Nitric oxide and the resolution of inflammation: implications for atherosclerosis

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The ubiquitous free radical, nitric oxide (NO), plays an important role in many biological processes including the regulation of the inflammatory response. Alterations in NO synthesis by endogenous systems likely influence inflammatory processes occurring in a wide range of diseases including many in the cardiovascular system (e.g. atherosclerosis). Progression of inflammatory conditions depends not only upon the recruitment and activation of inflammatory cells but also upon their subsequent removal from the inflammatory milieu. Apoptosis, or programmed cell death, is a fundamental process regulating inflammatory cell survival and is critically involved in ensuring the successful resolution of an inflammatory response. Apoptosis results in shutdown of secretory pathways and renders effete, but potentially highly histotoxic, cells instantly recognisable for non-inflammatory clearance by phagocytes (e.g., macrophages). However, dysregulation of apoptosis and phagocytic clearance mechanisms can have drastic consequences for development and resolution of inflammatory processes. In this review we highlight the complexities of NO-mediated regulation of inflammatory cell apoptosis and clearance by phagocytes and discuss the molecular mechanisms controlling these NO mediated effects. We believe that manipulation of pathways involving NO may have previously unrecognised therapeutic potential for limiting or resolving inflammatory and cardiovascular disease.

Key words: nitric oxide - apoptosis - inflammation - resolution

The inorganic free radical, nitric oxide (NO), was first identified as an endothelium-derived endogenous messenger responsible for the regulation of vascular tone (Furchgott & Zawadzki 1980, Palmer et al. 1987). However, since then it has become clear that NO is the signalling molecule responsible for several diverse physiological and pathophysiological processes. Synthesised from L-arginine by three isoforms of the enzyme nitric oxide synthase (NOS), NO is now known to control vascular smooth muscle tone, inhibit platelet and inflammatory cell adhesion and activation, and to be a transmitter at non-adrenergic non-cholinergic (NANC) synapses (Moncada et al. 1991, Quinn et al. 1995). Recent studies have revealed that NO can also modulate apoptosis, or programmed cell death, in a variety of cell types, including human inflammatory cells (Taylor et al. 2003). Apoptosis of inflammatory cells is a highly regulated process whereby cellular death occurs without the disruption of the cell membrane and subsequent release of the pro-inflammatory and histotoxic contents of the dying cell (Haslett 1997, Rossi et al. 2003). Apoptotic cells are instantly recognised and ingested by phagocytes, such as macrophages, using mechanisms that down-regulate pro-inflammatory mediator release and increase the release of agents with anti-inflammatory potential from the ingesting cell (Meagher et al. 1992, Fadok et al. 1998, Liu et al. 1999). Hence, apoptosis represents a non-inflammatory mechanism to remove potentially damaging pro-inflammatory cells from the site of inflammation and is therefore critical to the successful resolution of the inflammatory response. Pharmacological manipulation of the rate of apoptosis in inflammatory cells, such as granulocytes and macrophages, may represent a potential therapeutic strategy for the treatment of chronic inflammatory disorders (Ward et al. 1999, Gilroy et al. 2004).

NO can be both pro- and anti-apoptotic, depending on local concentrations and the specific cell type in question (Quinn et al. 1995, Kim et al. 1999, Taylor et al. 2003). Current evidence suggests that lower concentrations of NO produced by the constitutive endothelial and neuronal isoforms of NOS (eNOS and nNOS) are cytoprotective, whilst supraphysiological concentrations produced by the inducible NOS isoform (iNOS) trigger cell death (Nicotera et al. 1997). This paradox may be explained, at least in part, by the free radical nature of NO and hence the ease with which it will react with other radicals, particularly reactive oxygen species, present in the milieu to form various NO-related species in vivo. For example, NO combines rapidly with inflammatory cell derived superoxide anions (O₂⁻) to form highly cytotoxic peroxynitrite (ONOO⁻) (Maxwell & Lip 1997).

