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Forum Review

The Functions of Nitric Oxide-Mediated Signaling and Changes in Gene Expression During the Hypersensitive Response

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ABSTRACT

Nitric oxide (NO) is a highly reactive molecule that rapidly diffuses and permeates cell membranes. In animals, NO is implicated in a number of diverse physiological processes, such as neurotransmission, vascular smooth muscle relaxation, and platelet inhibition. It may have beneficial effects, e.g., as a messenger in immune responses, but it is also potentially toxic when the antioxidant system is overwhelmed and reactive oxygen intermediates (ROI) accumulate. During the last few years, NO has been detected in several plant species, and an increasing number of reports on its function have implicated NO as an important effector in plant growth, development, and defense. The broad chemistry of NO involves an array of interrelated redox forms with different chemical reactivities and numerous potential biological targets in plants. NO signaling functions depend on its reactivity. ROI are key modulators of NO in triggering cell death, but the nature of the mechanisms by which this occurs in plants is different from those commonly observed in animals. This review focuses on the signaling functions of NO, when channeled through the cell death pathway by ROI. *Antioxid. Redox Signal.* 5, 33–41.

BACKGROUND

Nitric oxide (NO), produced by mammalian cells, is implicated in a large number of diverse physiological processes such as neurotransmission, vascular smooth muscle relaxation, and regulation of vasoprotection, including inhibition of platelets, leukocyte adhesion, and mast cell degranulation (75). Macrophages and other circulating cells also produce NO, which mediates their bactericidal and tumoricidal effects (78). However, excessive and unregulated NO production contributes to adverse pathophysiological conditions, including several lethal and debilitating diseases, such as immune-type diabetes, multiple sclerosis, and neurodegeneration. In contrast to conventional signaling molecules, which act by binding to specific receptor molecules, NO manifests its biological actions via a wide range of chemical reactions (37). Its broad chemistry involves an interplay between three species differing in physical properties and chemical reactivity: the nitrosonium cation (NO^+), NO, and the nitroxyl anion (NO^-) (78). Due to the diverse chemical reactivity of these NO species, there are many potential targets for NO action. Similar to reactive oxygen intermediates (ROI), NO species are derived from the interaction of free radicals to form more persistent species that have multiple biological effects. NO plays a dual role in plant biology and may be beneficial in modulating a number of signaling pathways, but it is also potentially cytotoxic when ROI accumulate (75).

The presence of NO in plants has been known for some time (82). Recent studies have implicated NO as an important effector of plant growth, development, and defense responses. Despite the increasing number of reports, current understanding remains limited by the lack of experiments based on genetic manipulation of NO levels in plants. Most information to date is based on data obtained by exogenous application of NO scavengers and NO donors. Although some compounds

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release NO, others either are iron-nitrosyl substances with strong NO^+ character, or release equimolar amounts of NO and superoxide anion (O_2^-) and should therefore be considered as sources of peroxynitrite ($ONOO^-$). Data obtained using these compounds become even more difficult to interpret considering that unexpected players can appear on the already crowded field of redox signals. For example, under appropriate conditions, some molecules can cleave nitrosylating compounds such as *S*-nitrosoglutathione to produce NO and O_2^-. Furthermore, most of the data on the role of NO in plants have been obtained using NO donors in the presence of variable (often unknown) amounts of ROI. NO signaling functions fully depend on its reactivity (6), with ROI being the key mediator of NO function in triggering cell death (26). This discussion will not cover the modulatory role of NO in plant physiology as occurs, for example, during plant growth and development (for a review, see 7). Rather it focuses on our current understanding of NO signaling functions when ROI channel NO through the cell death pathway, as demonstrated during the hypersensitive resistance response to pathogens.

PRODUCTION OF NO DURING THE HYPERSENSITIVE RESPONSE

The innate immune systems of organisms as diverse as vertebrates, invertebrates, and plants show several similar characteristics with regards to the involvement of NO (60). In the mammalian immune system, NO cooperates with ROI to induce apoptosis of tumor cells and macrophage killing of bacteria (62, 75). In plants, a similar mechanism has evolved to prevent tissue invasion by pathogens (25).

