Nitric Oxide Signaling in Plant-Pathogen Interactions

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Summary

Nitric oxide (NO), first characterized as an endothelium-derived relaxation factor, is involved in diverse cellular processes including neuronal signaling, blood pressure homeostasis, and immune response. Recent studies have also revealed a role for NO as a signaling molecule in plants. As a developmental regulator, NO promotes germination, leaf extension and root growth, and delays leaf senescence and fruit maturation. Moreover, NO acts as a key signal in plant resistance to incompatible pathogens by triggering resistance-associated hypersensitive cell death. In addition, NO activates the expression of several defense genes (e.g. pathogenesis-related genes, phenylalanine ammonia lyase, chalcone synthase) and could play a role in pathways leading to systemic acquired resistance.

Keywords

Cell death; hypersensitive response; nitric oxide; reactive oxygen species; systemic acquired resistance.

INTRODUCTION

The plant resistance response to pathogen attack begins with specific recognition by plant receptors of the invading pathogen. The avirulent pathogen elicits a set of plant defense responses (Fig. 1), that usually results in the so-called hypersensitive reaction (HR), which is characterized by collapse of the infected cells. This hypersensitive cell death delimits the infected zone and avoids the multiplication and spread of the pathogen (1). One of the earliest events in the HR is the rapid accumulation of reactive oxygen species (ROS) and nitric oxide (NO) through the activation of enzyme systems similar to neutrophil NADPH oxidase (2; 3) and nitric oxide synthase (NOS; 4). Both NO and ROS are necessary to trigger host cell death and are also components of a highly amplified and integrated defense system that involves salicylic acid (SA), activation of ion fluxes, changes in protein phosphorylation patterns, extracellular pH, membrane potential, oxidative cross-linking of plant cell wall proteins, and perturbations in the level of cytosolic Ca$^{2+}$ (5). These events trigger the expression of disease and resistance mechanisms that mediate a systemic signal network involved in the establishment of a systemic immunity, called systemic acquired resistance (SAR), which confers resistance to secondary infection by either the same or a diverse pathogen. SAR is associated with the failure of virulent pathogens to progress in the immunized tissue and is correlated with the expression of different families of the so-called SAR genes (6).

NO SYNTHESIS IN PLANTS

In animal cells, the biosynthesis of NO is primarily catalyzed by a family of nitric oxide synthases (NOS), which oxidize L-arginine to form L-citrulline and NO. There are different isoforms of NOS, although each enzyme has an N-terminal oxygenase and a C-terminal reductase domain (7). In plant cells, there are different potential sources of NO. It may be generated either non-enzymatically via the conversion of NO2 by carotenoids, or enzymatically via nitrate reductase (8) and nitric oxide synthase (4). The major pathogen-induced NOS is a variant form of the P protein of the glycine decarboxylase complex (GDC) and it is specifically induced during the resistance response to pathogen infection (4). The purified protein is approximately 120 kDa and most likely is active as a dimer. The activity of this plant iNOS is suppressed by P protein inhibitors such as carboxymethoxylamine and aminoacetonitrile and, like the animal NOS, is sensitive to inhibitors like L-NMMA or guanidine (4). Moreover, like the
animal NOS, it requires H\textsubscript{2}B, FAD, NADPH, O\textsubscript{2} as well as Ca\textsuperscript{2+} and CaM (4). The localization of this plant iNOS and the regulation mechanisms of its activity during the disease resistance response are still unknown.

The production of NO in animal cells is catalyzed by different NOS isoforms, either constitutive or inducible, and plants may possess different NOS isoforms as well. Thus, iNOS may not be the only source of NO in plants and the existence of constitutive NOS would help to explain the basal NOS activity detected under diverse conditions as in the legume Mucuna hasjoo (9), in Lupinus albus nodules (10) and in pea leaf peroxisomes (11). Inhibitors of mammalian NOS reduce the generation of NO that precedes apoptosis in callus cells and foliar tissues of Kalanchoe daigremontiana and Taxus brevifolia (12) and also decrease the production of NO in Arabidopsis, parsley and tobacco cell cultures after treatment with cytokines (13). NOS activity has also been detected in the soluble fractions of root tips and young leaves of maize seedlings (14).

**NO AND HYPERSENSITIVE CELL DEATH**

It has been shown that plant cells accumulate NO in response to infection with bacterial, viral and fungal pathogens. Elicitation of epidermal tobacco cells induces overproduction of NO within minutes (15). Similarly, high levels of NO have been detected in tobacco plants 24 h after infection with tobacco mosaic virus (TMV) (4). Furthermore, NO accumulation has been detected under conditions in which the generation of ROS is also activated (15–17) and this is concomitant with the avirulent gene-dependent oxidative burst that occurs immediately prior to the onset of hypersensitive cell death (16, 18).

