose of this review is to discuss critically the evidence to support such an hypothesis. We will also go on to consider the molecular mechanisms that might underlie the inhibitory effects of NO on VSMC proliferation, with the following important caveats. Firstly, any direct action of NO on an increase in VSMC numbers may be mediated at a variety of levels, for example, on the signal transduction pathways, on energy production or by promoting cell death. Secondly, in the more complex in vivo models, effects of NO on endothelial cells (ECs), platelets and inflammatory cells, rather than directly on VSMCs may be responsible for modulating VSMC proliferation. Thirdly, NO is highly unstable, with a half-life measured in seconds [2,7,8]. It reacts rapidly with oxygen species (O_2, O_2^- and H_2O_2) to produce, nitrite, nitrate or the highly reactive species, peroxynitrite (ONOO) and

with thiol groups to produce nitrosothiols [9]. Hence, the effects of NO and the pathophysiological influences on it may also be mediated indirectly, potentiated or inhibited through the availability of these oxygen species. Irrespective of the physiological role of NO in VSMC proliferation, a pharmacological effect of NO may still be exploited to reduce neointima formation and prevent adverse vessel-wall remodeling. We will therefore also consider the experience with this approach either in conventional or gene therapy.

2. Alterations in NO formation and NO synthases that may influence VSMC proliferation in pathological states

During atherogenesis, VSMCs are activated to a synthetic state, which allows them to proliferate and migrate to the intima and produce extracellular matrix [10,11]. This leads to neointima formation and the fibrous component of atherosclerosis [12,13]. Atherosclerosis is associated with a reduced endothelium-dependent vasodilatation, which has been ascribed to reduced NO formation [4,14]. Although endothelium-dependent responses are attenuated in aortic tissue from cholesterol-fed rabbits, total arterial NO production is actually increased [15]. This increased NO production is likely to be the result of the induction of inducible nitric oxide synthase (iNOS) in VSMCs [16–19]. Indeed, cholesterol loading of arterial VSMCs upregulates cytokine-induced NO formation [20]. Current evidence indicates that, despite this, the decrease in endothelium-dependent relaxation is a consequence of increased superoxide production, which quenches the increased levels of NO [21–23]. In a recent elegant study, Luoma et al. [24] found high expression of iNOS in VSMCs from both human and rabbit atherosclerotic lesions, which was co-localised with epitopes of oxidised low-density lipoprotein (LDL) and ONOO-modified proteins. These data

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consolidate the view that the local increase in levels of iNOS increases NO, which, together with increased superoxide, leads to the generation of ONOO and other nitrated oxygen species [9].

In animal models of neointima formation (arterial injury, vein grafts), where VSMC proliferation and migration are central events, NOS activity and NO release are also markedly altered. After balloon injury, there is inevitable endothelial loss, which ipso facto results in the loss of endothelial constitutive NOS (eNOS). Endothelial re-growth occurs rapidly [25], although several studies have indicated that the regrown endothelium in injured coronary arteries is dysfunctional [26,27]. This leads to agonist-selective impairment of endothelium-dependent vasodilat-ation, which suggests a defect in receptor–effector cou-pling. The loss of endothelial eNOS may be compensated for by increased iNOS expression [28]. In balloon-injured rat carotid arteries, iNOS expression is induced by inter-leukin 1-β (IL-1β) [29]. Medial and neointimal VSMCs are the main cell type expressing iNOS [30].

The situation is similar in vein grafts. For example, Cross et al. [31,32] and Ku et al. [33,34] found that porcine vein graft rings, pre-contracted with noradrenaline, failed to show endothelium-dependent relaxation in re-sponse to acetylcholine or histamine. Impaired endo-thelium-dependent relaxation was also demonstrated in explanted human coronary vein grafts, with no response to acetylcholine but a significant relaxation to A23187 [35,36]. Since acetylcholine operates via receptor-mediated NO release and A23187 by receptor-independent mecha-nisms, these data also suggest that while ecNOS may be preserved in human vein grafts, disruption may occur at some point upstream in the signalling pathway linked to the receptor. Consistent with this, in porcine vein grafts, there is a marked reduction in cGMP formation in the medial and intimal regions, even though eNOS content and activity is actually increased [37,38].

Haemodynamic forces, which are altered by atheroma and as a consequence of restenosis and vein graft thicken-ing, also exert a powerful influence on endothelial struc-ture and function. For example, high shear stress induces activation of signal transduction mechanisms, including phosphoinositide (PI) turnover [39,40]. Thus, although there is rapid endothelial regrowth in recently implanted vein grafts, its functional integrity may be compromised by the impact of arterial haemodynamic forces. Increased ecNOS may constitute an adaptive response to shear stress. There also appears to be a marked increase in iNOS in vein-grafts [38]. Although a decrease in NO activity is associated with neointima formation [41], increased NO formation in itself may be deleterious to vein grafts. For example, there is increased accumulation and infiltration into vein grafts of leukocytes, including neutrophils [42,43], which generate large amounts of superoxide, thereby transforming NO into ONOO [44]. Indeed we have recently detected large amounts of nitrated tyrosine (an

index of ONOO [44]) in porcine vein grafts (P. Gadsdon and J.Y. Jeremy, unpublished observations).

