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Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure

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Abstract

The purpose of this review is to summarize the regulation of apoptosis by nitric oxide (NO) and to discuss the potential role that NO plays in cardiomyocyte apoptosis during myocardial ischemia/reperfusion and development of heart failure. NO is an important regulator of apoptosis within the mammalian system, capable of both inducing and preventing apoptosis, depending upon the level of NO production and environmental milieu. This bifunctional capacity is well illustrated in the heart. It appears that high levels of NO produced by inducible nitric oxide synthase (iNOS) promote apoptosis while basal levels of NO production from endothelial nitric oxide synthase (eNOS) protect cardiomyocytes from apoptosis. Since permanent loss of cardiomyocytes due to apoptosis contributes to the development of heart failure, inhibition of cardiomyocyte apoptosis may have therapeutic implications. Given its pro- and anti-apoptotic capacity within the heart, NO may serve as a valuable therapeutic target in myocardial ischemia and heart failure.

Keywords: Nitric oxide; Apoptosis; Cardiomyocytes; Myocardial infarction; Heart failure

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; I/R, ischemia and reperfusion; AP-1, activator protein-1; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; NF-κB, nuclear factor-kappa B; IκB, inhibitor of kappa B; NMDA, *N*-methyl-D-aspartate; TNF-α, tumour necrosis factor-alpha; TNFR, tumor necrosis factor receptor; SMT, *S*-methylisothiourea; TUNEL, terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling; IAP, inhibitor of apoptosis protein; cIAP1, cellular inhibitor of apoptosis protein-1; XIAP, X-linked inhibitor of apoptosis protein.

Contents

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1. Introduction

Cellular death plays a central role in homeostasis of an organ and disease processes. One form of cell death whereby the cell undergoes an ordered disassembly followed by death and phagocytosis is known as apoptosis (Kerr et al., 1972). Morphological features of apoptosis include cell shrinkage, chromatin condensation, membrane blebbing, and preservation of organelle ultrastructural integrity. The other form of cell death is necrosis. Cells die from necrosis as a result of gross and overt cellular injury causing depletion of cellular energy. Necrosis is characterized by cell swelling, membrane lysis, and release of the intracellular contents, leading to an inflammatory response, with edema and damage to the surrounding cells (Yaoita et al., 2000).

Two major signaling mechanisms of apoptosis have been well described; the death receptor pathway and the mitochondrial death pathway (Joza et al., 2002). Accordingly, the death receptor pathway involves activation of cell surface "death receptors", which belong to a specialized subset of the tumor necrosis factor receptor (TNFR) superfamily (Ashkenazi & Dixit, 1998). Upon activation, intracellular adapter proteins are recruited by these receptors, which subsequently stimulate caspase-8 autoproteolytic activation and initiate a well-defined caspase cascade leading to apoptotic cell death. Alternatively, increases in mitochondrial permeability lead to cytochrome *c* release and activation of caspase-9, which initiates the downstream caspases resulting in apoptosis (Joza et al., 2002).

Apoptosis plays a crucial role in development and homeostasis of multicellular organisms. For instance, synaptogenesis, morphogenesis, tissue turn over and resolution of an immune response all require apoptotic cell death (Ellis et al., 1991). While failure to undergo apoptosis after sustaining of severe DNA damage may lead to carcinogenesis, unscheduled apoptosis is an essential component of disease processes such as Alzheimer's and Parkinson's (Nijhawan et al., 2000). Emerging evidence also demonstrated that apoptosis contributes to the pathogenesis of many cardiovascular diseases (Bennett, 2002). As adult heart muscle has very limited ability to regenerate, loss of cardiomyocytes due to apoptosis may play an important role in myocardial ischemia and development of heart failure (Nadal-Ginard et al., 2003).

Nitric oxide (NO) is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalyzed by a family of nitric oxide synthase (NOS). There are at least 3 distinct NOS isoforms, derived from separate genes: neuronal NOS (nNOS, or NOS1), inducible NOS (iNOS, or NOS2), and endothelial NOS (eNOS, or NOS3). The 3 isoforms are similar in structure, utilizing L-arginine, oxygen and NADPH as substrates and requiring cofactors such as calmodulin and tetrahydrobiopterin (Stuehr, 1999; Alderton et al., 2001). While eNOS and nNOS are Ca^{2+}-dependent enzymes, iNOS is usually induced by cytokines and its enzyme activity is Ca^{2+}-independent. Strictly speaking, eNOS and nNOS are not "constitutive" as the expression of eNOS, for example, is up-regulated by shear stress, exercise, pregnancy and down-regulated by pro-inflammatory cytokines (Kelly et al., 1996). While activation of nNOS and eNOS has physiological and homeostatic effects, induction of iNOS and high levels of NO production are often, but not always, associated with pathological conditions (Bolli, 2001; Jugdutt, 2002).

The eNOS isoform, which was originally characterized in large conduit vessel endothelium, is expressed within the heart in the endocardium and in the endothelium of the coronary vasculature, including capillary and venular endothelium. It is also expressed in cardiac myocytes and in specialized cardiac conduction system, including sinoatrial and atrioventricular nodal tissues (Balligand & Cannon, 1997). Under normal physiological conditions, NO released from eNOS in the heart has several major roles including coronary vasodilation, regulation of platelet and neutrophil functions, and tonic inhibition of mitochondrial O_2 consumption (Boveris et al., 2000). NO may also play a role in muscarinic-cholinergic inhibition of β-adrenergic-stimulated chronotropy, inotropy, atrioventricular nodal conduction, and cardiac myocyte L-type Ca^{2+} currents (Massion et al., 2003).

A number of cellular constituents of cardiac muscle, including the endothelium and smooth muscle of the cardiac microvasculature, the endocardial endothelium, tissue macrophages, and cardiac myocytes, are capable of expressing iNOS in response to LPS and and/or cytokine stimulation (Muller et al., 2000). Myocardial iNOS induction has been demonstrated to cause contractile dysfunction in various preparations including isolated myocytes, perfused working hearts, and in vivo animal preparations. The physiological outcomes of iNOS induction are not limited to a reversible decline in myocyte contractile function. Some beneficial effects of NO from iNOS are apparent. For example, NO generated by iNOS has been shown to suppress viral replication and infection (Lowenstein et al., 1996). In this regard, Coxsackievirus replicates to higher titers, where viral clearance is hindered in $iNOS^{-/-}$ mice as compared to

wild-type controls (Zaragoza et al., 1998). More importantly, myocarditis induced by Coxsackievirus is much more severe in infected iNOS$^{-/-}$ mice than the wild-type mice (Zaragoza et al., 1998), suggesting iNOS is crucial for the host response to Coxsackievirus.

NO has both pro- or anti-apoptotic effects depending on its concentration, source of production and pathological conditions. Transient production of NO by eNOS and nNOS as a result of increases in intracellular Ca^{2+} due to receptor activation or phosphorylation of eNOS by AKT/PKB has been attributed to physiological NO effects (McCabe et al., 2000). On the other hand, overproduction of NO by iNOS can be injurious to host and foreign cells alike (Li & Forstermann, 2000). The pro- and anti-apoptotic effects of NO closely tie into the delicate regulation of cell cycle checkpoint control and the normal function of mitochondria that controls the cellular redox state. The purpose of this review is to summarize the molecular mechanisms of NO in the regulation of apoptosis. Furthermore, the potential role that NO plays in cardiomyocyte apoptosis during myocardial ischemia/reperfusion (I/R) injury and the development of heart failure will also be discussed.

2. Pro-apoptotic mechanisms of nitric oxide

2.1. Effects of nitric oxide on death receptors

Death receptors belong to the TNF receptor gene superfamily of single-pass transmembrane receptor proteins, which contain conserved intracellular death domains and extracellular cysteine-rich domains. These latter domains allow the formation of external disulfide bridges during arrangement of activated death receptor homotrimers. Intracellular death domains permit for engagement of the receptor with intracellular proteins that also contain death domains and set into motion the apoptotic process (Ashkenazi & Dixit, 1998).

