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Plant Physiol. Biochem. 40 (2002) 605–610

Plant Physiology and Biochemistry

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Review

Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response

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Received 9 January 2002; accepted 12 February 2002

Abstract

The mechanisms involved in plant defense show several similar characteristics with the innate immune systems of vertebrates and invertebrates. In animals, nitric oxide (NO) cooperates with reactive oxygen intermediates (ROI) to kill tumor cells and is also required for macrophage killing of bacteria. Such cytotoxic events occur because unregulated levels of NO determine its diffusion-limited reaction with O_2^- generating peroxynitrite ($ONOO^-$), a mediator of cellular injury in many biological systems. In soybean suspension cells, unregulated NO production during the onset of a pathogen-induced hypersensitive response (HR) is not sufficient to activate the hypersensitive cell death, which is triggered only by fine tuning the NO/ROI ratio. Furthermore, that hypersensitive cell death is activated following interaction of NO with H_2O_2, rather than O_2^-. Increasing O_2^- levels reduces NO-derived toxicity, and the addition of $ONOO^-$ to soybean suspensions does not affect cell viability. Consistently with the fact that $ONOO^-$ is not an essential mediator of NO/RO-induced cell death, during the HR superoxide dismutase (SOD) accelerates O_2^- dismutation to H_2O_2 and therefore minimizes the loss of NO by reaction with O_2^- and triggers hypersensitive cell death through the NO/H_2O_2 synergism. Consequently, the rates of production and dismutation of O_2^- generated during the oxidative burst play a crucial role in modulating NO signaling through the cell death pathway, which proceeds through mechanisms different from those commonly observed in animals.

Keywords: Cell death; Hypersensitive response; Nitric oxide; Reactive oxygen intermediates

1. Introduction

A widespread feature of plant disease resistance is the hypersensitive response (HR), which is characterized by the formation of necrotic lesions at the infection site and by the restriction of pathogen growth and spread. Following this local resistance response, tissue distal to the infection site develops a systemic acquired resistance (SAR) to secondary infection by the same or by different pathogens. Several lines of evidence suggest that the death of host cells during the HR results from the activation of suicide processes [18,20]. In fact, the HR is thought to be a form of programmed cell death, a genetically programmed process well known in animals, which is characterized by a distinct set of morphological and biochemical features [18,20,36].

Similarly to what is observed in the macrophage action during the immune response, one of the earliest events in the HR is the rapid accumulation of reactive oxygen intermediates (ROI) and nitric oxide (NO). The synthesis of these species occurs through the activation of enzyme systems similar to neutrophil NADPH oxidase [23,28] and nitric oxide synthase (NOS) [9,12]. We have previously shown that H_2O_2 is necessary to trigger localized host cell death, but is not alone sufficient for an efficient response [9]. Nitric oxide cooperates with ROI in the induction of hypersensi-

Abbreviations: DDC, sodium diethyldithiocarbamate; HR, hypersensitive reaction; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PR, pathogenesis-related; ROI, reactive oxygen intermediates; SAR, systemic acquired resistance; SIN-1, 3-morpholinosydnonimine N-ethylcarbamide; SNP, sodium nitroprusside; SOD, superoxide dismutase

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PII: S0981-9428(02)01397-9

tive cell death and functions independently of ROI in the induction of various defense genes including pathogenesis-related proteins and enzymes of the phenylpropanoid metabolism that are involved in the production of lignin, antibiotics, and the secondary signal salicylic acid [9,12].

The striking similarity between animal and plant disease response mechanisms prompted us to establish further parallels between mechanisms of action of the NO/ROI synergism. In a recent study [10], we investigated the role of different ROI in modulating NO signaling through the cell death pathway by altering the levels of NO and ROI alone and in combination. More specifically, in soybean suspension cells it was observed that only H_2O_2 increases the killing potential of NO, whereas the $ONOO^-$ generated by reaction of NO with O_2^- is not an essential mediator of NO/ROI-induced cell death. It was also demonstrated that during the HR, SOD triggers NO killing by scavenging O_2^- with subsequent H_2O_2 production, and thus minimizing NO/O_2^- interaction and maximizing H_2O_2 potentiation of NO-mediated cell death. Although O_2^- is not the ROI that synergizes with NO to promote cell death, it is the sensor molecule in triggering cell death in the *Arabidopsis lsd1* mutant [22] and functions as critical modulator of NO signaling during the HR. Thus, O_2^- can either promote the HR by providing H_2O_2, or repress the HR cell death by scavenging NO.