3. Evidence that NO inhibits VSMC proliferation

3.1. Pharmacological studies in vitro

Early studies demonstrated that NO donor agents, including sodium nitroprusside (SNP), nitrosothiols, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-mor-pholinosydon imine (SIN-1) inhibit the proliferation of isolated rat and rabbit VSMCs in tissue culture [45–48]. The effective concentrations of NO donor agents were higher than in studies of vasodilator action but occurred at similar intracellular cGMP levels [47]. Below 1 mM SIN-1, cells remained viable, as judged by ATP concentrations, although additional effects of higher concentrations of SIN-1 on proliferation were accompanied by loss of cell viability [48]. Not all preparations of VSMCs showed equal sensitivity to NO donors and 8-Br-cGMP. Indeed, synthetic state, isolated rabbit smooth muscle cells were inhibited, whereas contractile cells interacting with their native extracellular matrix within aortic explants were refractory [49]. On the other hand, human saphenous VSMCs within organ cultures were inhibited, arguing that the difference was not simply one of contractile versus synthetic cells [50].

In several studies, the effects of low, but not higher, concentrations of NO-donors were mimicked by 8-Br-cGMP [47], indicating the involvement of cGMP-depen-dent protein kinase. However, NO can also directly inhibit the synthesis of RNA and proteins in VSMCs [51]. NO also directly inhibits mitochondrial respiration, which, in turn, may influence growth [52]. Sarkar et al. [53,54] recently suggested multiple sites for the effect of the NO donors, SNAP and *S*-nitroso-glutathione (SNOG) on the cell cycle in rat aortic VSMCs. Individually, these elicited a 50% reduction in the fraction of cells in the S and G_2+M phases and a corresponding increase in the G_1 fraction, suggesting that NO inhibits S-phase entry of VSMCs. Addition of both donors together, however, immediately blocked replication reversibly in the S-phase, an effect that was not mimicked by exogenous cyclic GMP. These experiments implied that NO inhibition of VSMC proliferation is associated with two distinct and reversible cell cycle arrests, an immediate cGMP-independent S-phase block and a cGMP-dependent shift back from the G_1–S boundary to a quiescent G_0-like state [53,54].

The studies with lipid-soluble cGMP analogues imply that NO inhibits VSMC proliferation by activating the cGMP-dependent protein kinase (protein kinase G). How-ever, NO-induced elevation of cGMP in VSMCs can activate adenosine 3′,5′cyclic monophosphate (cAMP)-de-pendent protein kinase [55], which also is a potent inhibitor of VSMC replication [47,56–58].

3.2. Time course of the effects of NO on proliferation

Most in vitro experiments have studied the effect of NO, NO-donor drugs or cGMP analogues added initially to cells stimulated to enter the cell cycle from quiescence. However, a similar inhibitory effect is obtained when the addition of NO-donors or 8-Br-cGMP is delayed for 6 h after growth-factor stimulation (Taylor and Newby, unpublished observations, 1999). Hence, the most important site of action of NO and cGMP may not be on early signal transduction events but later in the G_1 phase of the cell cycle. Similar results have been observed in the case of cAMP-elevating agents and several other physiological inhibitors of VSMC proliferation, including transforming growth factor-β and interferon-γ [11,59]. This is not to say that the effects on early signalling events are irrelevant and, indeed, they may have more important roles in the induction of early response genes (for example, metalloproteinases).

3.3. Pharmacological studies in vivo

In vivo effects of substrates for NO synthase and NO donors may not be mediated through direct inhibition of VSMC proliferation but also through inhibition of platelet and leukocyte activation. Both of these cells can play a role in atherogenesis and neointima formation [6,59] (see sections on platelets and leukocytes below). Conversely, inhibitors of NO formation, such as L-NAME (L-nitro-arginine-methyl ester) can induce leukocyte activation [60].

Direct, long-term inhibition of NO formation promotes atherosclerosis and neointima formation in the hypercholesterolaemic rabbit thoracic aorta [61]. However, this observation has recently been challenged [62]. Several studies have demonstrated that the oral administration of L-arginine or NO donors inhibit the in vivo proliferation of VSMCs in laboratory animals [63,64]. Administration of L-arginine (which, as a substrate, increases endogenous NO formation) to hypercholesterolaemic rabbits augments vascular NO formation and reduces atheromatous lesions in rabbits [65]. Interestingly, dietary correction of hypercholesterolaemia in the rabbit also normalises increased endothelial superoxide production and, hence, generation of ONOO [66]. In cholesterolaemic rabbits, the administration of L-arginine reduces intimal thickening in iliac arteries denuded of endothelium with a balloon catheter [67–69]. Similar inhibitory effects of L-arginine administration on intimal thickening were obtained in the rat arterial injury model [70–74]. Chronic inhibition of NO formation with L-NAME in rats also caused a significant increase in the vessel wall-to-lumen ratio [74] and accelerated neointima formation in hypercholesterolaemic rabbits [41].

Administration of the NO donor (SIN-1) inhibited VSMC proliferation but not final neointimal thickening in balloon-injured pig carotid arteries [30]. The effect may have been direct or through platelet inhibition because SIN-1 inhibits both the adhesion of platelets and mural thrombus formation in balloon-injured pig arteries [75].

Few systematic pharmacological studies of the effects of NO on neointima formation have been carried out in man. However, in the ACCORD study, administration of the NO donors, linsidomine and molsidomine, was associated with a modest improvement in the long-term angiographic result after angioplasty but had no effect on clinical outcome [76].