Many prominent death receptors have been characterized, which include CD 95 (Fas ligand), tumor necrosis factor-alpha receptors 1 and 2 (TNFR1 and TNFR2), death receptor 3 (DR3, Apo 3), and death receptors 4 and 5 (DR4 and DR5). The ligands that activate these receptors are also related gene products that belong to the TNF gene superfamily. They are Fas ligand, tumour necrosis factor-alpha (TNF-α), Apo 3 ligand, and Apo2 (TRAIL) ligand that bind to Fas, TNFR1, DR3 and DR4, and DR5, respectively (Nagata, 1997). Activation of death receptors leads to recruitment of adaptor proteins such as Fas-associated death domain which recruit and activate caspase-8 and other downstream caspases and result in apoptosis (Chinnaiyan et al., 1995). This death receptor-mediated apoptotic mechanism has been demonstrated in many mammalian cell types. In cardiomyocytes, however, physiological levels of TNF-α do not induce apoptosis. Cardiomyocyte apoptosis occurs only in response to high concentrations of TNF-α (Song et al., 2000). Interestingly, TNF-α induces cardiomyocyte apoptosis through iNOS expression and NO production. NO production from iNOS appears to be a major pathway by which TNF-α induces cardiomyocyte apoptosis as inhibition of iNOS completely abrogate the apoptotic effect of TNF-α (Song et al., 2000).

A direct effect of NO-mediated apoptosis has been attributed to up-regulation of Fas and Apo-2 ligands in target cells. For instance, NO donor glycerol trinitrate induces apoptosis in Jurkat cells that are sensitive to CD95 activation. Specifically, NO up-regulates mRNA expression of CD95 (APO-1/Fas) and TRAIL/APO-2 ligands (Chlichlia et al., 1998). Furthermore, NO triggers apoptosis in freshly isolated human leukemic lymphocytes, which are also sensitive to anti-CD95 treatment. In these cells the ability of NO to induce apoptosis is completely blocked by a broad-spectrum caspase inhibitor and correlated with caspase-8 activation (Chlichlia et al., 1998).

Treatment with NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) in mesangial cells results in a potent and dose-dependent increase in ceramide, an important signaling molecule in TNFα-mediated apoptosis (Huwiler et al., 1999). NO-induced ceramide production is not only due to activation of acidic and neutral sphingomyelinases, the ceramide-generating enzymes, but also inhibition of acidic and neutral ceramidases, the ceramide-metabolizing enzymes (Huwiler et al., 1999). Thus, NO may enhance TNFα-mediated apoptosis through increased ceramide production.

2.2. Effects of nitric oxide on mitochondrial pathway of apoptosis

A central role of mitochondria as an orchestrator of apoptosis has been established in many different systems. There are at least 3 emergent pathways for which mitochondria can initiate the apoptotic program. Mitochondria can directly cause cell death via disruption of electron transport chain and depletion of ATP or they can release pro-apoptotic proteins and cofactors such as cytochrome *c* that activate the caspase cascade (Liu et al., 1996; Bossy-Wetzel et al., 1998). In addition, mitochondria can release apoptosis inducing factor and endonuclease G that induce apoptosis independent of caspase activation (Cregan et al., 2004). Finally, aberrant function of mitochondria can result in release of large amount of reactive oxygen species that accelerate both apoptotic and necrotic cell death depending on the availability of ATP as the deciding factor in the mode of cell death (Jacobson & Raff, 1995).

NO has been shown to regulate mitochondrial permeability transition and cytochrome *c* release (Balakirev et al., 1997). NO accelerated an onset of swelling in Ca^{2+}-loaded mitochondria in a cyclosporin-A-sensitive manner acting as an inducer of permeability transition. This was apparently a result of irreversible alteration of mitochondrial function due to the inhibition of respiratory chain in the presence of Ca^{2+}. It appears that NO's effect on mitochondrial permeability

and cytochrome *c* release depends on NO concentrations. Low levels (nM) of NO reversibly inhibited mitochondrial transition pore opening, which can be readily achieved in vivo by NOS. On the other hand, at higher than physiological concentrations, i.e., release rates (>2 μM/sec), NO accelerated mitochondrial transition pore opening and cytochrome *c* release (Balakirev et al., 1997; Brookes et al., 2000). The induced release of cytochrome *c* and complete dissipation of the inner mitochondrial membrane as well as collapse of oxidative phosphorylation lead to accelerated formation of large amounts of reactive oxygen species with the deleterious consequences (Brown & Borutaite, 1999).

NO increases Bax protein expression and results in increases in Bax to Bcl-2 ratio. This may represent an important mechanism by which NO induces apoptosis in cardiomyocytes, cardiac fibroblasts and vascular smooth muscles (Ing et al., 1999; Tian et al., 2002; Chae et al., 2004). Interestingly, NO has also been shown to impede the ATP-dependent activity of 26S proteasome that functionally degrades Bax and inhibitor of kappa B (IκB; Glockzin et al., 1999). While Bax is directly pro-apoptotic, IκB inhibits the release and transactivation activity of nuclear factor-kappa B (NF-κB), which can up-regulate a number of pro-survival pathways (Aggarwal, 2000). Thus, NO may increase Bax/Bcl-2 ratio and decrease NF-κB activity via inhibition of 26S proteasome activity. It appears that proteasome inhibition increases apoptosis in dividing cells, but decreases apoptosis in nondividing cells (Glockzin et al., 1999). Thus, inhibition of proteasome activity by NO may lead to both apoptotic and anti-apoptotic effects depending on the proliferation status of the cell.

Regulation of NF-κB activity can also be achieved in a more direct way. For instance, NO can directly inhibit the DNA binding activity of NF-κB family proteins (Matthews et al., 1996; Park et al., 1997). This represents another mechanism by which NO decreases NF-κB activity. Specifically, NO inhibits the DNA binding activities of p50 and/or p65 homodimers, as well as p50/p65 heterodimers. These effects are mediated by *S*-nitrosylation of C62 residue of p50 subunit (Matthews et al., 1996). Thus, one could envisage a negative feedback mechanism between NO and NF-κB. Activation of DNA binding and the nuclear translocation of NF-κB result in expression of many NF-κB responsive genes including iNOS and IκB. NO produced by newly expressed iNOS might then act in concert with newly synthesized IκB to inhibit the DNA binding activity of NF-κB and shut off the transcription of NF-κB responsive genes allowing for a return to cellular NF-κB/IκB steady state homeostatic levels (Matthews et al., 1996). The negative feedback control between NO and NF-κB may have physiological implications in the heart as NF-κB has recently been demonstrated to protect cardiomyocytes from apoptosis (Misra et al., 2003). However, whether the inhibitory effect of NO on NF-κB may increase cardiomyocyte apoptosis remains to be determined.

While the dissipation of the mitochondrial inner membrane potential is accelerated by pro-apoptotic members of the Bcl family of proteins, the predominant contributing factor to mitochondrial collapse is the formation of the permeability transition pore (Zamzami et al., 1996). The components of the permeability transition pore are the adenine dinucleotide transporter and the voltage-dependent anion channel that come together at the inner and outer mitochondrial membrane contact sites and form a large channel (Bernardi et al., 1994). Formation of this channel cannot only lead to dissipation of the inner membrane potential and loss of proton motive force but also facilitates the release of cytochrome *c* to the exterior of the mitochondria, leading to formation of the so-called apoptosome apparatus (Green & Reed, 1998).

