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Review

Nitric oxide – an endothelial cell survival factor

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Abstract

Due to its unique position in the vessel wall, the endothelium acts as a barrier and thereby controls adhesion, aggregation and invasion of immune competent cells. Apoptosis of endothelial cells may critically disturb the integrity of the endothelial monolayer and contribute to the initiation of proinflammatory events. Endothelial cell apoptosis is counteracted by nitric oxide synthesised by the endothelium nitric oxide synthase (eNOS). Thus, nitric oxide inhibits endothelial cell apoptosis induced by proinflammatory cytokines and proatherosclerotic factors including reactive oxygen species and angiotensin II. The apoptosis-suppression may contribute to the profound anti-inflammatory and anti-atherosclerotic effects of endothelial-derived NO. Furthermore, the support of endothelial cell survival by NO may further play a central role for the pro-angiogenic effects of NO.

Keywords: angiogenesis; atherosclerosis; Akt

Abbreviations: NO: nitric oxide; NOS: nitric oxide synthase

Introduction

The endothelium is uniquely positioned at the interface between the vessel wall and the flowing blood. There it exerts multiple important functions including the regulation of coagulation and the modulation of the vessel tone.[1] Moreover, the endothelium acts as a barrier and thereby controls the adhesion and invasion of immune competent cells (for review see[2]). The integrity of the endothelial monolayer is crucial to preserve these functions.[3] Programmed cell death or apoptosis of endothelial cells can critically disturb the integrity of the endothelium and, thereby, contribute to endothelial injury during inflammation. The following section, therefore, will focus on the role of endothelial cell apoptosis in physiologic and pathophysiologic conditions. Moreover, the specific effect of nitric oxide, an important endothelium-derived factor, will be addressed.

Effect of NO on endothelial cell apoptosis

Effect of NO *in vitro*

Nitric oxide (NO) is released from the endothelium following stimulation of the endothelial NO-synthase (NOS III). The physiologically most important stimulus for endothelial NO-synthesis is the viscous drag (shear stress) generated by the streaming blood at the luminal endothelial cell surface,[4–7] leading to a continuous formation of low concentrations of NO. The release of NO is crucial to the normal function of the endothelium.[8,9] Thus, endothelial-derived NO mediates vasodilation and inhibits platelet aggregation.[10] Moreover, NO prevents adhesion of neutrophils[11,12] and expression of macrophage chemotactic proteins,[13] thereby limiting inflammation.

The role of NO in regulating apoptosis is controversial. Stimulation of the expression of the inducible form of the NO-synthase by lipopolysaccharide or inflammatory cytokines has been shown to induce apoptosis of several cell types, mainly macrophages. In contrast, the majority of the studies demonstrated an anti-apoptotic and cell protective effect of NO in endothelial cells (Figure 1). NO-donors in physiologically relevant concentrations blocked endothelial cell death induced by various stimuli including the proinflammatory stimuli LPS or TNFα and reactive oxygen species.[14–16] Moreover, endothelial cell apoptosis induced by the pro-atherosclerotic factors angiotensin II or oxidized LDL was also completely prevented by NO-donors.[17,18] Finally, exogenous NO prevented serum withdrawal and UV-induced apoptosis of endothelial cells.[19,20] Similar protective effects were reported for endogenously generated NO. Thus, shear stress-induced activation of the endothelial NOS potently prevents endothelial cell apoptosis induced by TNFα, reactive oxygen species, angiotensin II or oxLDL.[15,16,21] Moreover, the endogenous generation of higher concentrations of NO by the inducible NO-synthase (iNOS) also exerts protective effects on endothelial cells. Overexpression or induction of the iNOS by UV irradiation abolished apoptosis.[20,22] In contrast, pro-apoptotic effects of NO were only observed at high concentrations of exogenous NO-donors.[23] Taken together, these studies suggest that NO either endogenously produced or exogenously applied in physiologically relevant concentrations acts as a endothelial cell survival factor *in vitro*.