3.4. Gene transfer and transgenics

A number of studies have established that gene transfer of eNOS successfully influences endothelium-dependent relaxation [77–79]. Kullo et al. [78] studied rabbit carotid arteries exposed to adenoviral vectors encoding eNOS (ADeNOS). Over-expression of eNOS resulted in diminished contractile responses as well as enhanced endothelium-dependent relaxations [78]. In another study, the same authors introduced adenoviral vectors encoding eNOS into the periarterial sheath (adventitia) of carotid arteries, which enhanced endothelium-dependent relaxation when assessed four days later [79]. Both luminal and adventitial delivery represents a convenient means of introducing gene vectors into blood vessels, which may be useful clinically.

Gene transfer and transgenic models have provided the most persuasive evidence for a role of NO in moderating VSMC proliferation [80–82]. For example, transfer of both eNOS and neuronal NOS (nNOS) inhibits VSMC proliferation and neointima formation in balloon injury and vein-grafting models [83–86]. Interestingly, adventitial expression of the recombinant eNOS gene restores NO production in arteries without endothelium [87]

Over-expression of human ecNOS in syngeneic rat arterial SMCs using a retrovirus, which were then seeded onto balloon-injured carotid arteries, inhibited neointimal formation by 37% and induced marked dilatation at two weeks compared with vector alone [82]. Orally administered N-ω-nitro-L-arginine blocked these changes. Varenne et al. [88] investigated the effect of intramural injection of adenovirus carrying the eNOS cDNA on pig coronary arteries subjected to angioplasty. In eNOS-transfected animals, neointimal thickness, lumenal area and % restenosis were all reduced compared to animals treated with control vector [88]. Mice display a hyperplastic response of the arterial wall in response to external carotid artery ligation [89]. Mice with targeted disruption of the eNOS gene show a greater increase in wall thickness compared to wild-type mice[89].

Given the larger quantities of NO generated, it is not clear if iNOS functions through the same mechanisms as eNOS to limit VSMC proliferation. Porcine arteries infected with human iNOS cDNA and subjected to balloon

injury exhibited a threefold increase in total NO synthesis and a 15-fold level of cGMP (despite only 1% transfer efficiency) as well as reduced intimal thickness [90,91]. The administration of a NOS inhibitor reversed these effects [90,91].

The effect of transfer of human iNOS cDNA was investigated in a rat aortic allograft transplantation model [92]. After 28 days, control allografts were found to have intimal thickening despite significant increases in both iNOS mRNA and protein. Inhibition of NO production with iNOS inhibitor, however, further increased intimal thickening by 57%. In this model, cyclosporine suppressed the expression of and promoted intimal thickening. Conversely, transduction with iNOS cDNA using adenoviral vector inhibited completely the development of allograft atherosclerosis. These data suggest that early immune-mediated upregulation in iNOS expression partially suppresses allograft arteriosclerosis.

In injured rat carotid arteries, transfer of the iNOS gene initiated at the time of vascular injury prevented intimal hyperplasia, a response reversed by the administration of L-*N*-6-(1-iminoethyl)-lysine [93]. In the same study, but using a different animal model, human iNOS gene transfer to injured porcine arteries also reduced intimal hyperplasia by 51.8% [93].

When hearts from wild-type mice were transplanted into syngeneic recipient mice, there was increased luminal stenosis and increased intimal/medial ratios [94]. Allografts taken from iNOS knock-out recipients had increased neointima formation compared to wild-type controls [94]. It was concluded that iNOS plays a protective role in the development of arteriosclerosis in transplantation, suppressing neointimal VSMC accumulation [94].

4. Mechanisms underlying the inhibitory effects of NO on VSMC proliferation

The success of various mechanisms in explaining the inhibitory effects of NO on proliferation can be judged against a series of increasingly stringent criteria (Table 1). Stated briefly, these are that addition of NO should produce the effect, other agents that produce the same effect should also inhibit VSMC proliferation, antagonism

of the effect should reverse the inhibitory action of NO and that a precise molecular mechanism for the action of NO should be defined.

4.1. Effects on the signal transduction pathways

To place the effects of NO on signal transduction into context, a brief and simplified overview of the principal intracellular signalling mechanisms involved in VSMC proliferation is warranted. VSMC proliferation is promoted by the concerted action of several distinct signal transduction pathways (Fig. 1). These include phospholipase C isoforms (which link to Ca^{2+} release from intracellular stores and activation of protein kinase C) and the ras, Raf-1, MAP kinase cascade [95–97]. Ultimately, these pathways lead to transcription of the nuclear transcription factors, *c-fos* and *c-jun*, by mechanisms involving pre-existing transduction factors, including the serum response factor. *C-fos* and *c-jun* together constitute the activator protein-1 complex that binds to the promoter region of many other early response genes. Coupling of tyrosine kinase receptors to the soluble tyrosine kinases, *c-src*, *c-yes* and *c-fyn* [98] leads to induction of the *c-myc* transcription factor [99]. Increased transcription of the *c-myc* oncogene is required for VSMC proliferation [100,101]. Agents such as angiotensin II, 5-hydroxy-tryptamine and thrombin, which act through G-protein-linked receptors, trigger the same pathways also, through less completely understood mechanisms [102,103]. Inflammatory cytokines, including interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) act through receptors that couple transiently with cytosolic serine kinases (janus kinases, JAKs), which, in turn, activate signal transducers and activators of transcription (Stats) [104].

Inflammatory cytokines also cause translocation of the nuclear factor-κB transcription factor (NF-κB) to the nucleus [105]. The events shown to be affected by NO/cGMP are circled in Fig. 1A and discussed below.

