ABSTRACT

Nitric oxide synthase (NOS) is a highly regulated enzyme that produces nitric oxide, a critical messenger in many physiological processes. In this perspective, we explore the role of proteolytic degradation of NOS, in particular the inducible and neuronal isoforms of NOS, as a mechanism of regulation of the enzyme. The ubiquitin-proteasome and calpain pathways are the major proteolytic systems identified to date that are responsible for this regulated degradation. The degradation of NOS is affected by diverse agents, including glucocorticoids, caveolin, neurotoxic compounds, and certain NOS inhibitors. Some irreversible inactivators of NOS enhance the proteolytic degradation of the enzyme, and this property may be of great importance in understanding the biological effects of these inhibitors, some of which are being developed for clinical use. Analogies with the regulated degradation of liver microsomal cytochromes P450, which are related to NOS, provide a framework for understanding these processes. Finally, a new perspective on the regulation of NOS by hsp90-based chaperones is presented that involves facilitated heme insertion into the enzyme.

Proteolysis of NOS

Selective proteolytic degradation of NOS is a mechanism for regulation of the enzyme. For example, transforming growth factor-β enhances the degradation of iNOS in interferon-γ-treated mouse peritoneal macrophages and causes suppression of NO release from these cells (Vodovotz et al., 1993). The glucocorticoid-mediated suppression of iNOS expression in IL-1β-treated rat glomerular mesangial cells (Kunz et al., 1996) and interferon-γ-treated murine macrophage cell line RAW 264.7 (Walker et al., 1997) is due, in part, to increased degradation of iNOS. Based on studies with the protease inhibitor N-acetyl-Leu-Leu-norleucinal, Walker et al. (1997) concluded that calpain is the key protease responsible for this glucocorticoid-mediated decrease in iNOS. Consistent with this notion, purified calpain preferen-

ABBREVIATIONS: NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; MG132, carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal; P450, cytochrome P450; apo-NOS, heme-deficient form of NOS.
tially degrades the monomeric form of iNOS in vitro (Walker et al., 2001). N-Acetyl-Leu-Leu-norleucinal, however, also inhibits the proteasome and cathepsins in addition to calpains (Rock et al., 1994), and the role of these other proteases has not been ruled out. In fact, Felley-Bosco et al. (2000) have shown with the use of lactacystin, a highly selective inhibitor of the proteasome, that cavelolin-1 down-regulates iNOS via the proteasome pathway in human colon carcinoma cells. Based in large part on the finding that iNOS degradation is blocked by lactacystin, MG132, and N-acetyl-Leu-Leu-norleucinal, but not by the calpain inhibitor calpastatin, Musial and Eissa (2001) concluded that iNOS is primarily regulated by the proteasome pathway in RAW 264.7 and HEK 293 cells. Consistent with proteasomal degradation, iNOS is found as ubiquitin-conjugates, and the formation of conjugates is critical in degradation (Kolodziejski et al., 2002).

The regulated proteolytic degradation of iNOS appears to be important in the development of salt-induced hypertension in Dahl/Rapp rats. An inbred strain of Dahl/Rapp rats that is susceptible to salt-induced hypertension has a S714P mutation in iNOS, whereas Dahl/Rapp rats that are resistant to salt-induced hypertension do not. This mutation in iNOS does not change the $K_m$ or $V_{max}$ for arginine but confers to the mutated protein a shorter half-life than the wild-type iNOS when transfected into COS cells (Ying et al., 2001). This increased turnover can be largely prevented by clasto-lactacystin, implicating a role of the proteasome in this process. Ying et al. (2001) suggest that this enhanced degradation is the biochemical mechanism for the lower NO production in the salt-sensitive Dahl/Rapp rats that renders them susceptible to hypertension. The administration of L-arginine to the salt-sensitive Dahl/Rapp rats prevents the salt-induced hypertension and increases NO production. Although the exact mechanism for this effect is still unknown, perhaps one mechanism is substrate-induced stabilization of the enzyme that decreases the proteolytic degradation of iNOS.

Calpain is activated under excitotoxic conditions in neurons. Neurotoxic-induced calpain activation in fetal rat cerebrocortical cells has been shown to lead to the proteolytic cleavage of nNOS (Hajimohammadzadeh et al., 1997). In in vitro studies with the use of brain or muscle homogenates, nNOS is rapidly degraded by a process that is attenuated by a calpain-specific inhibitor (Laine and Ortiz de Montellano, 1998). Moreover, purified m-calpain degrades nNOS (Laine and Ortiz de Montellano, 1998). Interestingly, the nNOS$_\mu$ form, which is expressed in striated muscle and has a 34 amino acid peptide insertion between the calmodulin- and flavin-binding domains (Silvagno et al., 1996), is degraded by calpain albeit at a slightly slower rate than the principal brain nNOS isoform. The rapid proteolytic degradation by calpain has been suggested as a reason for the absence of nNOS in skeletal muscle sarcolemma of muscular dystrophy patients (Laine and Ortiz de Montellano, 1998).