NO as a mediator of inflammatory cell apoptosis

The pro- and anti-apoptotic actions of NO have been well documented in many cell systems. For example, high concentrations of either exogenous or endogenous iNOS-derived NO have been shown to induce apoptosis in murine macrophage cell lines (Albina et al. 1993, Sarh et al. 1993). However, pre-treatment with low concentrations of exogenous NO protects RAW 264 cells against cell death upon subsequent exposure to higher concentra-
tions of NO which would normally be cytotoxic (Yoshioka et al. 2003). However, despite the apparent reduced capacity of human macrophages in comparison to murine macrophages (Albina 1995, Schneemann & Schoedon 2002), to generate iNOS derived NO (Thomassen & Kavuru 2001), human macrophages do undergo apoptosis in response to exogenous NO. For example, the NO donors, S-nitroso glutathione (GSNO), and spermine diazeneum diolate (SPER/NO) induce apoptosis in primary human monocyte-derived macrophages (von Knetthen et al. 1999). Exogenously delivered NO from NO donors (e.g., sodium nitroprusside; SNP and GSNO) induce apoptosis in human neutrophils (Fortenberry et al. 1999, Singhal et al. 1999). However, it has also been established that NO may have anti-apoptotic potential in neutrophils; low concentrations of NO generated from the spontaneous NO donors, SPER/NO and DEA/NO, reduce the rate of neutrophil apoptosis (Taylor et al. 2001). In contrast, the same study showed that the oxatrazole derivative, GEA-3162, at equivalent concentrations produced no such inhibition. However, it was demonstrated that GEA-3162 decomposes to co-generate both NO and O$_2^-$, which then react to form ONOO$^-$(Taylor et al. 2004). This suggests that the pro- or anti-apoptotic effects of NO may be critically governed by the specific NO-related species generated.

Interestingly, the production of ONOO$^-$ may be of particular importance at sites of inflammation where the concentration of reactive oxygen species is likely to be elevated (Crow & Beckman 1995). However, the precise role of ONOO$^-$ in inflammatory cell apoptosis remains to be fully elucidated. There is some evidence to suggest that ONOO$^-$ at higher concentrations increases apoptosis in murine RAW 264.7 cells (Sandoval et al. 1997), whilst at lower concentrations it may have a protective effect against lipopolysaccharide (LPS) and interferon (IFN)-γ induced apoptosis in these cells (Sciavittaro et al. 1997). A scavenger of ONOO$^-$, uric acid, had no effect on apoptosis induced by the NO donors GSNO or SPER/NO in RAW 264.7 macrophages, but abolished apoptosis induced by the ONOO$^-$ generator SIN-1, suggesting that ONOO$^-$ is a mediator of apoptosis, at least not in this cell type (Brockhaus & Brune 1999).

As is the case with macrophages, there are conflicting reports about the ability of ONOO$^-$ to induce or suppress apoptosis in neutrophils. Several investigators have demonstrated that SIN-1 and GEA-3162 increases the rate of apoptosis in human neutrophils (Blaylock et al. 1998, Ward et al. 2000, Taylor et al. 2004). Conversely, Blaylock et al. (1998) reported SIN-1 produced no significant increase in neutrophil apoptosis. However, this may be due to experimental differences and the exact amounts of ONOO$^-$ present rather than a true difference in the effect of ONOO$^-$ (Taylor et al. 2003).

**NO and apoptosis in the resolution of inflammation**

The ability of NO to induce apoptosis is particularly relevant during the resolution phase of inflammation. In a mouse model of kidney inflammation, activated macrophages have been shown to induce apoptosis in neighbouring mesangial cells prior to their ingestion by phagocytes (Duffield et al. 2000). The ability of these activated macrophages to induce apoptosis is greatly reduced in the presence of the NOS inhibitor N-É-monomethyl-L-arginine (L-NMMA), suggesting that macrophage-directed apoptosis of mesangial cell apoptosis occurs via a NO-dependent mechanism (Duffield et al. 2001). Similarly, several studies have demonstrated that activated macrophages infiltrating murine tumours induce apoptosis via a NO-dependent pathway in both activated anti-tumour T cells and in the tumour cells themselves (Saio et al. 2001, Chattopadhyay et al. 2002). Thus, it appears that macrophages have the capacity to induce apoptosis of nearby cells by the liberation of NO to enhance the clearance of apoptotic cells and thereby promote the resolution phase of inflammation (Figure).