These early events, within the first 6 h after the addition of growth factor, promote transition of cells from quiescence (defined as G_0) into the G_1 phase of the cell cycle. They prime cells for further progression through the cell cycle (see Fig. 1B), which may then be sustained by other growth factors, including epidermal growth factor (EGF)

Table 1
Criteria for judging the significance of mechanisms proposed to mediate the action of NO on VSMC proliferation

1. Addition of NO at the time points and concentrations that inhibit proliferation regulates the mechanism in the appropriate direction (i.e. activation or inhibition).
2. Modulation of the same pathway in the same sense by other agents also causes inhibition of proliferation.
3. Modulation of the mechanism in the opposite sense using pharmacological or molecular biological methods antagonises the effects of NO.
4. The biochemical mechanisms (e.g. phosphorylation events) regulating the mechanism in response to NO are defined.
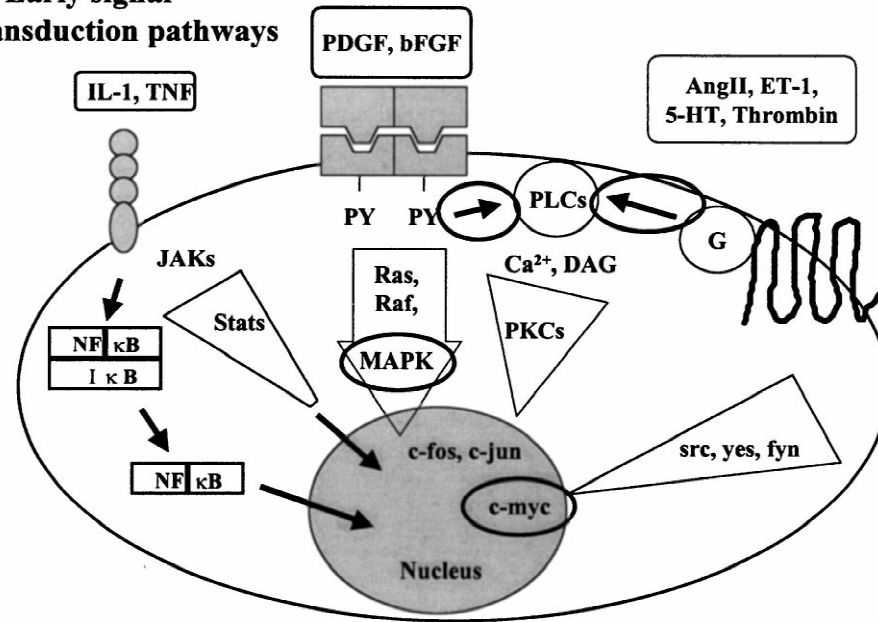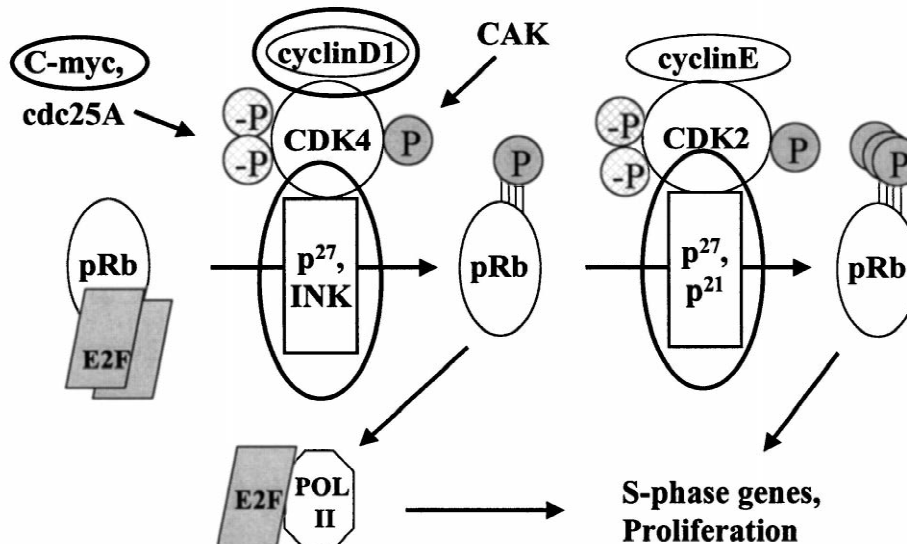
Fig. 1. Possible sites for cGMP-mediated inhibition of VSMC proliferation. (A) Early signal transduction events. Peptide growth factors, for example, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF, FGF-2) stimulate tyrosine phosphorylation of receptors (PY) and couple to effector mechanisms, which include phospholipase C (PLC) isoforms, which generate the second messengers Ca^{2+} and diacyl glycerol (DAG). These activate isoforms of protein kinase C (PKC). PY activates the *c-ras, c-raf,* mitogen-activated protein kinase (MAPK) cascade, the final result of which is transcription and activation of the early response genes *c-fos* and *c-jun.* PY also activates soluble tyrosine kinases, *c-src, c-yes* and *c-fyn,* which induce the nuclear transcription factor *c-myc.* Angiotensin II (Ang II), endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and thrombin, acting through G-protein-linked receptors, trigger the same pathways through less completely understood mechanisms. Inflammatory cytokines, including interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), act through receptors that couple transiently with cytosolic serine kinases (janus kinases, JAKS), which, in turn, activate signal transducers and activators of transcription (Stats). Inflammatory cytokines also cause translocation of nuclear factor-κB transcription factor (NF-κB) to the nucleus. Events potentially regulated by NO/cGMP are circled in bold. (B) Late G_1 events. The retinoblastoma protein (pRb) binds to transcription factors, including E2F, to prevent gene transcription, including that of DNA polymerase II (POL II), which is required for DNA replication. Cyclins D and E and their kinase partners CDK4 and CDK2, respectively, phosphorylate pRb, which reduces its affinity for E2F and other transcription factors. CDK4 and CDK2 are regulated by phosphorylation by cyclin-activating kinase (CAK) and by dephosphorylation by the phosphatase, cdc25A. This phosphatase may be induced by the *c-myc* transcription factor. Events potentially regulated by NO/cGMP are circled in bold.