In many biological systems, the cytotoxic effects of NO and ROS derive from the diffusion-limited reaction of NO with O\textsubscript{2} to form the peroxynitrite anion ONOO\textsuperscript{-}, which then interacts with many cellular components (19, 20) and may modulate the signaling functions of NO (21). Oddly, whereas exposure of animal cells to ONOO\textsuperscript{-} produces concentration dependent cell death in the range from 1 to 1000 \mu M (22–24), exposure of soybean suspensions cultured cells to a wide range of concentrations of ONOO\textsuperscript{-} (either commercial or in a continuous steady-state generation, SIN-1) does not affect cell viability despite a strong increase in nitrated proteins (25). ONOO\textsuperscript{-} does not appear to be an essential intermediate of NO-induced cell death in plants, but it is expected to have important physiological and signaling functions in the HR since the ONOO\textsuperscript{-} donor SIN-1 was found to induce accumulation of the transcript encoding PR-1 in tobacco leaves (18).

As the formation of ROS and NO is a normal event in plant cellular metabolism (26) and their concentration is also subject to significant variation during exposure to various stresses (27, 28), it may be hypothesized that ONOO\textsuperscript{-} is continuously formed in healthy cells. Consequently, plant cells may have developed specific mechanisms to overcome the toxicity of ONOO\textsuperscript{-}, and may have adopted different NO/ROS signals for triggering cell death during the hypersensitive response. Based on the lack of a killing effect of ONOO\textsuperscript{-} in plant cells, it is clear that the relative rates of production of NO and O\textsubscript{2}\textsuperscript{-} are critical in modulating the effects of NO, which can paradoxically offer protection against oxidative damage by intercepting reactive species and converting them to less damaging and/or more easily detoxified products. Consequently, an equilibrium model for the interactions of NO and ROS has been proposed (25; Fig. 1). In this model, the relative rates of O\textsubscript{2}\textsuperscript{-} dismutation to H\textsubscript{2}O\textsubscript{2} and reaction with NO to generate ONOO\textsuperscript{-} are critical in the integration of the signal system to deliver NO and H\textsubscript{2}O\textsubscript{2} that are required as co-activators of hypersensitive cell death.

How NO and ROS actually kill is still unclear. However, a great deal of experimental evidence indicates that NO induces cell death by triggering an active process, in which proteases appear to play a crucial role. Cystatin-sensitive proteases have been found to be critical regulators for HR in a soybean model system (29) and overexpression of AtCYS1, a cysteine protease inhibitor, was found to block cell death activated by either avirulent pathogens or by nitrosative stress in A.
**thaliana** suspension cultured cells and in transgenic tobacco plants (30). Furthermore, Ac-YVAD-CMK, an irreversible inhibitor of mammalian caspase-1, a class of cysteine proteases involved in apoptosis, was shown to block NO-induced cell death (17).

**NO-MEDIATED ACTIVATION OF DEFENSE GENES**

NO functions together with ROS in triggering hypersensitive cell death, but is also involved in other defense functions complementary to, and independent of, ROS. There is evidence that NO stimulates a signal transduction pathway for the production of phytoalexin in higher plant cells (31). Moreover, the first enzyme of phenylpropanoid biosynthesis pathway, phenylalanine ammonia-lyase (PAL), is induced after infiltration of tobacco leaves and cells with commercially available NOS or NO donors (18, 32) and inhibition of NOS activity clearly reduces its transcript accumulation (16). It has also been shown that the response of soybean cotyledons to elicitors from *Diaporthe phaseolorum* f. sp *meridionalis* triggers the biosynthesis of antimicrobial flavonoids via NOS-like activity. Furthermore, the use of NOS inhibitors caused a marked reduction in the expression of chalcone synthase (CHS; 33), the first enzyme of the branch specific for flavonoids and isoflavonoid-derived antibiotics (34).

As accumulation of PAL and PR1, a pathogenesis-related protein, has been detected in tobacco cell suspensions treated with a membrane permeable analogue of cGMP, and taking into account that induction of PAL in tobacco suspension cells by NO can be suppressed by several inhibitors of guanylate cyclase, activation of a NO-dependent defense gene is thought to be mediated by cGMP (18). However, PAL expression is not fully blocked by inhibitors of cGMP production, implying that different modes of PAL induction operate downstream of NO. Although the involvement of cGMP in several plant signal transduction pathways has been demonstrated (35), it remains to be determined whether or not NO is the physiological activator of plant guanylate cyclase.