**Mechanism of action of NO**

The classical pathway by which NO exerts many of its actions is via activation of the enzyme soluble guanylate cyclase (sGC) (Moncada et al. 1991) and resultant conversion of guanosine 5'-triphosphate (GTP) to the second messenger 3', 5'-cyclic guanosine monophosphate (cGMP) (Ignarro et al. 1999). However, recent studies have established that NO can also act via cGMP-independent pathways in various systems, particularly during the inhibition of platelet aggregation and regulation of inflammatory cell apoptosis (Gordge et al. 1998, Sogo et al. 2000, Ward et al. 2000, Crane et al. 2002).

It is generally thought that lower concentrations of NO inhibit apoptosis via cGMP-dependent mechanisms, whilst higher concentrations are cytotoxic on account of cGMP-independent signalling. For example, Yoshoka et al. (2003) demonstrated that pre-treatment of RAW 264 cells with a low concentration of the NO donor SNP inhibited cell death upon subsequent exposure to higher concentrations of NO. This protection was negated in the presence of sGC inhibitors and could be mimicked by cGMP analogues, suggesting that the cellular protection was conferred by cGMP.

Conversely, at higher concentrations, NO has been shown to induce apoptosis in rabbit macrophages – an effect which was unaffected by antagonism of cGMP-dependent kinases and not mimicked by cGMP analogues, suggesting that the pro-apoptotic action of NO is cGMP-independent (Wang et al. 1999). The peroxynitrite generators, SIN-1 and GEA-3162, have also been shown to produce a marked concentration-dependent induction of apoptosis in isolated human neutrophils (Ward et al. 2000). Again, this induction was unaffected by inhibitors of sGC, and cGMP analogues failed to elicit a pro-apoptotic response suggesting that a mechanism independent of cGMP signalling also featured in neutrophils. Interestingly, superoxide dismutase (SOD), the enzyme responsible for converting O$_2^-$ to hydrogen peroxide (H$_2$O$_2$), antagonised the actions of SIN-1 and GEA-3162, whilst “authentic” peroxynitrite mimicked their effects. This result may, therefore, highlight the critical importance of NO-related species in determining an anti- or pro-apoptotic response, with the final outcome depending on the balance between reactive oxygen and nitrogen species.
Atherosclerosis

Atherosclerosis is a multi-factorial condition with a complicated aetiology, and, in combination with the associated cardiovascular syndromes, such as myocardial infarction and stroke, is a major cause of morbidity and mortality. However, it is now widely recognised that there is an inflammatory component to the disease pathogenesis and progression (Ross 1999a, b, Ludewig et al. 2002).