and insulin-like growth factor-1 (IGF-1). Progress through the cell cycle is controlled by cyclin-dependent protein kinases (cdks) and their cyclin catalytic partners [106–108]. The G_1-phase-specific D and E cyclins are key regulators of passage from G_1 to S-phase, where DNA synthesis commences and cells become committed to replicate [103]. Cyclin D and E mRNAs are induced by growth factors, including PDGF [109,110], and are suppressed by antiproliferative agents [111,112]. Cyclin D1 expression is regulated positively by the MAP kinase p42/p44 and negatively by the MAP kinase p38/HOG pathways [113,114]. Induced over-expression of the D cyclins accelerates cell cycle progression and shortens the cycle in many cell types [115,116]. A principal substrate for the G_1 cyclins is the retinoblastoma (pRb) tumour suppressor gene [116], which, in its hypophosphorylated form, suppresses S-phase progression. Cyclin-dependent phosphorylation of pRb negates its action, allowing replication to progress. The activity of the cdks is itself regulated positively and negatively by phosphorylation at different sites [117,118]. Indeed, the essential role of *c-myc*, in cell cycle progression may be explained by its ability to induce the cdc25A protein phosphatase, which dephosphorylates and activates cdks99. Several cdk inhibitors, which are either selective for D cyclins (INK1-4) or nonselective for D and E cyclins (p27^{cip1}, p21^{waf1}), play an additional important role in regulating cdk phosphorylation of pRb [118,119]. In principle, NO/cGMP could interfere at any stage of this signal transduction cascade, although the events for which there is direct evidence (see below) are circled in Fig. 1B.

4.2. Calcium

There is no doubt that Ca^{2+} plays a key role in vascular contraction and that the NO–cGMP axis mediates its vasodilator effects in part by inhibiting Ca^{2+} entry and mobilisation [120]. Circumstantial evidence points to a similar regulatory role of Ca^{2+} in VSMC proliferation [121]. Vasoconstrictor agonists and growth factors generally cause an immediate (within seconds to minutes) increase in cytosolic Ca^{2+}. Recent studies with thapsigargin, a potent and selective inhibitor of the endoplasmic reticulum Ca^{2+}-sequestering ATPase, demonstrate the crucial role of Ca^{2+} stores in VSMC replication [122–124]. In isolated human VSMCs, thapsigargin, at concentrations less than 10 nM, inhibited the release of Ca^{2+} from intracellular pools [122–124], although it did not acutely alter resting cytosolic Ca^{2+} [122–124]. This highlights the importance of release from intracellular stores, which may include the endoplasmic reticulum and the nuclear envelope [125].

With regard to the NO–cGMP axis, NO has been shown in intact vascular preparations to inhibit agonist-induced Ca^{2+} mobilisation [126]. In turn, cyclic GMP influences the transport of Ca^{2+} in and out of intracellular stores as well as across plasma membranes by modulating the Ca^{2+}–Mg^{2+}-dependent ATPase activity of the Ca^{2+} pump [127]. Likewise, many studies have shown that Ca^{2+}-channel blockers, which inhibit Ca^{2+} influx, also inhibit the proliferation of VSMCs and neointima formation following experimental arterial injury [128]. Felbel et al. [128] found that cGMP kinase blocks Ca^{2+} influx from the extracellular space and Blatter and Weir [129] showed that both nitroprusside and cGMP lowered cytosolic Ca^{2+} in VSMCs. On this theme, Clementi et al. [130] reported that PDGF-BB induced Ca^{2+} responses are differentially modulated by NO: inhibition of IP$_3$-sensitive Ca^{2+} stores and augmentation of store-dependent Ca^{2+} channels (SDCs) and second messenger-operated Ca^{2+} channels (SMOCs). In this context, Ca^{2+}-channel blockers such as nifedipine and verapamil, which prevent Ca^{2+} entry via blockade of these channels, are potent inhibitors of VSMC proliferation and migration [130–132]. As mentioned, cAMP-dependent kinases, in particular PKAs, can be activated by cGMP [55]. In turn, PKA phosphorylates Ca^{2+}–Mg^{2+} ATPases (Ca^{2+} pumps) at both the plasma membrane and the sarcoplasmic reticulum, which results in cytosolic Ca^{2+} being pumped out of the cell or being re-sequestered back into the sarcoplasmic reticulum [56]. In VSMCs, SNAP decreased cytosolic Ca^{2+} levels and elicited phosphotyrosine dephosphorylation [133]. These effects are mimicked by the extra- and intracellular Ca^{2+} chelators EGTA and BAPTA and by the Ca^{2+} channel blocker [133]. Both BAPTA and nifedipine also decreased DNA synthesis [133], providing further evidence to link membrane Ca^{2+} channels, Ca^{2+} levels and dephosphorylation to the control of mitogenesis [133]. In contrast, Assender et al. [47] found no effect on basal or agonist-stimulated intracellular Ca^{2+} by NO and 8-Br-cGMP at concentrations that inhibited VSMC proliferation (failed criterion 1 of Table 1).