A widespread feature of this disease resistance is the hypersensitive response (HR), characterized by a rapid, localized cell death at the infection site that contributes to limit the growth and spread of the invading pathogen. HR is an example of programmed plant cell death (40). One of the earliest events in HR is the rapid accumulation of ROI through the activation of an enzyme system similar to neutrophil NADPH oxidase (46, 50). Activation of the oxidative burst is a central component of a highly amplified and integrated signaling system that also involves salicylic acid (SA) and perturbations of cytosolic Ca^{2+}. HR is responsible for the activation of mechanisms involved in the establishment of plant immunity. Hydrogen peroxide (H_2O_2) is also required for localized host cell death, but alone it is not sufficient to trigger an efficient response (25). Several lines of evidence now indicate that NO synthase (NOS), a key enzyme involved in mammalian macrophage action, is probably conserved in plants and might be activated during plant–pathogen interactions (65). Tobacco mosaic virus infection of resistant, but not susceptible, tobacco results in elevated NOS activity (30). Soybean and *Arabidopsis* cell suspensions inoculated with *Pseudomonas syringae* PV produce NO with a pattern similar to H_2O_2 accumulation. In this case, an initial rapid, but transient, stimulation of NO accumulation is induced by both avirulent and virulent *Pseudomonas syringae* PV strains. This is followed by sustained production of NO, however, only in the cells inoculated with the avirulent strain. This is concomitant with an avirulence gene-dependent oxidative burst that precedes the onset of hypersensitive cell death (19, 25).

The induction of hypersensitive cell death by avirulent *Pseudomonas syringae* strains is prevented either by scavenging of endogenous NO with carboxy-PTIO or by blocking NOS with the competitive inhibitor N^{ω}-nitro-L-arginine (L-NNA) in soybean suspension cells (25). Coinfiltration of *Arabidopsis* promotes the development of the spreading chlorosis observed with the isogenic virulent bacteria. Moreover, the development of disease symptoms in leaves with blocked NO accumulation is accompanied by enhanced bacterial growth, although at lower levels than observed with the isogenic virulent strain (25).

Treatment with the NOS inhibitors L-NNA or N^{ω}-monomethyl-L-arginine (L-NAME) at concentrations up to 1 m*M*, however, did not reduce the generation of NO in *Arabidopsis* cell suspensions following bacterial challenge (19). It is worth noting that L-NNA and L-NAME are competitive inhibitors of NOS and, hence, their effects depend on the concentration of free arginine. We have found that above a certain threshold, L-NNA alone causes cell death in soybean cell suspensions, whereas at concentrations below this threshold, L-NNA prevents HR cell death triggered by avirulent pathogens (Fig. 1). Careful analysis of the dual effect of L-NNA showed that the threshold values for L-NNA vary among different cell preparations, but intrabatch suspensions showed no variations. This suggests that even slight differences in growth that are frequently observed between different preparations of cells in suspension may cause wide variations in the free concentrations of intracellular arginine. The rate of synthesis of arginine is limited by the availability of mitochondrial ATP and ammonium. As cultured cells in suspension normally grow in ammonium-limiting conditions, well grown batches of cells may exhibit ammonium deficiency or limitation (58). This trend remains consistent despite the fact that data on the percentage of dead cells with respect to L-NNA concentration vary among different preparations. L-NNA always inhibits HR-induced cell death at concentrations immediately below the threshold. In *Arabidopsis* leaves, challenged with avirulent *P. syringae* PV, 200 µ*M* L-NNA consistently blocks HR formation, suggesting that the concentration of arginine in plants is more stable than that cell suspensions.