Cyclic ADP ribose (cADPR) has been implicated as another second messenger for NO signaling in animals, acting in a cGMP-dependent signaling cascade to mediate calcium mobilization (36). Treatment with cADPR induces PAL and PR-1 expression in tobacco (18), which can be inhibited by ruthenium red, indicating its calcium dependence. Moreover, a cADPR antagonist suppressed NO induction of PR-1; however, this effect was incomplete, indicating that NO activation of defense responses may occur through more than one pathway (37).

Nitrosylation of key components of cell metabolism is another mode of NO-signaling in animals (38) that may have parallels in plants. Strong evidence indicates that calcium release can also be regulated by channel S-nitrosylation or oxidation (39, 40). The existence of multiple mechanisms of NO action makes the dissection of specific pathways rather difficult, but might explain the incomplete inhibition observed when individual metabolic steps are blocked (37).

Both positive and negative regulation of plant defense responses operating downstream of the generation of NO and ROS have also been attributed to mitogen-activated protein kinase (MAPK) cascades. A MAPK was recently found to be activated by NO in *Arabidopsis* (17), but its role in the induction of defense genes has not yet been investigated. However, research performed during the past few years has revealed that at least two MAPKs function as early positive regulators in plant defense signaling (41). The tobacco SIPK (SA-induced protein kinase) and WIPK (wounding-induced protein kinase) are activated upon infection, treatment with elicitors, and in response to other types of abiotic stress; homologues have also been shown to function somewhat differently in diverse species (*Arabidopsis*, parsley, alfalfa). SIPK is typically induced by SA and by H$_2$O$_2$ (42) and shows SA-mediated NO inducibility (43), while WIPK is not induced by either SA or NO. The NtMEK2 kinase represents an upstream link between SIPK and WIPK as it can specifically activate both kinases (42). A number of other kinases, and kinase kinases, are being identified that might constitute a complex signaling network leading to resistance, which may at least partially overlap other responses to a number of different stresses (44).

**NO and Systemic Acquired Resistance**

NO was also shown to induce accumulation of SA and its conjugates (18), although the underlying mechanisms have not been fully elucidated. SA is required in incompatible interactions for the amplification of early signal(s) deriving from plant-pathogen recognition, resulting in the stimulation of an oxidative burst, defense gene expression, and hypersensitive cell death (5). It has been shown that in tobacco, NO-donors reduce the size of lesions caused by TMV on both treated and non-treated leaves (45). Furthermore, treatment of tobacco plant with either NOS inhibitors or NO scavengers attenuated, but did not abolish SA-induced systemic acquired resistance (45). Experiments with transgenic tobacco plants *NahG*, which are unable to accumulate SA, show that NO had no effect on lesion size following TMV infection (45). From these results, it is likely that NO plays an important role in the induction of SAR signaling pathway(s) in tobacco, although its activity is fully dependent on the function of SA.

The establishment of SAR involves the existence of a putative systemic signal that migrates from infected to systemic, non-infected leaves. Compelling evidence indicates that SA, although necessary both for local resistance and for SAR induction, is not the long-distance signal molecule that triggers systemic resistance (46). In mammals, NO circulates in the blood as either a S-nitroso protein adduct or as low molecular weight S-nitroso thiols such as nitroso glutathione (GSNO). This molecule, believed to act as both an intra- and intercellular NO carrier, is a powerful inducer of plant defense
genes (18). Since glutathione is a major metabolite in the phloem, where the SAR signal is transmitted, it can be hypothesized that excess NO produced during the HR binds to glutathione; in this form, it may act as a long distance SAR signal (47). Recently a GSNO-catabolizing enzyme and its encoding gene (GS-FDH) have been characterized, and a mutant yeast affected in gene functionality show enhanced susceptibility to nitrosative stress (48). This gene has also been identified in pea and Arabidopsis (49, 50), suggesting that plants may be able to modulate the bioactivity and signaling function of this stabilized form of NO.

CONCLUSIONS

Reminiscent of the mammal immune system, in which ROS function together with NO in macrophage killing of bacteria, the plant hypersensitive disease resistance response is characterized by the rapid accumulation of ROS and NO through the activation of enzyme systems similar to neutrophil NADPH oxidase and NO synthase. This defense mechanism induces hypersensitive cell death, triggers the expression of resistance genes and mediates a systemic signal network involved in the establishment of plant immunity. NO signaling functions depend on its reactivity and ROS are key modulators of NO in triggering cell death, although through mechanisms different from those commonly observed in animals. The recent identification of a plant NOS will soon pave the way towards the characterization and manipulation of mechanisms modulating NO signaling. Thus, the understanding of NO signaling functions at the biochemical, cellular and molecular levels will soon make it possible to discern several important physiological and pathological processes in plants, as has already been demonstrated in mammals.

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