Overall, the preceding studies provide strong evidence that NO inhibits VSMC proliferation by preventing an increase in cytosolic Ca^{2+}. However, the precise relationship between NO, Ca^{2+} mobilisation and VSMC proliferation remains to be fully elucidated, particularly in light of the studies that showed no effect of NO on Ca^{2+}.

4.3. MAP kinase

A recent study by Yu et al. [134] demonstrated that sodium, 8-bromo cGMP as well as a phosphodiesterase type V inhibitor (which elevates cGMP levels through inhibition of its hydrolysis) all attenuate growth factor-stimulated VSMC replication via a cGMP-dependent mechanism. It was also demonstrated that sodium nitroprusside and 8-Br-cGMP decreased the activity of MAP kinase, MAP kinase kinase and their regulatory proteins

Ras and Raf-1, which couple peptide growth factors to the MAP kinase cascade [134] (criterion 1 of Table 1). SNAP inhibited VSMC proliferation and increased protein tyrosine phosphatase activity, in particular, in proteins of 70–85 kDa and ~215 kDa molecular mass [135]. SNAP also increased protein tyrosine phosphatase (PTPase) activity in VSMC homogenates, indicating that phosphotyrosine dephosphorylation was likely to be the result of increased PTPase activity. 8Br-cGMP and atrial natriuretic peptide elicited similar effects [135] (criterion 2 of Table 1).

4.4. c-myc

Bennett et al. [136,137] observed that 8-Br-cGMP, as well as 8-Br-cGMP, heparin and interferon-γ, at concentrations that caused a similar 50% inhibition of rat aortic VSMC proliferation, all inhibited the expression of c-myc (criterion 1 of Table 1). Studies with antisense oligonucleotides directed against c-myc demonstrate that this inhibition is sufficient to cause cell cycle arrest (criterion 2 of Table 1). Moreover, when c-myc was over-expressed using a retroviral construct, the inhibitory effects 8-Br-cGMP and the other agents was completely reversed (criterion 3 of Table 1) [136,137]. Taken together, these data represent a strong case for considering c-myc, which is active late in the late G_1 phase of the cell cycle, as a possible mediator of the effects of NO/cGMP on VSMC proliferation. However, as with the other proposed mechanisms, the precise phosphorylation events required have not been defined (criterion 4 of Table 1)

4.5. G_1 cyclins

NO has also been shown to exert an impact downstream of these aforementioned cytosolic events. Ishida et al. [138] have demonstrated that the NO donor, SNAP, induces cyclin-dependent kinase inhibitor $p21^{Sdi1/Cip1/Waf1}$. (criterion 1 of Table 1). Thus, NO ultimately inhibits the G_1/S transition by inhibiting Cdk2-mediated phosphorylation of the retinoblastoma protein, and p21 induction is involved in the Cdk2 inhibition.

Less effort has been devoted to identifying the cGMP-independent effects of NO on VSMC proliferation. NO also exerts direct effects on ribonucleotide reductase, an enzyme essential in DNA synthesis [139–141]. Inhibition of this enzyme by NO (criterion 1 of Table 1) may result in cell cycle arrest at the G_1–S boundary

5. Indirect influences of NO on VSMC proliferation

In a complex event, such as atherosclerosis or restenosis after angioplasty, both cell death and direct inhibition of cell proliferation may contribute to decreasing the extent of hyperplasia [142]. Furthermore, indirect effects on other cell types, such as endothelial cells, which generally suppress VSMC proliferation [143,144] and macrophages, which are stimulatory [145], can also influence the final response.

5.1. Effects on VSMC death

Cell death and apoptosis are prominent features of advanced atherosclerotic lesions, often resulting in the formation of hypocellular fibrous zones and a lipid-rich necrotic core [146–148]. Apoptosis also occurs in restenotic lesions after balloon angioplasty [149,150]. The main significance of VSMC apoptosis is to counteract the increase in neointimal cell number resulting from neointimal migration and proliferation. Apoptosis is generally held to be silent, i.e., it does not result in growth-factor release or provoke an inflammatory response. Unlike necrosis, it therefore should not provoke compensatory VSMC proliferation or migration nor induce iNOS in neighbouring cells. However, recent evidence suggests that bFGF can be released from cells dying by apoptosis and could therefore elicit proliferation in remaining healthy cells [151]. This might explain why the early occurrence of apoptosis [152] is strongly correlated with later hyperplasia [147].

Inflammatory mediators may also directly stimulate VSMC apoptosis through the generation of NO. For example, incubation of VSMCs with IL-1β results in NO release and concomitant cytotoxicity, an effect reversed by N^G-monomethyl-L-arginine [151]. Geng et al. [153] also demonstrated that apoptosis of VSMCs induced with INF-γ, TNF-α and IL-1β is mediated by NO. NO derived from iNOS also induces macrophage apoptosis [154,155], which, in turn, may influence VSMC proliferation.

5.2. VSMC migration and matrix deposition

It is the currently held consensus that neointima formation after arterial injury or vein graft surgery involves migration of medial VSMCs towards the lumen where they continue to proliferate and secrete matrix proteins [11]. The mechanisms underlying migration have been reviewed [156,157]. There is much less data concerning the effects of NO and its derivatives on VSMC migration or matrix formation than proliferation. However, NO has been shown to inhibit VSMC migration when stimulated with angiotensin II through a cGMP-dependent mechanism [158]. NO modulates basal and endothelin-induced increases in collagen levels [159], NO down-regulates the synthesis of type IV collagen and fibronectin but stimulates the production of laminin by rat mesangial cells [160]. In a

study in which porcine coronary EC and VSMCs were grown in co-culture, NO produced by the endothelium inhibited VSMC production of collagen types I and III but had no effect on collagen type I [161]. These effects were blocked by L-NAME. As noted previously, proliferating VSMCs in intact vascular tissues secrete metalloproteinases, which proteolyse matrix proteins, thereby allowing them to migrate [11]. Trachtman et al. [160] have found that NO stimulates the activity of a 72-kDa neutral matrix metalloproteinase (gelatinase) in cultured rat mesangial cells. If also true for VSMCs, this would constitute a pathway by which NO might promote migration.