NO can react with superoxide anion to generate a potent reactive nitrogen species, peroxynitrite. Both NO and peroxynitrite inhibit mitochondrial superoxide dismutase which acts to convert superoxide into hydrogen peroxide (Macmillan-Crow & Cruthirds, 2001). Inhibition of this enzyme will lead to accumulation of superoxide and augmentation of peroxynitrite formation. Peroxynitrite can in turn oxidize a range of biomolecules including lipids, nucleic acids, and proteins. It can oxidize not only protein thiols but also glutathione and therefore change the redox potential of the cellular environment to promote apoptosis (Keller et al., 1998). Both NO and peroxynitrite have been shown to oxidize the adenine nucleotide translocator, one of the key components of the permeability transition pore complex and thus accelerate its pro-apoptotic function (Vieira et al., 2001).

Peroxynitrite and possibly NO can inhibit iron-sulfur centers of mitochondrial enzymes such as the electron transport chain complex I–III, *cis*-aconitase (Krebs cycle), and glyceraldehydes-3-phosphate dehydrogenase (glycolysis), a combined effect that would lead to complete collapse of ATP production (Bolanos et al., 1995; Padgett & Whorton, 1995). Furthermore, high NO concentrations can act to inhibit ribonucleotide reductase thus blocking deoxyribonucleotide and DNA synthesis, which is essential for cell survival (Roy et al., 1995). Therefore, prolonged NO overproduction may favor necrotic cell death as complete absence of ATP can lead to mitochondrial but also cellular swelling, rupture and lysis (Nicotera et al., 1999).

2.3. Effects of nitric oxide on cell cycle progression, p53, and DNA repair

Whether a cell remains dormant or commits itself to cell cycle progression on route to cell division depends on a number of molecular events. Proteins known as cyclins bind and activate cyclin-dependent protein kinases, which are vital to progression of cell cycle (Nigg, 1995). Accordingly, in G1 phase of the cell cycle progression program, cyclin D activates cyclin-dependent protein kinase 4/6 resulting in

phosphorylation and inactivation of retinoblastoma protein, which in the hypo-phosphorylated form inhibits the formation of transcription factors that are needed for synthesis of S phase factors and S phase entry. Therefore, phosphorylation of retinoblastoma protein and release of factors E2F and DP-1 proteins lead to transactivation of S phase genes and production of S phase proteins. The phosphorylation and inactivation of retinoblastoma protein constitutes the passage of cell cycle restriction checkpoint after which the cell is committed to pass through the rest of the cell cycle, which results in mitosis and cell division (Sherr, 1994).

Sustained DNA damage by reactive oxygen, NO or peroxynitrite can inhibit the cell cycle progression program, which affords the cell a window of opportunity to repair itself and resume the cell cycle. On the other hand, sustained and superfluous damage prompts the cell to undergo apoptosis. NO generated from an NO donor or from iNOS overexpression up-regulates p53 expression (Forrester et al., 1996). Following its induction, p53 acts as a transcription factor and induces the genetic transcription of a number of products including GADD 45, MDM 2, and importantly p21cip1/waf (Kastan et al., 1992; Harper et al., 1995). In cell cycle arrest, p21cip1/waf binds directly to cyclin/cyclin-dependent protein kinase complexes and acts as cyclin-dependent protein kinase inhibitor preventing cell cycle progression. In this regard, NO inhibits the 26S mediated degradation of p21cip1/waf prolonging cell cycle arrest and increasing the probability of apoptosis (Kibbe et al., 2000).

In addition, in sustained NO/peroxynitrite-mediated DNA damage and following cell cycle arrest, a nuclear repair protein known as poly ADP-ribose polymerase is activated (Cuzzocrea et al., 1998). This protein undertakes a futile repair cycle both on DNA and proteins (including itself), where it ADP-ribosylates these constituents. This repair cycle inadvertently depletes the cells of reduced NADH and leads to depletion of ATP (Szabo, 1998). Therefore, this process can serve as a fine-tuning mechanism that dictates the fate of cellular death. Since apoptosis is an energy consuming process, cellular ATP depletion would lead to cell lysis. Strong pro-apoptotic signals, however, activate executor caspases that cleave poly ADP-ribose polymerase inhibiting its pro-necrotic activity (DiPietrantonio et al., 2000).

2.4. Effects of nitric oxide on caspase activity

Caspases (*c*ysteine *asp*artate prote*ases*) are a family of related cysteine proteases (Raff, 1998). Activation of caspases involves proteolytic cleavage of the pro-domain and rearrangement of the large and the small domains to form a heterodimer. The active caspase, however, normally rearranges, where 2 heterodimers bind to form an active tetramer (Thornberry & Lazebnik, 1998). Apoptotic signals from death receptor and mitochondrial pathways activate upstream caspase-8 and -9, respectively. The executioner caspases include caspase-3, -6, and -7. Prominent substrates of these caspases are inhibitor of caspase-activated deoxyribonuclease /DNA fragmentation factor 45 that bind and inhibit caspase-activated deoxyribonuclease. Upon activation, caspase-activated deoxyribonuclease relocates into the nucleus cleaving DNA, which manifests as a distinct ladder pattern on an agrose gel (Liu et al., 1997a, 1997b; Enari et al., 1998).

NO-mediated initiation of caspase activation has been demonstrated. Apoptosis induced by NO donor sodium nitroprusside (SNP) in vascular smooth muscle cells is associated with caspase-3 activation, where it was shown that the effects of SNP were blocked by NO scavenger hemoglobin (Chae et al., 2004). A universal caspase inhibitor, z-VAD-fmk, dose-dependently inhibited NO-induced apoptosis in these cells. Moreover, exposure to SNP induced the release of cytochrome *c* from the mitochondria to the cytosol and increased the ratio of Bax/Bcl-2 protein expression. These data suggest an emerging picture, where NO-induced apoptosis in rat vascular smooth muscle cells involves an increase in the ratio of Bax/Bcl-2 gene expression, leading to release of cytochrome *c* from the mitochondria to the cytosol, followed by caspase-3 activation and apoptosis (Chae et al., 2004).

Caspase activation during NO stimulation may also result from down-regulation of X-linked inhibitor of apoptosis protein (XIAP), which inhibits caspase activity (Manderscheid et al., 2001). In lipopolysaccharide (LPS)/interferon-γ-stimulated RAW 264.7 macrophages, iNOS expression down-regulated cellular inhibitor of apoptosis protein-1 (cIAP1) and XIAP protein levels, which correlated temporally with the induction of apoptosis. This effect was at least partially reversed by L-NAME, an inhibitor of NOS activity. However, in rat mesangial cells, iNOS induction was not associated with any significant change in inhibitor of apoptosis protein (IAP) levels. These cells were more resistant to NO-induced apoptosis (Manderscheid et al., 2001). It is possible that NO-induced apoptosis requires IAP down-regulation/degradation. Differences in IAP levels between rat mesangial cells and RAW 264.7 macrophages, resulting from differences in specific degradation pathways, may explain the different sensitivities to NO-induced apoptosis.

2.5. Summary

NO induces apoptosis by modulating multiple sites along the signaling pathways (Fig. 1). In the death receptor pathway, NO increases Fas expression and ceramide production, which lead to caspase activation. In the mitochondrial pathway, NO increases Bax/Bcl-2 ratio resulting in increased mitochondrial permeability and cytochrome *c* release. NO also inhibits the electron transport chain complex I–III to reduce energy production and increase p53, both of which contribute to the pro-apoptotic