In studies using pulse-chase techniques on HEK 293 cells under nonexcitatory conditions, nNOS degradation has been shown to be inhibited by lactacystin (Noguchi et al., 2000). Moreover, nNOS is found in ubiquitin conjugates in lactacystin-treated HEK 293 cells or rat brain homogenates (Bender et al., 2000a), strongly suggesting the ubiquitin-proteasome pathway in regulating the degradation of nNOS in vivo. Studies with the use of reticulocyte lysates and purified NOS indicate that the monomeric form of nNOS is preferentially ubiquitinated (Bender et al., 2000a). Consistent with these reports on the proteasomal degradation of nNOS, treatment of NT-2 and SK-N-MC cells with inhibitors to the proteasome increase nNOS protein by approximately 4-fold along with an increase in NO formation, as indicated by higher levels of nitrite/nitrate and 3-nitrotyrosine (Lee et al., 2001). The evidence to date shows that calpain and proteasome are the major proteolytic pathways regulating the turnover of iNOS and nNOS.

### Inhibition and Turnover of NOS

As described above, NOS enzymes are regulated, in part, by post-translational proteolysis. Interestingly, certain types of NOS inhibitors enhance the degradation of NOS protein. Ignarro and coworkers (Wei et al., 1998) were the first to report that certain S-isothiourea-based NOS inhibitors, namely S-aminomethylisothiourea, could cause a loss in immunodetectable iNOS protein in RAW 264.7 cells, whereas others, namely S-ethylisothiourea, had no effects on protein levels and may have actually stabilized the iNOS protein. The loss of iNOS protein caused by S-aminomethylisothiourea was due to decreased protein translation as well as accelerated degradation, as determined by pulse-chase studies. These authors conjectured that these effects were due to nonspecific effects of S-aminomethylisothiourea on total protein synthesis and degradation. The exact mechanism for the different actions of S-aminomethylisothiourea and S-ethylisothiourea still remains undefined. The accelerated degradation of nNOS has been observed with several other NOS inhibitors, including N$^G$-methyl-L-arginine, N$^G$-(1-iminoethyl)-L-ornithine, and guanabenz (Nakatsuka et al., 1998; Noguchi et al., 2000). The loss of nNOS activity per se is not the signal for proteolytic removal since other inhibitors, such as N$^G$-nitro-L-arginine and 7-nitroindazole, did not enhance degradation of nNOS and may have actually stabilized the protein (Nakatsuka et al., 1998; Noguchi et al., 2000). It is noteworthy that N$^G$-methyl-L-arginine, N$^G$-(1-iminoethyl)-L-ornithine, and guanabenz are irreversible, metabolism-based, or suicide-type inactivators, S-ethylisothiourea and 7-nitroindazole are rapidly reversible inhibitors, and N$^G$-nitro-L-arginine is a slowly reversible inhibitor. There is precedence for metabolism-based or suicide inactivators to cause the enhanced proteolytic turnover of the affected enzyme. Especially pertinent are the examples described below from the liver microsomal cytochrome P450 enzymes, which are related to NOS (Alderton et al., 2001).

Metabolism-based inactivators or suicide inactivators are chemically inert molecules that mimic the natural substrate of the enzyme and become metabolized to a highly reactive intermediate that can covalently alter important active site entities and result in inactivation of the enzyme. In effect, the compound causes the enzyme to catalyze its own demise. The liver microsomal cytochromes P450 are responsible for bioactivation of many chemicals to reactive intermediates. In some cases, the cytochromes P450 are the initial targets of these reactive intermediates and result in the covalent alteration, inactivation, and destruction of the enzyme by such a metabolism-based or suicide process. This metabolism-based inactivation is known to proceed by three major pathways: the covalent alteration of the heme, the covalent alteration of...
the protein, and cross-linking of the heme to the protein (Osawa and Pohl, 1989).