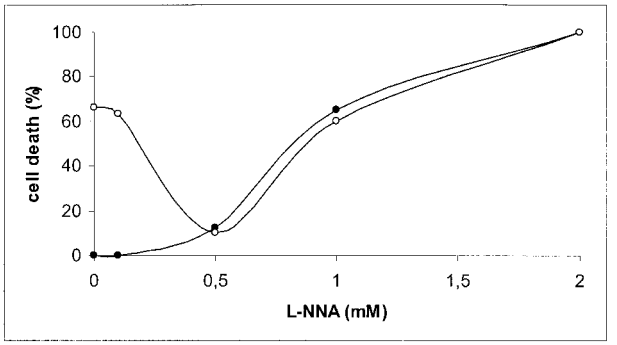

FIG. 1. Cell death in soybean suspension cultures. Filled circles: cells are treated with the NOS inhibitor L-NNA. Open circles: cells are treated with L-NNA in the presence of 10^8 *Pseudomonas syringae* pv. *glycinea* carrying the avirulence gene *avrA*.

It is of interest to note that a complementary DNA sequence with high homology to PIN, a protein inhibitor of NOS, has been identified in plants (44). Moreover, NOS activity has been detected in a number of plant tissues (7). However, analysis of the complete sequence of the *Arabidopsis* genome has not revealed the presence of putative NOS genes (22). NOS are bimodal enzymes with C-terminal domains that have high homology with many plant cytochrome P450 reductases. Consequently, several anti-NOS antibodies may cross-react with some oxidoreductases (7). Recently, an NADPH- and Ca^{2+}-dependent NOS activity was detected in pea peroxisomes. Moreover, an immunoreactive protein of 130 kDa was identified from the same material using a polyclonal antibody against 14 residues from the C-terminus of inducible NOS that are absent in the NADPH-cytochrome P450 reductases (4). A partial clone of a putative plant NOS has been isolated from a pea cDNA library (22), but the identity of this gene remains to be verified. The clear demonstration of a plant NOS is essential. Only then will the opportune manipulation of NO accumulation in plants be possible at the genetic level. This is necessary to eliminate unpredictable side reactions and other effects associated with the use of pharmacological manipulation of NO levels, thereby permitting a more precise characterization of NO and NOS function in plants. Indeed, there are other potential sources of NO in plants. For example, NO may be generated nonenzymatically via the conversion of NO_2 by carotenoids, or enzymatically via nitrate reductase (81). The physiological implications of NO production by these sources remain under extensive investigation (84).

NO AND CELL DEATH

The role of NO in the activation of mechanisms leading to hypersensitive cell death has been characterized by the investigation of the effects of treatment of cultured soybean cells with the NO donor sodium nitroprusside (SNP). This compound potentiates the induction of cell death by exogenous H_2O_2 in a synergistic manner and causes an almost 10-fold increase in ROI-induced cell death (25). NO also potentiates cell death induced by endogenous ROI. The addition of the protein phosphatase type 2A inhibitor, cantharidin, and SA gives rise to a massive oxidative burst resulting in the accumulation of >30 μ*M* H_2O_2. Although this causes a relatively weak induction of cell death, the response is markedly enhanced by the simultaneous addition of NO generators (25). The NO donors SNP and Roussin's black salt induce death in *Arabidopsis* cells when present at concentrations that release NO in amounts similar to those generated by cells challenged by avirulent bacteria (19). NO-induced cell death is inhibited by cordycepin or cycloheximide, indicating that RNA processing and protein synthesis are necessary for cell death. Experiments with Ac-YVAD-CMK, an irreversible inhibitor of mammalian caspase-1, also suggest that NO triggers a form of programmed cell death (19). Furthermore, NO-induced cell death requires the synthesis of cyclic GMP (cGMP), a well known second messenger of NO, in animal systems (27). cGMP is produced by guanylate cyclase, which is activated either by NO binding to the heme-iron or through *S*-nitrosylation at critical cysteine residues (77). This second messenger can both directly and indirectly regulate different physiological functions of animal cells by activation of different types of calcium channels (81). One such example of this type of action in plants might be the observed requirement for a nucleotide-gated channel (AtCNGC2) in the activation of cell death responses in *Arabidopsis*. *Dnd1* mutants, which are defective in AtCNGC2, fail to develop HR, although resistance is unaffected (20, 52).