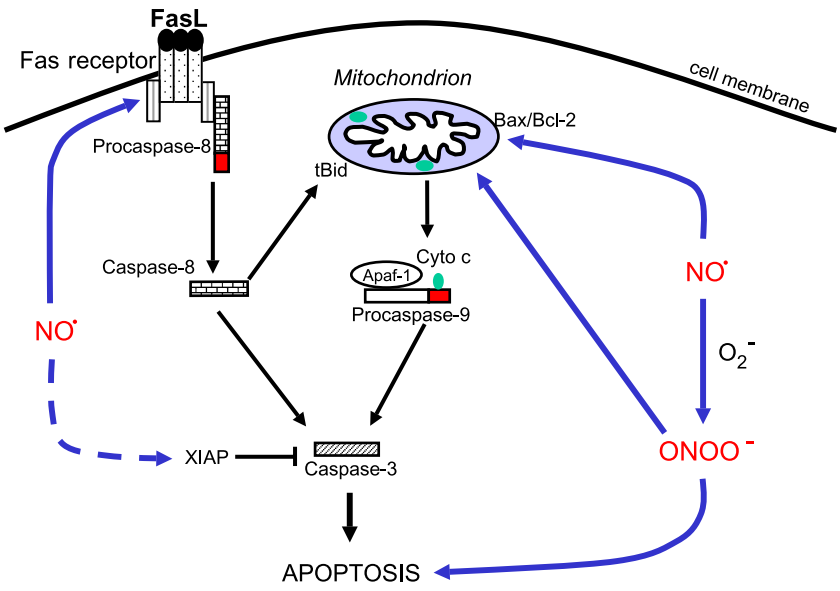

Fig. 1. Pro-apoptotic mechanisms of NO. NO induces apoptosis by modulating multiple sites along the signaling pathways. In the death receptor pathway, NO increases Fas expression. Fas-mediated receptor stimulation leads to activation of caspase proteolytic cascades including activation of caspase 3 and 9. Caspase 3 protease cleaves and activates the pro-apoptotic BCL homology containing protein tBid, which in turn triggers the mitochondrial apoptotic pathway. In the mitochondrial pathway, NO increases Bax/Bcl-2 ratio leading to increased mitochondrial permeability and cytochrome *c* (Cyto *c*) release. The release of Cyto *c* is rightly regulated and once released it recombines with the apoptosis associated factor-1 (Apaf-1) to recruit and activate the procaspase-9 in a so-called apoptosome complex. NO can react with superoxide anion (O_2^-) and generate peroxynitrite ($ONOO^-$), which induces apoptosis through increases in cellular oxidative stress, mitochondrial cytochrome *c* release and DNA damage. NO also downregulates X-linked inhibitor of apoptosis protein (XIAP). Effects of NO are indicated by blue lines. Solid lines indicate stimulation while dashed lines indicate inhibition.

effects of NO. In addition, NO increases caspase activity through down-regulation of IAP.

3. Anti-apoptotic mechanisms of nitric oxide

In contrast to its pro-apoptotic effects, NO also has prominent anti-apoptotic effects (Kolb, 2000). These effects could conceivably be attributed to cGMP/PKG-mediated Bcl-2 expression, linking the NO signaling pathway with inhibition of mitochondrial permeability. However, in most cases, protection from NO against apoptosis is clearly independent of cGMP. Although NO can up-regulate cell protective proteins such as heme oxygenase-1 and metallothionein (Spahl et al., 2003; Pae et al., 2004), the most immediate and effective mechanism of NO-mediated inhibition of apoptosis is *S*-nitrosylation of key apoptotic executor proteins (Kim et al., 1997a, 1997b; Li et al., 1997).

S-nitrosylation can modulate a number of proteins that are intricately involved in the apoptotic process. Most importantly, NO-mediated *S*-nitrosylation inhibits activation of caspases. Studies have shown that NO donor, SNAP inhibited recombinant human caspase-1, -2, -3, 4-, 6-, 7-, and -8 in a concentration-dependent manner (Li et al., 1997). The SNAP-induced inhibition was reversed by dithiothreitol, which effectively removes the thiol-bound NO groups from the effected proteins (Mohr et al., 1996). These results suggest that SNAP inhibits caspase activation via an *S*-nitrosylation-mediated mechanism (Li et al., 1997). More direct evidence demonstrated that inhibition of caspase-3 by NO is mediated via cysteine 163 *S*-nitrosylation in the catalytic site (Rossig et al., 1999). Thus, NO-mediated inhibition of this caspase prevents the activity of chief death substrates such as DNA fragmentation factor. Apart from *S*-nitrosylation, NO also inhibit caspase-3 activation via cGMP-dependent mechanism (Kim et al., 1997a, 1997b). Although the possibility of a direct effect on a caspase-3-like protease is not excluded, an interaction of cGMP-dependent kinase with the apoptotic signaling pathway upstream of caspase-3-like protease activation seems more likely (Kim et al., 1997a, 1997b).

Intracellular Ca^{2+} is an important second messenger in the regulation of apoptosis (Nicotera & Orrenius, 1998). Under normal conditions, Ca^{2+} continuously cycles between the sarcoplasmic reticulum and/or its equivalent endoplasmic reticulum, cytosol and mitochondria. Ca^{2+} is pumped into the sarcoplasmic reticulum by SERCA2a and released by ryanodine Ca^{2+} release channels. Ca^{2+} enters mitochondria by a Ca^{2+} uniporter and is released by Na^+/Ca^{2+} exchanger (Demaurex & Distelhorst, 2003). This close connection between sarcoplasmic reticulum, cytosol, and mitochondria enables Ca^{2+} signals not only to fine-tune cellular metabolism but also to modulate mitochondria-mediated apoptosis. Ca^{2+} content in the sarcoplasmic reticulum determines the cell's sensitivity to apoptotic stimuli. For example, overexpression of plasma membrane Ca^{2+}-ATPase decreased Ca^{2+} concentrations in the endoplasmic reticulum of HeLa cells, protecting the cells from

apoptosis (Pinton et al., 2001). In contrast, overexpression of SERCA channels, resulted in a rise in endoplasmic reticulum Ca^{2+} level sensitizing the cells to apoptotic stress (Pinton et al., 2001). Consistent with this notion, over activation of *N*-methyl-D-aspartate (NMDA) receptors causes intracellular Ca^{2+} overload leading to neurotoxicity and apoptosis (Tenneti et al., 1998).

Of relevance, NO protects neurons from apoptosis via *S*-nitrosylation and inactivation of NMDA receptors (Lipton, 1999). NO also regulates Ca^{2+} channel activity. SIN-1 generates both NO and superoxide, which can then react at nearly diffusion-limited rates ($k = 3.7\times10^7$ M^{-1} s^{-1}) to produce peroxynitrite (Feelisch & Stamler, 1996). The effects of SIN-1 (1 mM) on L-type Ca^{2+} channels are not straightforward, in that it could either reversibly decrease or increase L-type Ca^{2+} currents in ferret right ventricular cardiomyocytes (Campbell et al., 1996). In the presence of superoxide dismutase, which scavenges superoxide and minimizes the formation of peroxynitrite, SIN-1 consistently decreased L-type Ca^{2+} currents in cardiomyocytes. Since 8-Br-cGMP also inhibited L-type Ca^{2+} channels, the inhibitory effect of NO have been postulated to be cGMP-dependent (Campbell et al., 1996). Recent studies using a novel transgenic mouse model overexpressing cGMP-dependent protein kinase type I in the heart have confirmed that this kinase is a critical downstream target for NO/cGMP inhibition of L-type Ca^{2+} currents in adult cardiac myocytes (Schroder et al., 2003). Since inhibition of L-type Ca^{2+} channels has been shown to protect cardiomyocytes from apoptosis (Gao et al., 2001), inhibition of L-type Ca^{2+} channels may represent one of the mechanisms by which NO protect cardiomyocytes from apoptosis.