Mechanism of NO-mediated inhibition of endothelial cell apoptosis

The mechanisms, by which NO inhibits apoptosis, may include several transcriptional and posttranscriptional events. Initially, the well-known activation of the guanylyl cyclase by NO leading to the formation of the second messenger cGMP was proposed to mediate the anti-

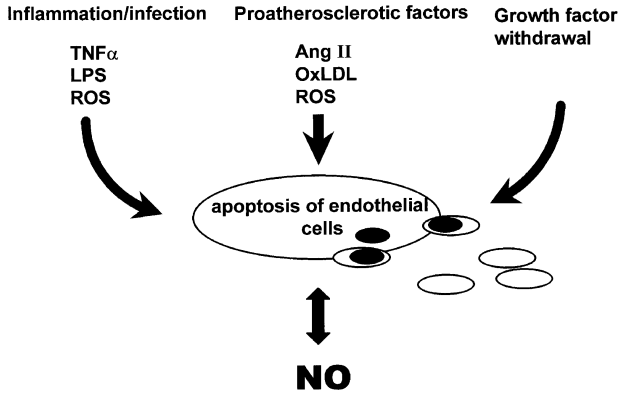

Figure 1 Nitric oxide inhibits endothelial cell apoptosis induced by various stimuli

apoptotic effects of NO. Membrane-permeable cGMP analogs reduced apoptosis in B-cells, eosinophils, and T-lymphocytes.[24,25] In contrast, other studies failed to demonstrate a protective effect for cGMP. Neither membrane-permeable cGMP analogs[14,26–28] nor pharmacological activation of the guanylyl cyclase[17] mimicked the NO effect. Beside the potential modulation of apoptosis by cGMP, NO directly interferes with the caspases, the key executioner of apoptosis signaling. The activation of these cysteine proteases by various stimuli, including TNFα, Fas-receptor activation, growth factor withdrawal, reactive oxygen species, as well as angiotensin II, has been shown to be inhibited by NO.[14,16,17,19,28–30] The inhibition of caspase activation appears to be mediated by S-nitrosylation of the essential cysteine residue located in the active site of the enzyme.[14,30] Thus, different purified caspases were shown to be S-nitrosylated by NO *in vitro*, which leads to inactivation of the enzymatic activity.[14,19,26,31,32] Recently, the direct interference of NO with the caspase cascade was further proven in intact cells by demonstrating that NO-donors stimulated the S-nitrosylation of overexpressed caspase-3 *in vivo*, thereby preventing cell death as well as enzyme activity.[33,34]

Furthermore, NO regulates the activation of kinases, which modulate the apoptotic cascade. Thus, NO blocked Fas- or ceramide-induction activation of the pro-apoptotic stress-activated jun-kinase (JNK), an effect that was mimicked by cGMP analogs.[35] In contrast, NO activates the mitogen-activated protein kinase ERK1/2[36] (personal communication). In endothelial cells, activation of MAP-kinases has been shown to stabilize the anti-apoptotic Bcl-2 protein,[37] which exerts potent anti-apoptotic functions and prevents the disruption of mitochondrial membrane function induced by several pro-apoptotic stimuli.[38] The NO-mediated increase of Bcl-2 protein, either by enhancing the protein half-live time[39] or by inhibition of caspase-dependent cleavage,[40] may prevent the mitochondrial cytochrome C release and thereby antagonize the amplification loop induced by pro-apoptotic stimuli.[41]

Long-term anti-apoptotic effects of NO might be elicited by transcriptional up-regulation of anti-apoptotic proteins. In detail, NO has been shown to increase the expression of heat shock protein 70 (HSP70),[42] the Bcl-2 protein and the cytoprotective proteins heme oxygenase-1 and ferritin.[43–45]

Taken together, NO interacts with several components of the apoptotic signal transduction pathway. In endothelial cells, the anti-apoptotic effect seems to be mainly due to inhibition of caspases and the interference with anti-apoptotic protein kinases. With regard to the various biochemical reactions, by which NO can interact with proteins (for example nitro-tyrosine formation, S-nitrosylation), and the interference with the oxidative flux in cells, it is not surprising that several steps in apoptotic signal transduction are inhibited by NO.