In animals, activated macrophages produce high levels of NO and of O_2^- (63). The toxicity of these compounds occurs through their diffusion-limited reaction that generates $ONOO^-$, which subsequently interacts with many cellular components (48) and may modulate the signaling functions of NO (5). Although $ONOO^-$ is relatively unreactive, its conjugate acid, peroxynitrous acid, is considerably versatile and able to perform both one- and two-electron oxidations of a variety of molecules (amines, thiols, some nitrogenous bases, ascorbate, etc.), thus damaging lipids and denaturing DNA and proteins (71, 78). The mechanisms of these oxidative reactions are not completely understood, but at least some are mediated by formation of tyrosyl radicals (3). Urate, a $ONOO^-$ scavenger that efficiently reduces the radicals centered on tyrosine residues (69), was shown to greatly reduce lesion formation in *Arabidopsis* leaves treated with an abiotic $ONOO^-$-generating system or with a $ONOO^-$ solution. However, it did not exert any protective effect against damage originating from NO plus H_2O_2 (1). This indicates that $ONOO^-$ is not an essential intermediate in cell death cooperatively induced by NO and H_2O_2. Exposure of animal cells to $ONOO^-$ produces concentration-dependent cell death in the range from 1 to 1,000 μ*M* (21, 34, 55). 3-Morpholinosydnonimine *N*-ethylcarbamide (SIN-1) is an NO donor that provides a continuous source of $ONOO^-$ because it gradually decomposes to yield equimolar amounts of NO and O_2^-. The viability of soybean suspensions remains unaltered following exposure to SIN-1 or to commercial $ONOO^-$ at concentrations of 1 m*M* and 5 m*M*, respectively. Nonetheless, SIN-1 induces the accumulation of the transcripts encoding pathogenesis-related 1 (PR-1) in tobacco leaves (31), and $ONOO^-$ induces protein nitration in soybean and *Arabidopsis,* leading to changes in cellular redox state (26). Therefore, although $ONOO^-$ is not an essential intermediate of NO-induced cell death, it is expected to have important physiological and signaling functions in plants.

In animals, the reaction between NO and H_2O_2 does not appear to be directly involved in cell killing. However, NO has been shown to cooperate with H_2O_2 to induce DNA fragmentation and cell lysis in murine lymphoma cells, hepatoma cells, and endothelial cells (32, 33), although the molecular mechanisms of this interaction are not clearly understood. *In vitro* studies have suggested that reaction of NO with H_2O_2 produces hydroxyl radicals in both the gaseous and liquid phases (45). Furthermore, it has been demonstrated that singlet oxygen, another highly reactive form of oxygen with cytotoxic potential (66), is produced. Alternatively, the toxicity of NO/H_2O_2 may be due to the production of a potent oxidant formed by a process dependent on a trace metal, H_2O_2, and NO (32).

NO AND IRON HOMEOSTASIS

The cellular modulation of free iron levels not only is a housekeeping function, but also has been proposed as a possible way to change the levels of reactive molecules involved in the defense response (47). Recent findings, discussed below, suggest that NO regulates both these events.

When iron is in a free uncomplexed form, *e.g.,* in heme or [Fe-S] clusters, it acts as a prooxidant to produce hydroxyl radicals from the less harmful H_2O_2 and O_2^- (during the Haber–Weiss reaction) (10). Ferritins are iron-storage proteins that allow cells to accumulate iron in a safe, biologically useful form. Their regulation has become a model for iron-regulated gene expression in both animals and plants (13, 39, 70). In animals, the synthesis of ferritin is mainly regulated at the translational level in response to iron (15). Animal ferritin mRNA contains a so-called iron responsive element (IRE) in its 5′ untranslated region that forms a stem-loop structure to which *trans*-acting iron regulatory proteins (IRPs) can bind (14). The IRE/IRP complex represses the translation of ferritin mRNA and stabilizes the transferrin RNA transcript (42). Aminolevulinate synthase and mitochondrial aconitase are also under translational control mediated by the IRE/IRP complex, which is thus coupled to iron availability (23, 74). To date, two different IRPs have been identified. IRP-1 is a cytosolic aconitase with a [4Fe-4S] cluster that disassembles when iron is deficient, switching the apoprotein to IRP activity (42). IRP-2 shares a high homology with IRP-1, but possesses neither a [4Fe-4S] cluster nor aconitase activity (41). Mammalian cytoplasmic and mitochondrial aconitases are both sensitive to inactivation by NO, but only cytoplasmic aconitase serves as an mRNA-binding regulator of ferritin translation when NO causes the disassembly of the [4Fe-4S] cluster (35). Recent findings show that NO, in the form of NO^+, causes a specific down-regulation of IRP-2 activity (8). In macrophage cytosolic extracts exposed to NO in the form of $ONOO^-$, IRP-1 loses its aconitase activity without increasing its capacity to bind the IRE. Nevertheless, $ONOO^-$ predisposes IRP-1 to bind IREs under a suitable reducing environment (9). Therefore, a number of uncertainties remain concerning the effect of NO on IRPs, partly because the reactivity of NO also depends on the redox state of the environment in which it is generated.