Tissue transglutaminase is an enzyme that catalyzes the cross-linking of intracellular proteins, thus assembling a protein scaffold that prevents leakage of intracellular components. Tissue transglutaminase is activated during the apoptotic cell death cascade and plays a key role in the formation of apoptotic bodies by stabilizing the integrity of the apoptotic cells before their clearance by phagocytosis (Volokhina et al., 2003). This specific function prevents the release of harmful intracellular components into the extracellular space, which are the key features of superfluous inflammation. Overexpression of tissue transglutaminase by cDNA transfection significantly increases spontaneous apoptosis in neuroblastoma cells, whereas reduced tissue transglutaminase expression results in a pronounced decrease of basal as well as retinoid-induced neuroblastoma cell death (Melino et al., 1994). NO donor SNAP dose-dependently inhibited tissue transglutaminase activity, an effect that was prevented by NO scavengers such as oxyhemoglobin and reducing agents such as glutathione and cysteine. As a marker of tissue transglutaminase activity, dithiothreitol reduces the cysteine residue of the enzymatic active site facilitating its detection in vitro. In the absence of dithiothreitol, the NO concentration required to achieve the same level of enzymatic inhibition is signifi-

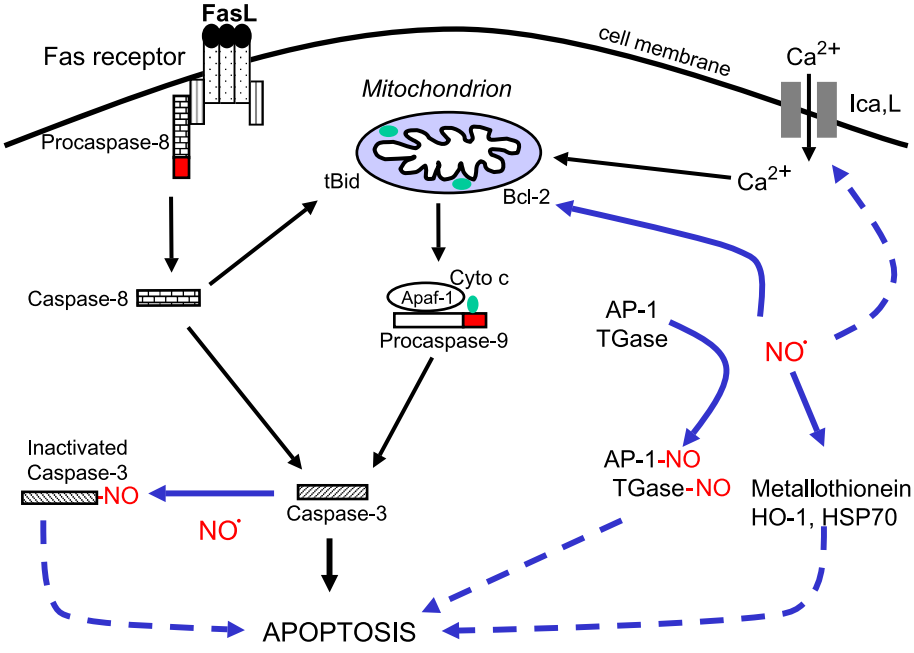

Fig. 2. Anti-apoptotic mechanisms of NO. The anti-apoptotic actions of NO involve both death receptor and mitochondrial pathways of apoptosis. NO inhibits apoptosis by *S*-nitrosylation of caspases, including executor caspase-3, activator protein-1 (AP-1) and tissue transglutaminase (TGase). NO also inhibits L-type Ca^{2+} channels and increases Bcl-2 expression, preventing cytochrome *c* release from the mitochondria. In addition, NO protects cells from apoptosis through upregulation of protective proteins such as heme oxygenase-1 (HO-1), heat shock protein 70 (HSP70) and metallothionein. Effects of NO are indicated by blue lines. Solid lines indicate stimulation while dashed lines indicate inhibition.

cantly increased. This indicates that NO is able to react with the thiol group of an active cysteine residue and suggests that the inhibition of tissue transglutaminase activity is due to *S*-nitrosylation (Bernassola et al., 1999).

NO inhibits mitochondrial cytochrome *c* release and decrease permeability transition pore opening via mitochondrial membrane depolarization and inhibition of Ca^{2+} accumulation (Brookes et al., 2000). Activation of transcription activator protein-1 (AP-1) is involved in cardiomyocyte apoptosis (Taimor et al., 2001). NO-mediated *S*-nitrosylation decreases AP-1 activity (Tabuchi et al., 1994) and may protect cardiomyocytes from apoptosis. Furthermore, NO up-regulates heat shock protein 70 that directly bind Apaf-1 and inhibit deoxy-ATP/cytochrome *c* mediated formation of apoptosome (Kim et al., 1997a, 1997b; Beere et al., 2000). These effects also contribute to the anti-apoptotic actions of NO.

In summary, NO has anti-apoptotic effects through both cGMP-dependent and -independent mechanisms (see Fig. 2). While the effects of NO on Bcl-2 expression and L-type Ca^{2+} channels are cGMP/PKG-dependent, *S*-nitrosylation of caspases, AP-1 and tissue transglutaminase is clearly a cGMP-independent effect. In addition, NO also protects cells from apoptosis through up-regulation of protective proteins such as heme oxygenase-1, heat shock proteins, and metallothionein.

4. Apoptosis in myocardial ischemia and reperfusion

Cardiomyocytes apoptosis occurs during myocardial ischemia in animals and humans. In this regard, apoptosis has been shown in the infarcted myocardium in rats following coronary artery occlusion. Cardiomyocyte apoptosis started at 2 hr and peaked at 4.5 hr following coronary artery occlusion in vivo. Specifically, 2 hr after myocardial infarction, apoptotic cell death involved 2.8 million cells and necrotic cell death only 90,000 myocytes in the rat heart (Kajstura et al., 1996). Furthermore, apoptosis continued to represent the major form of myocyte cell death, affecting 6.6 million myocytes at 4.5 hr. In contrast, myocyte necrosis peaked at 1 day, amounting to 1.1 million myocytes. These investigators concluded that myocyte apoptosis is the major form of myocardial damage after myocardial infarction, whereas necrotic myocyte cell death follows apoptosis and contributes to the progressive loss of cells with time postinfarction (Kajstura et al., 1996). The manifestation of apoptotic cell death is not confined to the ischemic episode alone. Reperfusion following myocardial ischemia accelerates cardiomyocyte apoptosis, which may contribute to reperfusion injury and myocardial dysfunction (Fliss & Gattinger, 1996). Cardiomyocyte apoptosis therefore occurs in both myocardial I/R, and contributes to the ischemic damage.

Consistent with data from animal studies, myocardial apoptosis has also been demonstrated in humans after acute myocardial infarction. Olivetti et al. (1996) studied myocardial samples obtained from the region adjacent to and remote from infarction in patients who died within 10 days from the initial clinical symptoms. Apoptosis was measured quantitatively by the terminal deoxynucleotidyl transferase assay and confirmed biochemically by DNA extraction and agarose gel electrophoresis. DNA strand breaks in myocyte nuclei were observed in all 20 infarcted hearts in both the regions bordering on and distant from the necrotic myocardium. However, the number of apoptotic nuclei was greater in the peri-infarcted region than in that away from infarction. Quantitatively, 12% of myocytes in the border zone showed DNA strand breaks, whereas 1% of cells were undergoing apoptosis in the remote myocardium (Olivetti et al., 1996). Thus, apoptosis appears to be a significant complicating factor of acute myocardial infarction increasing the magnitude of myocyte cell death associated with coronary artery occlusion. In a related study, Saraste et al. investigated myocardial samples from 8 patients who died from acute myocardial infarction within 6 to 120 hr after initial clinical symptoms. All patients had patent infarct-related arteries at autopsy as a result of successful thrombolysis, indicating these hearts were reperfused. Apoptotic cardiomyocytes (0.806%) were observed particularly in the border zones of histologically infarcted myocardium, whereas very few apoptotic cells (0.005%) were present in the remote noninfarcted myocardium (Saraste et al., 1997). This study provides evidence that in addition to overt necrosis, a subset of myocytes undergo apoptosis during ischemia-reperfusion injury in human hearts.