As in the case of NOS, it appears that structural changes and not the functional inactivation per se is the "trigger" for proteolysis of liver microsomal cytochromes P450 (Correia et al., 1987; Tierney et al., 1992). Moreover, the cross-linking of heme to protein plays a major role in the proteolytic recognition, whereas covalent alteration of the heme or the protein do not appear to be involved (Tierney et al., 1992; Korsmeyer et al., 1999; Wang et al., 1999). The formation of the heme-protein cross-link is known to lead to an opening of the heme binding cleft in myoglobin (Osawa et al., 1991) and to give a less globular or more unfolded state of the protein (Osawa and Pohl, 1989). This in turn results in a highly reactive altered heme site that catalyzes redox reactions, including the reduction of molecular oxygen to form peroxide, which can oxidatively damage the hemeprotein and form more of the heme-protein cross-linked adducts and eventually lead to destruction of the heme (Osawa and Korzekwa, 1991). The formation of the heme-protein cross-link and the subsequent effects on protein structure, including oxidative damage, probably play a role in proteolytic recognition. In addition, the propensity of cytochromes P450 to autoinactivate oxidatively during catalysis due to the formation of superoxide and hydrogen peroxide may be responsible for the "normal" proteolytic turnover of the P450 cytochromes (Tierney et al., 1992). In support of this notion, troleandomycin, a macrolide antibiotic inhibitor of P450 that forms a quasi-stable metabolite complex with the heme, has been shown to decrease the degradation of P450 3A (Watkins et al., 1986), perhaps by decreasing the formation of oxidants that damage the protein. Thus, certain reversible inhibitors, such as N\textsuperscript{\textcircled{2}}-nitro-L-arginine, may protect NOS from degradation by decreasing oxidative damage to the enzyme.

As in the case of NOS, cytochromes P450 are ubiquitinated (Korsmeyer et al., 1999; Wang et al., 1999; Banerjee et al., 2000) and thus are potential substrates for ubiquitin-dependent proteases, which are known to play an important role in degradation of other abnormal or damaged proteins. Several laboratories have now shown that cytochrome P450 3A isoforms and 2E1 are ubiquitinated and degraded by the proteasome (Yang and Cederbaum, 1996; Goasduff and Cederbaum, 1999; Korsmeyer et al., 1999; Wang et al., 1999; Banerjee et al., 2000). For cytochrome P450 3A, phosphorylation may serve as a trigger for ubiquitination (Korsmeyer et al., 1999). Thus, ubiquitination and proteasomal degradation appear to regulate the selective removal of inactivated and altered cytochrome P450 in a manner similar to that found for NOS.

Although it has been established for liver P450 cytochromes that heme-protein cross-linking is an important signal for proteolysis, the nature of the inactivated and altered NOS that are degraded is unknown. Bryk and Wolff (1999) reviewed suicide inactivators of NOS and described the evidence showing the alteration of the heme and protein as mechanisms for inactivation. More recently, the formation of heme-protein cross-linked adducts has been described for NOS (Jianmongkol et al., 2000; Vuletich et al., 2002), thus showing that all the major pathways described for the inactivation of liver microsomal cytochromes P450 also describe the inactivation of NOS. Due to the complexity of NOS, however, other mechanisms, such as the alteration of tetrahydrobiopterin, may also play a role in the inactivation process.

Although the exact covalent alteration that triggers NOS for proteolysis remains to be defined, it is known that the heme-deficient monomeric form of nNOS is selectively ubiquitinated. Thus, suicide inactivators may favor formation of the monomeric form of NOS to enhance degradation. In support of this, suicide inactivators of NOS have been shown to lead to degradation of the heme prosthetic group of NOS to form the heme-deficient enzyme, which is likely monomeric (Bryk and Wolff, 1999; Vuletich et al., 2002) (Fig. 1). If this is the case, any condition that favors a change in the ratio of the amount of dimeric to that of the monomeric form of NOS could, in turn, affect the overall proteolytic degradation of NOS. These conditions include heme availability (Albakri and Stuehr, 1996), tetrahydrobiopterin depletion (Reif et al., 1999), and use of dimerization inhibitors (McMillan et al., 2000).
It is still unclear why the monomeric form of NOS, once formed, is preferentially degraded. It is noteworthy that the monomeric form of NOS is more susceptible to trypsin hydrolysis (Panda et al., 2002), and perhaps there is greater accessibility of site(s) for ubiquitination. It is also possible the hsp70 component of the hsp90/hsp70-based chaperone machinery that is known to regulate NOS enzymes, as described below, directs the ubiquitination of NOS by recruiting ubiquitin ligases similar to that found for other hsp90-associated proteins (Cyr et al., 2002).