Iron is a strong inducer of ferritin accumulation in all plant systems tested (36, 53, 56, 57). A common scheme of iron regulation of ferritin expression in plant and animal cells is now emerging, although the targets appear to be different: DNA in plants and mRNA in animals. The mechanism of iron-dependent regulation of plant ferritin is based on gene derepression and is analogous to the IRP/IRE-mediated derepression of ferritin mRNA in animal cells. In fact, in soybean and *Arabidopsis*, the promoters of the ferritin genes contain FRE and IDRS sequences, respectively, which are responsible for transcriptional repression under low iron supply (68, 80). Indirect evidence suggests that NO is a possible regulator of iron homeostasis in plants as has been shown in animals. The NO donors NOC-9 [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)*N*-methyl-1-hexanamine] and SIN-1 inhibit cytosolic aconitase from tobacco (64); therefore, NO could play a role in the conversion of cytoplasmic aconitase into a functional IRP and/or may destabilize the [4Fe-4S] clusters. The consequent increase of free iron levels would contribute to the oxidative stress in the HR. However, even though plant aconitases show high homology especially in the IRP region (64) with mammalian aconitases, no IREs are present in plant ferritin genes and, to date, no IRP activity has been detected in plant cells. Thus, a direct involvement of aconitase in the regulation of plant ferritin still awaits conclusive demonstration.

To determine the contribution of NO in the regulation of ferritin levels, we have analyzed the effect of the NO donor SNP on the accumulation of ferritin transcript in *Arabidopsis* cells treated with iron, a strong inducer of ferritin transcript and protein accumulation. We found that SNP was unable to prevent accumulation of ferritin transcript upon iron overload. Furthermore, SNP alone caused a massive accumulation of ferritin transcripts within 3 h, and the NO scavenger carboxy-PTIO prevented accumulation of ferritin transcripts by iron overload. These results indicate that NO acts downstream of iron in the signaling cascade, leading to ferritin accumulation (Murgia, Delledonne, and Soave, unpublished observations). Although surprising, these results are supported by the findings that nitrate reductase can produce NO (85) and that ferritin accumulates in the nodules of nitrate-fed, dark-stressed bean and pea plants (59, 72).

We propose a dual function of NO in modulating free iron levels in plant cells. NO is a physiological regulator of ferritin levels, acting downstream of iron itself. The strict control of the free iron pool by the iron-storage protein ferritin prevents both iron toxicity and iron deficiency, which are equally deleterious to cell survival (38). On the other hand, NO inhibits cytoplasmic tobacco aconitase. This gives rise to the possibility that NO detaches iron directly from the [Fe-S] cluster of aconitase, thus increasing the pool of free iron and the formation of toxic hydroxyl radicals when ROI accumulate for killing. This might occur, for example, during the HR.