5.3. Effects on endothelial proliferation and death

Intact endothelium is a key inhibitor of VSMC proliferation [162,163]. It follows that regeneration of damaged endothelium is a major determinant of VSMC hyperplasia after balloon injury. In other circumstances, activated ECs are a source of VSMC mitogens [162,163].

Hansson et al. [164] demonstrated that ecNOS is rapidly upregulated in the ECs of vessels in response to injury. There is also a three-to sixfold increase in ecNOS protein and ecNOS mRNA in growing compared to growth-arrested bovine endothelial cells [164]. The increased release of NO may limit neointima formation under the repairing EC. On the other hand, Kourembanas et al. [165] demonstrated that in vitro suppression of NO formation with L-NAME caused a threefold increase in endothelin-1 (ET-1) and PDGF-B expression in human umbilical vein ECs. Both ET-1 and PDGF-B are potent promoters of VSMC proliferation and are chemoattractants for VSMCs [166–168]. Similarly, long-term blockade of NO synthesis in rats resulted in increased tissue angiotensin converting enzyme activity and angiotensin II formation, an established mitogen for VSMCs [169]. Thus, suppression of NO formation can increase VSMC proliferation either directly or through modulation of other growth promoters.

With regard to EC regeneration itself, data are ambivalent. Neither NOS inhibitors nor superoxide dismutase (SOD) affect EC proliferation in culture [170]. Similarly, vascular iNOS gene transfer, while inhibiting SMC proliferation, does not affect EC proliferation or viability [171]. In relation to programmed EC death, the addition of NO actually inhibits lipopolysaccharide-induced apoptosis in ECs by reducing caspase 3-like protease activity [171]. In a blood vessel, more rapid endothelial regrowth as a result of NO-mediated protection from apoptosis would indirectly inhibit VSMC proliferation (see below).

In contrast, there is compelling evidence for a key role for NO in angiogenesis, a process that quintessentially involves the proliferation, migration and tube formation of ECs [172–175]. Angiogenesis is regulated predominantly by vascular endothelial growth factor (VEGF), although many other growth and humoral factors also come into play [172]. VEGF augments NO release from the quiescent rabbit and human vascular endothelium [173] and induces NO-dependent relaxation in coronary arteries [174]. It has also been demonstrated that NO mediates VEGF-stimulated mitogenesis of microvascular ECs [175]. VEGF-stimulated NO release from simian virus 40 immortalised microvascular ECs induced cell migration whereas L-NAME or antisense oligonucleotides to ecNOS suppressed this effect of VEGF [176]. In an in vivo model of angiogenesis, the rabbit cornea, Ziche et al. [177] found that sodium nitroprusside potentiated the pro-angiogenic effect of substance P. The same authors found that angiogenesis stimulated by basic fibroblast growth factor (bFGF) was unaffected by inhibitors of NO formation [177,178]. This indicates that NO is one, but not the only, factor controlling angiogenesis in this system. Angiogenesis in the rabbit cornea in vivo and in vitro and migration of human umbilical ECs is greatly reduced by the absence of L-arginine [179]. VEGF transfer reduces intimal thickening via increased production of NO in carotid arteries [180].

5.4. Effects on blood platelets and inflammatory cells

NO donors may also influence VMSC behaviour indirectly through actions on platelets and leukocytes. It is well established that NO inhibits platelet and leukocyte adhesion to ECs [180–182]. On adhesion and aggregation, platelets generate a number of pro-mitogenic substances, including PDGF, TGF-β, epidermal growth factor (EGF), thromboxane A_2 (TXA_2), 5-HT, platelet-activating factor (PAF), platelet factor IV, fibrinogen, fibronectin, vWf, thrombospondin and β-thromboglobulin, as well as heparitinase, elastase, collagenases and cathepsins [183,184]. Neutrophils, T lymphocytes and monocytes release an array of substances that may also contribute to neointima formation, including peptide growth factors, leukotrienes (LTs), interleukins, histamine, TNF-α and PAF. There is increasing evidence that there is significant 'cross talk' between platelets and incoming leukocytes. For example, platelet–neutrophil complexes play a key role in angina [185,186]. Neutrophils, in addition to platelets, are the main constituents of intracoronary thrombus in acute myocardial ischaemia. In turn, microthrombi, which accumulate secondarily to platelet adhesion, have been suggested as being more relevant as a trigger to neointima formation than platelet accumulation alone.