Potential implications of the apoptosis-suppressive effect of NO

The *in vitro* studies indicate that NO prevents endothelial cell death. However, what are the biological consequences of the inhibition of endothelial cell apoptosis by NO? Endothelial cell apoptosis may contribute to the pathophysiology of several diseases (Table 1). Specifically, injury of the vascular endothelium is a critical event in acute inflammation (e.g. endotoxic shock) or during chronic inflammatory diseases (e.g. atherosclerosis, transplant vasculopathy). Moreover, the erosion of the atherosclerotic plaque, which leads to myocardial infarction, the fatal endpoint of atherosclerosis, may be due to apoptosis of the endothelial monolayer. In addition, endothelial cell apoptosis may counteract angiogenesis. Therefore, the following sections will discuss the potential involvement of the anti-apoptotic effect of NO in inflammatory diseases and angiogenesis.

Inflammation Due to their unique localization in the vessel wall, endothelial cells are continuously exposed towards pro-inflammatory and proatherosclerotic mediators. Inflammatory processes trigger the release of proinflammatory mediators including cytokines and reactive oxygen species leading to endothelial damage. Mechanistically, apoptosis of endothelial cells may contribute to endothelial injury. Indeed, pronounced stimulation of the immune system by infusion of endotoxin induced massive apoptosis of microvascular endothelial cells *in vivo*.[46] Endothelial damage thereby preceded non-endothelial damage,[46] suggesting that loss of vascular integrity may play a key role in the initiation of multiple organ failure that characterizes the endotoxic shock syndrome. Although the influence of NO on endothelial cell apoptosis during endotoxic shock is not known, inhibition of the NO-synthase has been shown to enhance apoptosis in the liver of endotoxin-treated animals.[47] Moreover, the

Table 1 Potential involvement of endothelial cell apoptosis

	Disease	Proposed effect of endothelial NO
Cardiovascular system	Atherosclerosis	Anti-atherosclerotic
	Plaque Erosion	
	Angiogenesis	Angiogenic
Immune-related disorders	Endotoxic shock	Protective
	Transplant rejection	
Cancer	Tumor angiogenesis	Promotion

endothelial-derived NO seems to protect against the pneumotoxic effects of LPS.[48] However, since LPS also leads to the induction of the high capacity NO-synthase (NOS II), further studies are required to elucidate the specific effects of NO derived from the eNOS.

Endothelial cell apoptosis may also contribute to the pathogenesis of chronic inflammatory diseases such as atherosclerosis.[49] Thus, endothelial cells in lesion-prone regions, where atherosclerotic lesions preferentially develop, are characterized by increased endothelial cell turnover rates suggesting a mechanistic link between endothelial cell turn-over and the susceptibility to atherosclerotic plaque development.[50] Since endothelial cells grow as monolayers, enhanced endothelial cell turn-over most likely is secondary to an increase of apoptosis. The continuous production of NO in endothelial cells provides a defense against the injurious insults and therefore protects against endothelial dysfunction and atherosclerotic lesion development. This is evidenced by various *in vitro* and *in vivo* studies. The regions predisposed for the development of atherosclerotic lesions are characterized by low or unsteady blood flow.[51] Since the shear stress exerted by the flowing blood is the physiological most important stimulus for the synthesis of NO,[52] one may speculate that the reduced NO-synthesis in regions with low or disturbed flow leads to increased endothelial cell apoptosis, which promotes the initiation of atherosclerotic lesion formation. Indeed, a reduction of blood flow by ligation of the vessel has been shown to induce endothelial cell apoptosis *in vivo*.[53] Although the specific contribution of NO within this scenario is still speculative, endothelial NO-synthesis without any doubt counteracts atherogenesis as recently demonstrated in eNOS knock-out mice.[54]

Angiogenesis The vessel growth (angiogenesis), which guarantees the oxygen supply of the tissue at higher demand, is antagonized by apoptosis of endothelial cells. Thus, the induction of endothelial cell apoptosis leads to rarefaction of vessels.[55] Moreover, pro-angiogenic growth factors such as the vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) not only stimulate the mitogenic response but also potently suppress apoptosis of endothelial cells,[56,57] suggesting that angiogenesis requires concomitant inhibition of cell death.