NO may also detach iron from other Fe-proteins, but not from the ferritin reservoir itself, as ferritin is absent in mature, unstressed leaves. The timing of these events also supports our hypothesis. After treatment with iron, ferritin transcripts can be detected within 3 h, whereas the protein is detectable after 9–12 h, reaching a peak several hours later (36, 57). Accordingly, NO stimulation of ferritin mRNA synthesis is a rapid event, whereas accumulation of the ferritin protein occurs much later and may reach a maximum at 24 h after SNP treatment (Murgia, Delledonne, and Soave, unpublished observations). Thus, ferritin cannot directly contribute to either the potentiation or the reduction of oxidative stress during the onset of the HR. In fact, the protein would begin to accumulate when the oxidative burst has already finished and the cell death program has already reached the point of no return. Nevertheless, once the oxidative burst has finished, ferritin could protect the surrounding cells from further oxidative damage (24, 61). The decrease of aconitase activity in tobacco leaf extracts to <20% within 1 h following treatment with the NO donor NOC-9 (64) indicates that the effect of NO on the [Fe-S] cluster is indeed very rapid. The measurements of changes in free iron levels in cells undergoing HR, as well as a more detailed investigation of the regulation of ferritin levels upon pathogen attack in different plant sys-

tems, should help to clarify the role of NO with respect to the control of free iron levels.

NO AND ACTIVATION OF GENES INVOLVED IN DEFENSE RESPONSES

NO functions together with ROI in triggering hypersensitive cell death, but it is also involved in other defense functions complementary to and independent of ROI. H_2O_2 drives oxidative cross-linking of tyrosine-rich structural proteins of the cell wall (12) and induces genes having a role in cellular protection, including glutathione *S*-transferase (*gst*) and glutathione peroxidase (*gpx*) (50) (Fig. 2). However, at least in soybean cells, ROI are not the primary signal for rapid induction of defense genes, such as those encoding enzymes involved in the synthesis of phenylpropanoids, phyodexins, lignin, and SA (54). Inhibition of NOS activity with L-NNA has little effect on the initial induction of *gst* in response to avirulent *Pseudomonas*. In contrast, the accumulation of transcripts encoding phenylalanine ammonia lyase (PAL), the first enzyme of the phenylpropanoid biosynthesis pathway, and chalcone synthase (CHS), the first enzyme of the branch specific for flavonoids and isoflavonoid-derived phyodexins, is markedly reduced (25, 28).

The role of NO in the induction of genes involved in cellular defense is further supported by experiments in tobacco (30). Infiltration of tobacco leaves with mammalian NOS results in a significant accumulation of PAL and of PR-1 protein, a well defined marker of plant disease resistance, whose expression during the defense response generally lags behind that of PAL. The same pattern of accumulation has been detected in tobacco cell suspensions treated with either NO donors or a membrane-permeable analogue of cGMP. NO induction of PAL in tobacco suspension cells can be suppressed by several inhibitors of guanylate cyclase, suggesting the involvement of cGMP-dependent components in NO-dependent defense gene activation (30). 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 (11), 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 in NO signaling in animals, acting in a cGMP-dependent signaling cascade to mediate calcium mobilization (27). Treatment with cADPR induces PAL and PR-1 expression in tobacco (30), which can be inhibited by ruthenium red, indicating their calcium dependence. Moreover, a cADPR antagonist suppressed NO induction of PR-1; however, this effect was incomplete, suggesting that NO activation of defense responses may occur through more than one pathway (47). In addition to the activation of cGMP-dependent signaling cascades, nitrosylation of key components of cell metabolism is another mode of NO signaling operating in animals (79) that may have parallels in plants. Strong evidence indicates that calcium release can also be regulated by channel *S*-nitrosylation or oxidation (2, 83). The existence of multiple mechanisms of NO action makes the dissection of specific pathways rather difficult and might ex-