5.5. Interactions of NO with reactive oxygen species, lipoproteins and homocysteine may directly or indirectly influence its effects on VSMC proliferation

It is by virtue of its interactions with reactive oxygen species generated by 'oxidative stress' that NO can become pro-atherogenic [187]. Briefly, NO reacts with

superoxide (O_2), hydroxyl (OH), peroxyl, alkoxyl, hydroperoxyl and hydrogen peroxide (H_2O_2) as well as with thiols [8]. Principal amongst the products of these reactions are peroxynitrite (ONOO) nitrite (NO_2^-), nitrate (NO_3^-), nitrosonium ions and nitroxide ions [187]. In the presence of transition elements (e.g. copper or iron), NO can also undergo reactions with phenolics, thiols and secondary amines to yield nitrosothiols and iron nitrotyrosyls [187]. Nitrosothiols act as naturally occurring NO donors. Indeed, the formation of *S*-nitrosothiols may account for the ability of ONOO to produce vascular relaxation [188]. Nitrosothiols also inhibit VSMC proliferation. At a mechanistic level, ONOO is ambivalent in that it can elicit events that are either pro- or anti-mitogenic. For example, ONOO elicits release of $[Ca^{2+}]_i$ in VSMCs but reduces IP_3-mediated release of Ca^{2+} in response to VEGF [21,189]. MAP kinase can be activated by reactive oxygen species, such as O_2^- or H_2O_2, and this may be mimicked by ONOO [190]. Although ONOO activates guanylate cyclase, it is 50–100 times less potent than NO [191]. Interestingly, acidic FGF enhances ONOO-induced apoptosis in primary murine fibroblasts [192].

In the context of ONOO, we have recently explored the pro-atherogenic effect of homocysteine, an established risk factor for accelerated atherosclerosis [193,194]. Homocysteinaemia in animal models results in endothelial denudation, VSMC proliferation, matrix protein deposition and intimal thickening [195–198]. Homocysteine inhibits EC growth in culture [199–201] but promotes proliferation of VSMCs [202–204]. In addition, homocysteine enhances VSMC collagen matrix production and an excessive accumulation of insoluble collagen [205–207]. Such observations are consistent with a role for homocysteine in the pathogenesis of atherosclerosis. In the majority of these in vitro studies, high concentrations of homocysteine (mM) were used to elicit effects. Since homocysteine levels become pathological in the 20–100 μM range, these data indicate that, in vivo, homocysteine interacts with other substances to promote angiopathy. For example, oxidation of homocysteine can promote the formation of H_2O_2 and O_2^-, [208] which augment VSMC proliferation but impair EC growth and function [193,194]. We have recently established that copper markedly augments the inhibitory effect of homocysteine on endothelium-dependent relaxation of the isolated rat aorta, an effect that is reversed by the presence of superoxide dismutase and catalase [193,194]. We proposed that O_2^- generated by homocysteine and copper interacts with NO to produce ONOO (Fig. 2) and that this cascade may be central to the angiopathic impact of homocysteine.

6. Concluding remarks

Although it is clear from current evidence that NO plays a key role in mediating vascular remodelling, its mechanisms of action are far from being straightforward. For example, NO elicits diametrically opposite effects on different cell types: namely, the inhibition of VSMC proliferation but the promotion of EC proliferation and microvessel formation. This selectivity is remarkable given
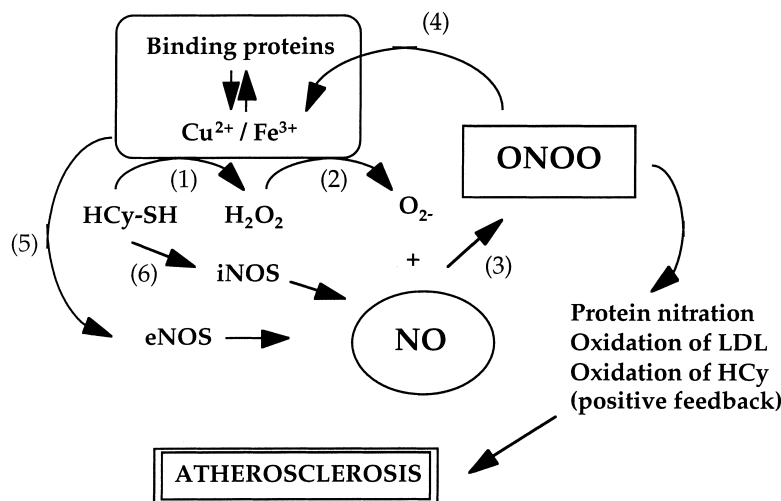
Fig. 2. Hypothetical model of the oxidant stress cascade mediated by homocysteine (HCy–SH): (1) transition metals (free and protein bound) accelerate oxidation of HCy to generate H_2O_2. (2) H_2O_2 is oxidised to superoxide (O_2^-), which also catalysed by transition metals. (3) Superoxide reacts with NO to produce peroxynitrite (ONOO). (4) ONOO dissociates copper and iron from binding proteins. (5) Free Cu^{2+} augments eNOS activity and (6) inducible NO (iNOS) is enhanced by HCy as well as cytokines and hypoxia. Together, increased local levels of NO provide 'fuel' for the generation of ONOO and perpetuation of the cascade. ONOO also accelerates the oxidation of HCy to generate more H_2O_2. This cascade can come into play in other risk factor scenarios, in particular, diabetes mellitus and lipidaemia, where superoxide generation is enhanced.

the common signal transduction systems shared by the two cell types. Nevertheless, elucidation of the differential mechanisms governing these effects of NO may provide invaluable insights into the fundamental systems that control proliferation of these important cell types. There are also several paradoxes that arise from the reactivity of NO with oxygen free radicals and the up-regulation of NO production by cytokines and growth factors. In certain pathological environments, normally protective NO can become pro-atherogenic. Under these circumstances, it may be that drug therapy with NO donors or even gene transfer of NOS may be ineffective or even deleterious. Certainly, plenty of research remains to be carried out on this perennially interesting molecule.

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