It is important to note that although significant myocyte apoptosis is observed in the myocardium after myocardial infarction, the quantification of apoptosis has varied dramatically between these studies ranging from 0.8% to 12% (Olivetti et al., 1996; Saraste et al., 1997). Although the reason for such variability is not completely clear, the inherent methodological limitation is likely an important factor. Terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling (TUNEL) assay, which identifies DNA breaks with free 3′-OH terminals, have been widely used. A clear conclusion from the use of such methods, however, is that the manner of tissue preservation used to prepare the samples can have dramatic effects on detection of strand breaks. Inherent technical considerations, such as fixation, embedding into paraffin, cutting, blocking of endogenous peroxidase with H_2O_2, various pretreatment protocols, and certainly tissue necrosis and autolysis, may cause “nonapoptotic” DNA fragmentation. On the other hand, inaccessibility of sites can prevent the detection of strand breaks (Willingham, 1999; Stadelmann & Lassmann, 2000). Thus, such techniques should be carefully validated in each laboratory with positive and negative controls. In addition, a multiparametric approach should be employed in each study, which includes TUNEL, immunohistochemistry for apoptosis-associated proteins and morphological criteria.

4.1. Effects of nitric oxide on cardiomyocyte apoptosis

Similar to other cell systems, NO has also been demonstrated to have both pro- and anti-apoptotic effects in cardiomyocytes. In this regard, induction of iNOS by cytokines or exogenous NO donors have been shown to increase cardiomyocyte apoptosis (Ing et al., 1999; Song et al., 2000). In response to a combination of the macrophage-derived cytokines, interleukin-1β, TNF-α, and interferon-γ, a time-dependent induction of apoptosis in the cultured neonatal cardiomyocytes was observed, beginning 72-hr posttreatment. In this experiment, apoptosis was preceded by a >50-fold induction of iNOS mRNA and the release of large amounts (5 to 8 nmol/μg protein) of NO metabolites (NO_x) into the medium. Cell death was completely blocked by a NOS inhibitor and attenuated by antioxidants and the caspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone (Ing et al., 1999).

Cytokines also caused an NO-dependent increase in the expression of the Bcl-2 homologs Bak and Bcl-x(L) in neonatal rat cardiomyocytes. The NO donor *S*-nitrosoglutathione induced apoptosis and increased cellular concentrations of Bak (Ing et al., 1999). These results suggest that high levels of NO production from iNOS induce cardiomyocyte apoptosis due to a mitochondria-dependent pathway. Interestingly, cardiomyocyte apoptosis was significantly increased in the myocardium of $eNOS^{-/-}$ mice during fetal and neonatal heart development, indicating basal NO release from eNOS protects cardiomyocytes from apoptosis (Feng et al., 2002). The mechanisms for these totally different effects of NO on cardiomyocyte apoptosis are still not fully understood. It is possible that concentrations and flux of NO and its interaction with other oxidants such as superoxide as well as molecules associated with apoptotic pathway may ultimately dictate detrimental or beneficial effects of NO in cardiomyocytes under physiological and pathophysiological conditions.

4.2. Effects of nitric oxide in myocardial ischemia and reperfusion

Studies aimed directly at assessing the effects of NO on myocardial I/R injury have yielded both pro- and anti-apoptotic effects depending on the source of NO. For instance, nonselective NOS inhibition with L-NAME was associated with increased apoptosis following myocardial I/R as measured by increased caspase-3 activity and Bax activation (Weiland et al., 2000; Czarnowska et al., 2001), suggesting an anti-apoptotic role of NO. The protective effects may be due to eNOS-derived NO as myocardial injury is exacerbated following I/R in $eNOS^{-/-}$ mice (Jones et al., 1999; Sumeray et al., 2000). Following myocardial I/R, iNOS is expressed in the ischemic region (Liu et al., 1997a, 1997b). Depending on experimental conditions, iNOS expression during myocardial I/R has protective but also detrimental effects. Experiments using 30 min of ischemia followed by 60 min of reperfusion demonstrated the protective effects of iNOS (Kanno et al., 2000; Zingarelli et al., 2002). In these experiments, $iNOS^{-/-}$ mice had higher mortality after reperfusion and extensive myocardial injury associated with marked apoptosis and infiltration of neutrophils (Zingarelli et al., 2002). Consistent with this notion, adenoviral iNOS gene transfer protected against I/R injury (Li et al., 2003). However, inhibition of iNOS by aminoguanidine in prolonged reperfusion for 48 hr decreased infarct size and improved myocardial function, suggesting the detrimental effects of iNOS (Wildhirt et al., 1999). It is well known that NO has anti-inflammatory effects. It is possible that in the early phase of reperfusion, NO inhibits neutrophil infiltration and results in myocardial protection while prolonged high levels of NO production from iNOS may increase oxidative stress through formation of peroxynitrite (Liu et al., 1997a, 1997b). Therefore, further investigation is warranted to elucidate these mechanisms.

4.3. Effects of nitric oxide in ischemic preconditioning

Ischemic preconditioning involves exposing the heart to brief periods of ischemic insult that, in turn, generates a cardioprotective effect against later, more prolonged ischemic episode. This phenomenon exhibits 2 distinct phases: an early phase, that is protective for 2–3 hr after the initial insult; and a late phase, a second window of protection, which is effective 1–3 days following the initial ischemic insult. Thus, ischemic preconditioning serves to protect against myocardial dysfunction normally associated with I/R events. The role of NO in ischemic preconditioning has recently been reviewed by Bolli (2001). While endogenous NO production does not have any effects on the protection induced by early phase ischemic preconditioning in most studies (Weselcouch et al., 1995; Post et al., 2000), there is consistent evidence that supports the protective effects of NO in late phase ischemic preconditioning. Most in vivo studies showed that iNOS plays a prominent role in the protective effect of late phase ischemic preconditioning (Guo et al., 1999; Salloum et al., 2003).

Interestingly, Bell et al. (2002) showed that infarct size was reduced to a similar extent in wild-type and $iNOS^{-/-}$ mice after delayed preconditioning triggered by adenosine A1 receptor activation. This protection was abrogated with the nonspecific NOS inhibitor, L-NAME, and could be mimicked in naive hearts with the NO donor, SNAP. Delayed protection appears to be mediated by eNOS as eNOS protein and NO_x production were increased in both wild-type and $iNOS^{-/-}$ hearts (Bell et al., 2002). Rui et al. (2003) studied delayed preconditioning in cultured neonatal mouse cardiomyocytes subjected to anoxia and reoxygenation. Consistent with the in vivo data from Bell et al., delayed preconditioning occurred in $iNOS^{-/-}$, but not $eNOS^{-/-}$ cardiomyocytes. Anoxia and reoxygenation increased NO production and mRNA for eNOS, but not

iNOS, in wild-type myocytes. These data suggest an important role of eNOS in the protective effects of delayed preconditioning (Rui et al., 2003). Therefore, NO production from either eNOS or iNOS can elicit delayed preconditioning depending on different experimental conditions.

The mechanisms by which NO protect myocardium from I/R injury are not fully understood. NO has been shown to inhibit Ca^{2+} flux into cardiomyocytes, antagonize the effects of adrenergic stimulation, and reduce oxygen consumption (Kelly et al., 1996; Balligand & Cannon, 1997). NO induces expression of heat shock proteins (Malyshev et al., 1996) and cyclooxygenase-2, both of which have been demonstrated to protect cardiomyocytes from I/R injury (Shinmura et al., 2000; Lin et al., 2001; Shinmura et al., 2002). Although many of these studies did not determine apoptosis as an end point for delayed protection, a recent study demonstrated that heat shock protein 20 inhibits β-agonist-induced apoptosis in cultured adult rat cardiomyocytes (Fan et al., 2004). The fact that apoptosis contributes to cell death during myocardial I/R suggests that NO produced by eNOS and iNOS may protect cardiomyocytes from apoptosis in the late phase of preconditioning.