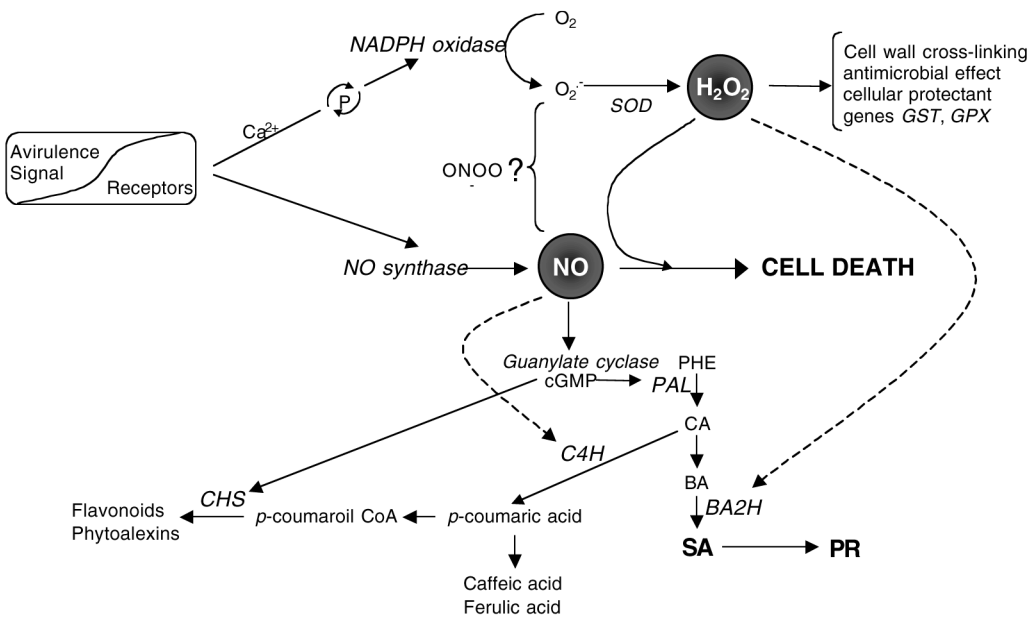

FIG. 2. Model of action of NO and H_2O_2 in the hypersensitive response. Abbreviations are as follows. BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; CA, cinnamic acid; Ca^{2+}, calcium influx; cGMP, cyclic GMP; CHS, chalcone synthase; C4H, cinnamic acid 4-hydroxylase; GPX, glutathione peroxidases; GST, glutathione *S*-transferases; H_2O_2, hydrogen peroxide; NO, nitric oxide; $ONOO^-$, peroxynitrite; P, phosphorylation-dependent step; PAL, phenylalanine ammonia lyase; PHE, phenylalanine; PR, pathogenesis-related proteins; SA, salicylic acid; SOD, superoxide dismutase.

plain the incomplete inhibition observed when individual metabolic steps are blocked (47).

NO is known to induce SA accumulation (30), although the underlying mechanisms of this induction have not been fully elucidated. SA is required for the amplification of early signal(s) derived from plant-pathogen recognition in incompatible interactions. SA accumulation stimulates the oxidative burst, enhances defense gene expression, and leads to hypersensitive cell death (76). A large body of evidence now indicates that SA is produced from the shikimate-phenylpropanoid pathway. In this pathway, PAL catalyzes the conversion of phenylalanine to cinnamic acid. Two possible routes that differ at the step involving hydroxylation of the aromatic ring (73) convert *trans*-cinnamic acid, the first important precursor of SA biosynthesis (18), to benzoic acid (BA). The conversion of BA to SA is catalyzed by a benzoic acid 2-hydroxylase (BA2H). Whereas NO stimulates PAL accumulation in tobacco, H_2O_2 activates BA2H activity *in vivo* and *in vitro,* resulting in a significant increase in SA levels (51). Furthermore, the prevention of the oxidative burst with diphenylene iodonium, a suicide inhibitor of NADPH oxidase, in elicited tobacco tissues favors BA accumulation (29) and strongly decreases SA.