An essential role of NO for growth factor-induced angiogenesis has been suggested by several *in vitro* studies. Thus, the angiogenic response to VEGF or bFGF was reduced by inhibition of endogenous NO-synthesis.[58,59] Moreover, exogenous NO-donors mimicked the effects induced by growth factors,[58] suggesting that NO may act as a crucial signal in the angiogenic response. Similar effects were observed when angiogenesis was stimulated by hypoxia.[60,61] The causal contribution of the eNOS was finally proven by demonstrating that eNOS knock out animals revealed an impaired angiogenesis in response to ischemia,[61] clearly confirming that NO is necessary for revascularization of the tissue. Since NO itself is unlikely to directly stimulate endothelial cell proliferation, one may speculate that its anti-apoptotic effect may be required for growth factor- or hypoxia-induced cell cycle progression *in vivo*. Mechanistically, the protein kinase Akt seems to play a central role. Growth factors as well as hypoxia are known to stimulate Akt.[60,62] This serine/threonine protein kinase is well known to prevent the induction of apoptosis.[63,64] Recent studies further demonstrated that Akt directly phosphorylates and thereby activates the eNOS.[65,66] With this information, one may speculate, that the angiogenic response induced by growth factors may be mediated by Akt-dependent activation of the eNOS, which in turn prevents apoptosis of endothelial cells and promotes angiogenesis (see Figure 2). Although the regulatory pathways may of course be more complex and may include several amplification as well as inhibitory feed back loops (see Figure 2), Akt may also regulate further targets beside eNOS (for example cell cycle regulatory proteins)[67,68] and other kinase cascades may participate as well. However, taken together compelling evidence suggest that NO plays a central role within this scenario.

Conclusion

In summary, apoptosis of endothelial cells may contribute to the pathophysiology of several inflammatory diseases. Overall, it appears that the effects of NO formation by the endothelial NOS protects against cytotoxicity and apoptosis. Most importantly, these *in vitro* observations have been supported by studies with gene knock-out animals, which demonstrate an atheroprotective and anti-inflammatory role of the eNOS.[54] Moreover, the anti-apoptotic activity of NO may contribute to angiogenesis, a process requiring the eNOS.[61] Why does NO exert protective effects in endothelial cells, whereas pro-apoptotic effects were mainly observed in macrophages? A plausible explanation might be that low concentrations of NO as generated by eNOS being anti-apoptotic and anti-inflammatory, whereas high concentrations generated by iNOS being pro-apoptotic. A novel hypothesis may be based on the very recent study by Mannick *et al*,[33] who demonstrated that several stimuli can counteract the caspase-inhibitory effect of NO and specifically denitro(sy)late caspase-3. Thus, the activation of specific, yet unknown

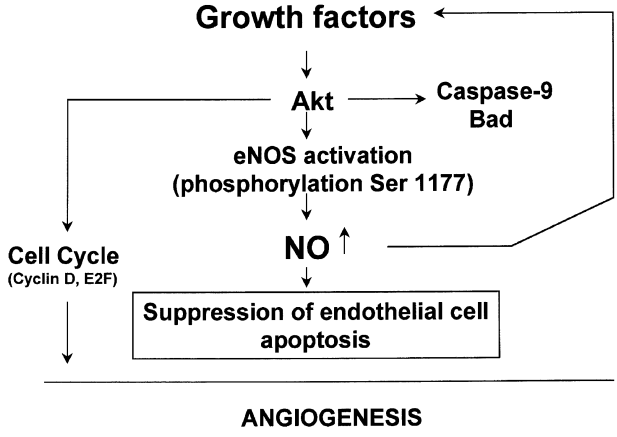

Figure 2 Potential involvement of the apoptosis-suppressive effect of NO in angiogenesis

mechanisms may unleash the inhibitory effect of NO on the apoptosis machinery. Thus, depending on balance between nitrosylation and denitrosylation, NO may modulate the caspase-activity. In endothelial cells, the continuous production of NO seems to switch off the activation of the caspase cascade via S-nitrosylation. The induction of denitrosylation may be controlled by the cellular redox status, which might not only differ between distinct cell types, but may also depend on the concentration of NO.

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