5. Apoptosis in heart failure

Chronic heart failure is the final clinical presentation for a variety of cardiovascular diseases, including myocardial infarction, hypertension, and cardiomyopathy, as a result of persistent stress to the myocardium and progressive cardiac remodeling. Recent studies from patients and animal models of heart failure have demonstrated that cardiomyocyte apoptosis is observed during development of heart failure. In this regard, apoptotic myocytes have been documented in patients with heart failure due to a variety of etiologies including ischemic heart disease, valvular heart disease, and dilated cardiomyopathy (Narula et al., 1996; Olivetti et al., 1997). Myocyte apoptosis has also been demonstrated in a pacing-induced heart failure dogs (Goussev et al., 1998; Leri et al., 1998). However, in most of the studies, the amount of apoptosis observed is low (less than 0.5%). It has been debated whether apoptosis plays a significant role in the development of heart failure. We recently studied a time course of myocyte apoptosis in the pacing-induced heart failure dogs (Moe et al., 2002). Although apoptosis was significantly increased over control after 1, 3, and 4 weeks of pacing, the amount of apoptosis measured at all time points was low and did not correlate with any parameters of cardiac dysfunction. However, when we calculated the amount of apoptosis over time, the accumulated myocyte apoptosis correlated significantly to the degree of myocardial dysfunction in this animal model of heart failure, suggesting that apoptosis plays an important role in the development of heart failure (Moe et al., 2002). More recently, Wencker et al. (2003) have provided definitive evidence for a causal relationship between ongoing low levels of cardiomyocyte apoptosis and development of heart failure. Using a transgenic mouse model that expresses a conditionally active caspase exclusively in the myocardium, they demonstrated that very low levels of myocyte apoptosis (23 myocytes per 10^5 nuclei, compared with 1.5 myocytes per 10^5 nuclei in controls) are sufficient to cause a lethal, dilated cardiomyopathy. Interestingly, these levels are 4- to 10-fold lower than those observed in failing human hearts. Conversely, inhibition of cardiac myocyte death in this murine model largely prevents the development of cardiac dilation and contractile dysfunction, which are hallmarks of heart failure (Wencker et al., 2003). Therefore, it appears that ongoing low levels of cardiomyocyte apoptosis contribute to the development of heart failure.

5.1. Nitric oxide production in heart failure

Heart failure is associated with increased in vivo NO production. The increased NO generation has been demonstrated in heart failure patients as well as animal models of heart failure (Winlaw et al., 1994; Stathopulos et al., 2001; Sugamori et al., 2002). High levels of NO are produced despite compensatory increase of endogenous NOS inhibitors in heart failure (Feng et al., 1998; Kielstein et al., 2003; Saitoh et al., 2003). While increased NO production in heart failure is well established, the contribution from each NOS isoform is still not clear. In the infarct rat model of heart failure, vascular expression of both eNOS and iNOS is up-regulated. However, vascular eNOS activity and endothelium-dependent relaxation are decreased (Feng et al., 1998; Bauersachs et al., 1999; Stathopulos et al., 2001), which may contribute to the development of heart failure postmyocardial infarction (see Fig. 3). Similar findings have been shown in the ventricular myocardium, where protein concentrations of eNOS, iNOS, and nNOS are all increased (Haywood et al., 1996; Fukuchi et al., 1998; Takimoto et al., 2000). In patients with heart failure, systemic NO production and TNF-α levels are increased in proportion to the severity of heart failure (Sugamori et al., 2002). Up-regulation of NOS isoforms and increased NO production may be one of the consequences of neurohumoral activation and expression of pro-inflammatory cytokines including TNF-α, which are characteristic features of heart failure.

5.2. Pro-apoptotic effects of nitric oxide in heart failure

NO production from iNOS has been shown to play an important role in the development of heart failure post-myocardial infarction. Using $iNOS^{-/-}$ mice, we recently demonstrated that iNOS expression following myocardial infarction impairs cardiac function and decreases survival (Feng et al., 2001). Furthermore, myocardial and plasma nitrotyrosine levels are increased in mice with heart failure, suggesting increased production of peroxynitrite, which

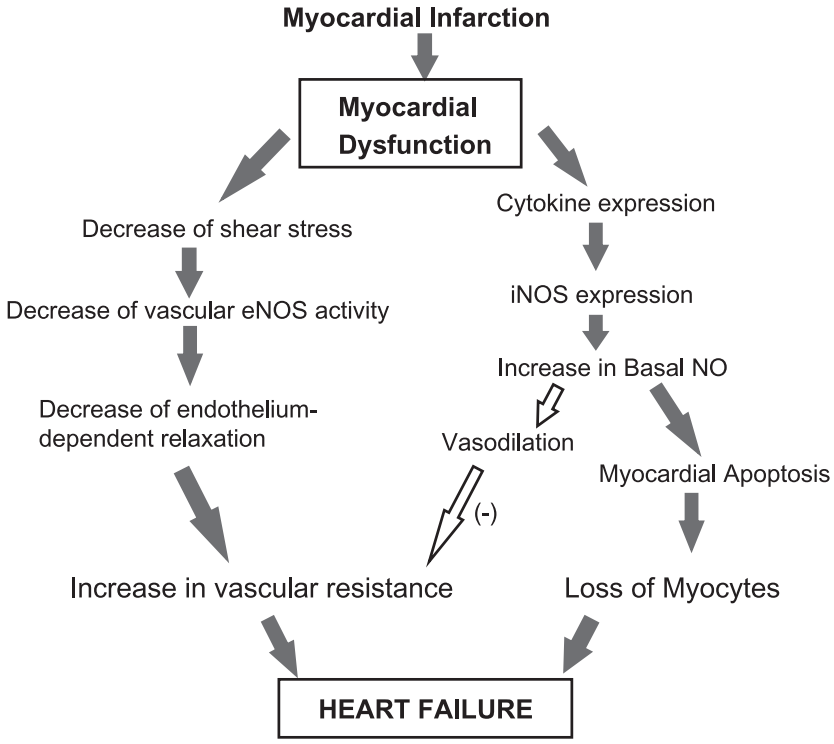

Fig. 3. Effects of NO in heart failure post myocardial infarction. We propose 2 major pathways that contribute to development of heart failure following myocardial infarction. (1) Decreased vascular eNOS activity and endothelium-dependent relaxation contribute to increased vascular resistance. (2) Increased cytokine expression including TNF-α induces iNOS expression. High levels of NO production from iNOS expression have multiple effects. NO thus produced can decrease vascular resistance by causing vasodilation and hypotension (green arrows), which may be beneficial. However, high levels of NO production can lead to myocardial apoptosis and myocyte cell loss, which are detrimental post myocardial infarction. Decreased endothelial function from decreased vascular eNOS activity and increased myocardial apoptosis from increased iNOS expression in the myocardium contribute to the development of heart failure.

may induce cardiomyocyte apoptosis. Indeed, studies by Sam et al. (2001) showed that 4 months after myocardial infarction, the abundance of apoptotic myocytes measured by in situ TUNEL staining was increased in wild-type mice but not in iNOS$^{-/-}$ mice. Improved contractile function and reduced apoptosis were associated with reduced mortality rate in iNOS$^{-/-}$ mice at 4 months after myocardial infarction. Thus, iNOS activity contributes to contractile dysfunction, and increased myocyte apoptosis in the remote myocardium in the late stages of heart failure postmyocardial infarction (Sam et al., 2001; see Fig. 3).

Acute heart allograft rejection is associated with myocardial iNOS expression and apoptosis. In this regard, C57BL/6J donor hearts transplanted into C57BL/6J.129J mice resulted in increased iNOS mRNA and protein expression, allograft rejection, and significant increase in total number of apoptotic cardiomyocytes. When iNOS$^{-/-}$ donor hearts (C57BL/6J) transplanted into iNOS$^{-/-}$ recipients (C57BL/6J.129J), allograft survival was significantly increased, which was associated with decreases in peroxynitrite generation and cardiomyocyte apoptosis (Szabolcs et al., 2001). Consistent with the animal data, increased iNOS expression, peroxynitrite generation and cardiomyocyte apoptosis have also been demonstrated in human cardiac allograft rejection. Apoptosis is a major form of myocyte death during human cardiac allograft rejection. Furthermore, evidence suggests that cardiac myocyte apoptosis is closely associated with myocardial iNOS expression and nitration of myocyte proteins by peroxynitrite (Szabolcs et al., 1998).