We have recently found that the transcript encoding cinnamate-4-hydroxylase (C4H) accumulates following infiltration of *Arabidopsis* leaves with the NO donor SNP in cell death-inducing conditions (Polverari and Delledonne, unpublished observations). C4H belongs to the cytochrome P450 family and catalyzes the conversion of cinnamic acid to *p*-coumaric acid. It is considered a key enzyme in the synthesis of phenolic compounds related to disease resistance, such as *p*-coumaric acid, ferulic acid, caffeic acid, and several other derivatives having strong antimicrobial activity. Several hydroxycinnamic acid derivatives participate in the composition of suberized tissues, often formed around infection sites and wounds, and in the polymerization of lignins and lignans. Phenolic compounds can be further oxidized by polyphenol oxidases and peroxidases to form highly toxic quinones. Hydroxycinnamates are also known to act as peroxyl-radical scavengers and are effective in increasing the resistance of low-density lipoprotein to oxidation (17). From *p*-coumaric acid, the phenylpropanoid pathway can also lead to the production of flavonoids and isoflavonoid phytoalexins via CHS, whose transcript accumulation is also modulated by NO (25). The finding that transcription of C4H increases following SNP infiltration gives further credence to the notion that defense responses related to the phenylpropanoid pathway are induced by NO in plants.

Both positive and negative regulation of plant defense responses operating downstream of NO and ROI generation have been attributed to mitogen-activated protein kinase (MAPK) cascades. A MAPK was recently found to be activated by NO in *Arabidopsis* (19), but its role in induction of genes involved in defense has yet to be 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 (16). 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. Orthologues of these kinases have been shown to function in a similar manner in various species (*Arabidopsis*, parsley, alfalfa). SA induces SIPK by H_2O_2 (86) and shows SA-mediated NO inducibility (49), whereas neither SA nor NO induces WIPK. The NtMEK2 kinase represents an upstream link between SIPK and WIPK as it can specifically activate both kinases (86). The action of NtMEK2 appears to be SA-independent and is not associated with H_2O_2 production. A number of recently identified other kinases and kinase kinases might act together in a complex signaling network leading to resistance, which may, at least partially, overlap other responses to a number of different stresses (67).

In addition to its involvement in HR and defense activation, NO could also play a role in pathways leading to systemic acquired resistance (SAR) (31). The establishment of SAR, an inducible plant defense response, often ensues HR and cell death responses and necrogenic-type plant infections. This response involves the existence of a systemic signal that migrates from infected to distal, noninfected leaves and requires SA. Compelling evidence indicates that SA, although necessary for both local resistance and SAR induction, is not the long-distance signal molecule that triggers systemic resistance (43). In mammals, NO circulates in the blood as *S*-nitroso adducts of proteins, or low molecular weight *S*-nitrosothiols such as nitrosoglutathione. The latter, believed to act as both an intra- and intercellular NO carrier, is a powerful inducer of plant defense genes (30). As glutathione is a major metabolite in the phloem, where the SAR signal is transmitted, it might be hypothesized that excess NO produced during the HR binds to glutathione in which form it could serve as a long-distance SAR signal (31).

In summary, although several hypotheses still await experimental demonstration, it is now clear that NO is an important component of plant defense systems. Much evidence supports the view that NO plays a key role in disease resistance responses. In addition, we are confident that an understanding of NO biosynthesis, regulation, and action will lead to new avenues of exploitation for improved resistance to a wide range of biotic and abiotic stresses.

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ABBREVIATIONS

BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; cADPR, cyclic ADP ribose; cGMP, cyclic GMP; C4H, cinnamic acid 4-hydroxylase; CHS, chalcone synthase; GPX, glutathione peroxidase; GST, glutathione *S*-transferase; H_2O_2, hydrogen peroxide; HR, hypersensitive reaction; IRE, iron-responsive element; IRP, iron regulatory protein; MAPK, mitogen-activated protein kinase; L-NAME, N^G-monomethyl-L-arginine; L-NNA, N^ω-nitro-L-arginine; NO, nitric oxide; NO^+, nitrosonium cation; NOS, nitric oxide syn-

thase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PAL, phenylalanine ammonia lyase; PR, pathogenesis-related; ROI, reactive oxygen intermediates; SA, salicylic acid; SAR, systemic acquired resistance; SIN-1, 3-morpholinosydnonimine *N*-ethylcarbamide; SIPK, salicylic acid-induced protein kinase; SNP, sodium nitroprusside; WIPK, wounding-induced protein kinase.

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