Atherosclerosis is characterised by the development of lipid-rich atherosclerotic plaques in the subendothelial space of conduit vessels, such as the coronary artery and aorta (Badimon et al. 1993). These plaques are usually eccentric, with the lipid rich core encapsulated by a fibrous, collagen-rich cap of smooth muscle cells and extracellular matrix (Davies 1997). The underlying causes of atherogenesis remain largely unknown, although a critical early stage is thought to be an insult to the endothelium, either physical or through oxidative stress. The consequences of this insult are multiple; firstly, in contrast to the situation in healthy endothelium, the injured endothelium becomes dysfunctional and production of NO by eNOS decreases, promoting vasoconstriction and platelet and inflammatory cell adhesion. Secondly, a protective inflammatory response is triggered. However, depending on the nature and duration of the insult, this protective response becomes excessive and over a period of years, comes to constitute the disease process itself (Ross 1999a, b). The inflammatory process begins with the expression of chemotactic and adhesion molecules for monocytes and lymphocytes, such as vascular cell adhesion molecule 1 (VCAM-1), on dysfunctional endothelial cells. Circulating monocytes adhere to the site of endothelial damage and translocate to the sub-endothelial space (Vogel 1997). Colony stimulating factors secreted from areas of endothelial damage induce monocytes to differentiate into macrophages, which then express scavenger receptors on their membranes, facilitating the internalisation of oxidised low density lipoprotein (ox-LDL). The accumulation of ox-LDL continues unchecked as, unlike LDL receptors, scavenger receptors are not downregulated by cells in the cholesterol-replete state (Maxwell & Lip 1997). In this lipid-laden state, macrophages are known as foam cells and it is an aggregation of these foam cells in the vessel intima which form the earliest recognisable lesion of atherosclerosis – the fatty streak (Ross 1993). The plaque continues to grow via the accumulation of further macrophage foam cells and eventually becomes overlaid with a layer of smooth muscle cells forming a fibrous, collagen-rich cap. The cap serves to keep the highly thrombogenic contents of the plaque separate from the circulation. However, if the plaque cap is compromised and the contents exposed to the circulation, platelets are rapidly recruited and activated resulting in thrombus formation, leading to the more serious acute cardiovascular syndromes (Badimon et al. 1993).

High concentrations of nitric oxide (NO) synthesised by iNOS in phagocytes, such as macrophages, induce apoptosis in neighbouring cells. In addition, apoptosis can also be induced by ONOO• generated as O2•− produced by phagocytes reacts with NO. Apoptotic cells are subsequently recognised and ingested by phagocytes, thus aiding the resolution of inflammation. Conversely, low concentrations of NO produced constitutively by eNOS in endothelial cells can inhibit apoptosis.
Inflammatory cell apoptosis in atherosclerosis

Recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind plaque growth and development. However, the plaque is dynamic and inflammatory cells are constantly turning over within the core. It is well established that apoptotic cells, particularly macrophages, are present in atherosclerotic plaques in both human and animal models of the disease. Apoptotic macrophages and smooth muscle cells have been identified by TUNEL staining in sections from human plaques by various authors (Bjorkerud & Bjorkerud 1996, Haunstetter & Izumo 1998). Because apoptotic cells are ingested by phagocytes without initiating any further pro-inflammatory response, it has been suggested that apoptosis may represent a mechanism to repress the plaque. NO is a particularly promising candidate for this strategy because, as well as the pro-apoptotic actions discussed above, it has several other powerful anti-atherogenic characteristics including a powerful inhibitory effect on platelet and inflammatory cell activation (Moncada et al. 1991, Armstrong 2001). Evidence is emerging in support of this hypothesis. For example, administration of L-arginine (the substrate for NOS) to hypercholesterolemic rabbits increases the number of apoptotic macrophages in intimal lesions by three fold. This increase in apoptosis was associated with a regression of the plaque, suggesting that manipulation of the NO synthase pathway may well represent a therapeutic approach to resolving the inflammatory response in the vessel wall (Wang et al. 1999). However, care must be exercised when considering this approach because NO is also known to induce apoptosis in smooth muscle cells (Labelle et al. 2004). Loss of cells from the fibrous cap during the latter stages of atherosclerosis may well be detrimental, destabilising the plaque and promoting rupture (Kockx & Knapp 2000)

Conclusion

Apoptosis of inflammatory cells is a tightly regulated process whereby cells are removed from the site of inflammation without triggering a subsequent pro-inflammatory response that would instigate further tissue injury. Pharmacological manipulation of apoptosis during chronic inflammatory conditions, such as atherosclerosis, may aid the resolution of inflammation and hence halt, or delay, disease progression. The ubiquitous signalling molecule and inducer of apoptosis, NO, is a likely candidate for such manipulation and may represent a novel therapeutic target for the treatment of such conditions.

REFERENCES