5.3. Anti-apoptotic effects of nitric oxide in heart failure

NO production from eNOS and NO-mediated vascular relaxation are important in maintaining normal cardiovascular function. In a recent study, we demonstrated that lack of NO production from eNOS increased the incidence of apoptosis associated with caspase-3 activation and DNA fragmentation in the myocardium of embryonic and neonatal hearts (Feng et al., 2002). Furthermore, deficiency of eNOS resulted in congenital septal defects and postnatal heart failure. Our results indicate that NO production from eNOS is important for cardiomyocyte survival and normal heart development (Feng et al., 2002).

Deficiency in eNOS has been shown to promote the development of heart failure postmyocardial infarction. Mice deficient in eNOS exhibit decreased myocardial function and increased mortality following myocardial infarction (Scherrer-Crosbie et al., 2001). NO production for eNOS has been shown to inhibit caspase activity. In this

regard, stimulation of mouse heart caspase-3 activity by upstream caspase-8 or -9 was effectively lower in myocardium of wild-type as compared to eNOS$^{-/-}$ mice (Mital et al., 2002). Furthermore, L-NAME increased caspase-3 activity only in wild-type hearts, indicating that endogenous NO inhibits caspase-3. Stimulated caspase-3 activity was lower in controls compared to cardiomyopathic hamsters with decreased eNOS activity. This was associated with progressive ventricular dysfunction, thinning, and dilatation (Mital et al., 2002). In failing human myocardium, enalaprilat which up-regulates eNOS, inhibited caspase-3, an effect that was reversed by L-NAME (Mital et al., 2002). These studies suggest that decreased eNOS activity in heart failure increases myocardial caspase-3 activity and consequently myocardial apoptosis.

Cardiomyocytes also express nNOS, which is normally located in the sarcoplasmic reticulum. The strategic location of nNOS in the sarcoplasmic reticulum suggest that nNOS is involved in the regulation of intracellular Ca^{2+} (Xu et al., 1999). Indeed, nNOS has been shown to inhibit L-type Ca^{2+} channels and Ca^{2+} release from ryanodine receptors and to promote sarcoplasmic reticulum Ca^{2+} uptake through Ca^{2+}-ATPase (Barouch et al., 2002; Zhou et al., 2002; Sears et al., 2003). Deficiency in nNOS resulted in elevations of intracellular Ca^{2+} and increases in inotropic function of cardiomyocytes (Sears et al., 2003). Interestingly, nNOS is translocated to sarcolemma in heart failure (Damy et al., 2004), which may have a pathological significance in Ca^{2+} handling and myocardial function. Whether nNOS is beneficial or detrimental to cardiomyocyte apoptosis during development of heart failure is currently unknown, and requires further investigation.

6. Therapeutic implications

Caspases are a group of cysteine proteases that play a crucial role in initiating and executing apoptosis. It is therefore indubitable that caspases are potential targets for anti-apoptotic treatment. Indeed, in rats that underwent 30 min of myocardial ischemia followed by 24 hr of reperfusion, a broad-spectrum caspase inhibitor, benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone, reduced both infarct size and cardiomyocyte apoptosis, with significant hemodynamic improvement in vivo (Yaoita et al., 1998). Furthermore, in a rabbit model of heart failure induced by rapid ventricular pacing, in vivo transcoronary adenovirus-mediated gene delivery of the potent caspase inhibitor p35 decreased myocardial caspase-3 activity and improved cardiac function (Laugwitz et al., 2001). Moreover, treatment with a caspase inhibitor prevented the development of heart failure in mice overexpressing caspase-8 (Wencker et al., 2003). These studies demonstrate the therapeutic potential of caspase inhibition during myocardial I/R and development of heart failure. However, there are potential safety concerns with chronic systemic use of apoptosis inhibition, because apoptosis is required in the prevention of cancer and autoimmune diseases (Kang & Izumo, 2003).

Since iNOS has been implicated in various pathophysiologic conditions including myocardial infarction and heart failure, selective iNOS inhibitors have been developed and tested in these disease conditions in animal studies. In this regard, long-term administration (2 months) of the selective iNOS inhibitor *S*-methylisothiourea (SMT) to rats with myocardial infarction significantly improved cardiac function. A significant drop in mortality, lung water content, infarct size, and cardiomyocyte hypertrophy was also associated with decreased iNOS activity (Saito et al., 2002). Inhibition of iNOS-dependent NO production has been shown to decrease cardiomyocyte apoptosis induced by cytokines in vitro (Ing et al., 1999). However, there are no reports to date on the effects of selective iNOS inhibition on cardiomyocyte apoptosis after myocardial infarction in vivo. Interestingly, cardiomyocyte apoptosis was significantly decreased following myocardial infarction in iNOS$^{-/-}$ mice (Sam et al., 2001). The therapeutic potential of iNOS inhibitors in myocardial infarction and heart failure requires further investigation.

During acute cardiac allograft rejection, iNOS expression is associated with myocardial inflammation, contractile dysfunction, and death of cardiomyocytes by necrosis and apoptosis (Szabolcs et al., 1998). Recently, McMillan et al. (2000) using combinatorial chemistry developed allosteric inhibitors of iNOS monomer dimerization (BBS-1 and BBS-2), which selectively block the enzyme activity of iNOS. Administration of BBS-1 and BBS-2 to rats that had undergone heterotopic abdominal heart transplantation was associated with significantly prolonged cardiac allograft survival and significant reductions in the myocardial inflammation and cardiomyocyte death by apoptosis and necrosis (Szabolcs et al., 2002). These data suggest that selective iNOS inhibitors may provide useful additions to the current therapeutic strategies for acute cardiac allograft rejection.

Endogenous NO production from eNOS has been shown to protect cardiomyocytes from apoptosis during normal heart development and in myocardial I/R (Jones et al., 1999; Feng et al., 2002). Recent studies from transgenic mice with cardiac specific eNOS overexpression further support the cardioprotective effects of eNOS during myocardial I/R (Brunner et al., 2003). Accordingly, factors stimulating eNOS activity, such as angiotensin-converting enzyme inhibitors acting through bradykinin, may protect the myocardium. In support, enalaprilat inhibited caspase-3 activity in the explanted failing human myocardium, and this action was reversed by L-NAME (Mital et al., 2002). These results suggest that activation of eNOS by angiotensin-converting enzyme inhibitors blocks myocardial apoptosis, contributing to the beneficial effects of this class of drugs in myocardial infarction and heart failure. Thus, eNOS may also be a potential therapeutic target for cardiomyocyte apoptosis in cardiac pathologies.

7. Conclusions

Apoptosis, or programmed cell death, is a highly regulated and evolutionarily conserved mechanism of cell death. The mechanism of apoptosis is multifactorial and complex. NO is an important regulator of apoptosis within the mammalian system, capable of both inducing and preventing apoptosis, depending upon the environmental milieu. This bifunctional capacity is well illustrated within the heart. A variety of cell types, including endothelium, vascular smooth muscle, and cardiomyocytes, can produce NO. It seems that high levels of NO produced by iNOS can induce apoptosis directly. Moreover, NO recombines with superoxide to cause apoptosis indirectly via generation of a potent oxidant peroxynitrite. Conversely, basal NO production from eNOS is essential for cardiomyocyte survival and can provide cardioprotective effects. Thus, NO appears to be an important regulator of apoptosis in the heart during normal physiological conditions as well as in myocardial I/R and heart failure. Since permanent loss of cardiomyocytes due to apoptosis contributes to the development of heart failure, inhibition of cardiomyocyte apoptosis may have therapeutic implications. Given the pro- and anti-apoptotic capacity of NO within the heart, this molecule may serve as a valuable therapeutic target in myocardial ischemia and heart failure.

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