Clearly, studies are needed to define the E2 and E3 ligases involved in ubiquitination of NOS, the sites of ubiquitination, and the role of chaperones in this process. Although sequence motifs are present in NOS that may be recognition sites for E3 ligases, it remains to be shown what ligases and sites are functionally important. On the other hand, if the chaperones direct the ubiquitination as in the case with CHIP (Cyr et al., 2002), a chaperone-associated ubiquitin ligase, then the recognition may be at the level of the chaperone. In this respect, the recognition site may involve exposed hydrophobic surfaces, as have been proposed for the recognition of steroid receptors by hsp90-based chaperones (Kaul et al., 2002) and not a sequence motif per se.

Role of hsp90-Based Chaperones on Turnover

Geldanamycin, a selective inhibitor of hsp90, causes the loss of cellular enzyme activity of iNOS (Joly et al., 1997), eNOS (García-Cardenas et al., 1998), and nNOS (Bender et al., 1999). In the case of eNOS (Garcia-Cardenas et al., 1998) and nNOS (Bender et al., 1999), the enzymes were found in a complex with hsp90 in vivo. In the case of nNOS, this loss of activity was shown to be followed by the enhanced proteasomal degradation of the enzyme (Bender et al., 1999). Taken together, these results suggest a role for hsp90-based chaperones in regulating the pool of active NOS enzyme, although the exact mechanism by which this occurs is not known. One proposed mechanism is for hsp90 to enhance the affinity of calmodulin for the enzyme (Song et al., 2001a,b). Another mechanism involves the allosteric modulation of the NOS by hsp90, as described in a recent perspective for eNOS (Fulton et al., 2001). We present here a different but not mutually exclusive perspective on the role of hsp90-based chaperones in facilitating functional heme insertion into nNOS as a mechanism for regulation.

As described in the above sections, the heme-deficient monomeric form of NOS is actively ubiquitinylated and degraded by the proteasome (Fig. 1). Thus, geldanamycin probably enhances degradation by favoring greater amounts of the nNOS to be in this monomeric state. Conversely, hsp90-based chaperones must favor the formation of greater amounts of the dimeric active state. One of the key elements of keeping NOS in this active dimeric state is the heme prosthetic group, which can initiate the dimerization of heme-deficient monomers (Baek et al., 1993; Bender et al., 2000b). Thus, we proposed that insertion of the hydrophobic heme into the heme-deficient apo-nNOS is facilitated by hsp90-based chaperones (Bender et al., 1999; Billecke et al., 2002). Consequently, the inhibition of hsp90-based chaperones would favor the monomeric form of NOS and lead to ubiquitination and enhanced proteasomal degradation.

In support of this notion, hsp90 inhibitors geldanamycin and radicicol decrease the heme-mediated activation of apo-nNOS during baculovirus-mediated overexpression in insect cells (Bender et al., 1999; Billecke et al., 2002). In this system, there is only a low level of endogenous heme, and exogenous heme must be added for incorporation of heme into the overexpressed apo-nNOS. In the presence of radicicol, the heme is not incorporated into apo-nNOS to form the P450 complex, indicating that hsp90-based chaperones are needed for functional heme reconstitution. As expected from the lack of heme insertion, the dimeric state of nNOS is not formed, and NO production is not observed. The lack of heme insertion into apo-nNOS does not appear to be due to a lack of heme entry into the cells (Billecke et al., 2002).

This notion of insertion of a hydrophobic heme into nNOS is consistent with the action of the hsp90-based chaperone machinery on the glucocorticoid receptor in which the hydrophobic ligand binding cleft is opened to allow access by steroid (Stancato et al., 1996; Xu et al., 1998; Giannoukos et al., 1999; Kaul et al., 2002). In a similar manner, the chaperone machinery may favor opening of the heme-binding cleft and facilitate heme entry. This may be important in other heme-regulated proteins that associate with hsp90-based chaperones. For example, hsp90 is required for the heme-mediated activation of the yeast transcriptional activator Hap1, which regulates genes involved in respiration and oxidant control (Lee et al., 2002). Heme insertion into Hap1 may be controlled by the hsp90-based chaperone machinery and be the basis for this regulation. Indeed, the presence of hydrophobic clefs is a universal feature of all properly folded proteins, and the ability of hsp90-based chaperone machinery to recognize these regions is consistent with the notion that cleft opening and facilitated binding of hydrophobic compounds is a primary function of the chaperone machinery. Moreover, it is likely that the hsp90-based chaperone machinery facilitates binding of a number of hydrophobic ligands and prothetetic groups by facilitating the opening and stabilization of hydrophobic clefs in native acceptor proteins.

References