2. NO/ROI regulation of cell death

Nitric oxide (NO) is a near ubiquitous intracellular and intercellular signaling molecule involved in the regulation of an impressive spectrum of diverse cellular functions. The effect of NO on animal cells depends on many complex conditions, such as the rate of its production and diffusion, the concentration of ROI and the level of enzymes involved in ROI scavenging, such as SOD and catalase [37]. When unregulated NO production occurs, cell death can occur through oxidative stress, disrupted energy metabolism, DNA damage, activation of poly (ADP-ribose) polymerase, and disregulation of cytosolic calcium [32].

We have previously reported that treatment of soybean cell suspensions with 500 μM sodium nitroprusside (SNP) generates a steady state NO concentration of about 1–2 μM, which markedly augmented the induction of cell death in the presence of ROI [9]. To determine if NO alone could induce cell death, we applied SNP in concentrations ranging from 0.1 to 20 mM [10]. It was found that in the absence of ROI, neither high nor low concentrations of NO affect cell viability. In contrast, when cells were rapidly agitated (100 rpm) to trigger a mechanically induced oxidative burst [41], SNP induced cell death at concentrations as low as 0.1 mM, with a maximal effect at 0.5 mM. However, addition of higher concentrations of the NO donor reversed the response and determined a dramatic reduction of cell death which, in the presence of 5 mM SNP, was completely abolished.

To determine if the induction of the cell death program was dependent on an equilibrium between NO and ROI levels, we tested the effect of various ROI levels at both high (cell death abolished) and low (cell death-inducing conditions) NO concentrations [10]. Enhancing O_2^- production, and thus the accumulation of H_2O_2, by the synergistic action of salicylic acid and the protein phosphatase type 2A inhibitor cantharidin, strongly reactivated the cell death program in the presence of high SNP concentrations. On the other hand, this augmentation of the oxidative burst dramatically reduced the cell death induced by 0.5 mM SNP in soybean cell suspensions when agitated at 100 rpm. In this case, the activation of cell death could be rescued by increasing the concentration of SNP up to 20 mM.

Thus, not only additional ROI was required to compensate the higher NO level, but additional NO was necessary to compensate for the increased levels of ROI. It should be stressed that cell death is activated only when the NO/ROI ratio is within a limited range, and not when the levels of either NO or ROI are increased independently.

These data indicate that any increase in the level of one individual component of the NO/ROI binary system must be compensated by an increased level of the other, in order to maintain the same magnitude of cell death from a well-defined NO/ROI balance. This may explain the fact that in different experimental systems and/or in different laboratories, NO has been observed to exert both toxic and protective effects. Different agitation speeds for the suspension cultured cells or other different experimental conditions which are difficult to standardize (filtering, washing and manipulation of the cells), may all provoke oxidative stress [41] and therefore changes in the redox state of the cells. In turn, these changes would affect that NO/ROI ratio. For example, elicitors able to induce hypersensitive cell death upon infiltration into plant leaves will not activate the cell death program in cultured cells [19,34]. Additionally, avirulent *Pseudomonas syringae* triggers the oxidative burst but fails to trigger the hypersensitive cell death when soybean suspension cells are agitated at high speed (Delledonne and Lamb, unpublished).

3. Peroxynitrite is not an essential intermediate in cell death induced by NO and ROI

In many biological systems, the cytotoxic effects of NO and ROI derive from the diffusion-limited reaction of NO with O_2^- to form $ONOO^-$, which then interacts with many cellular components [13,27]. Although excessive production of $ONOO^-$ can damage normal tissue, the reactive chemistry of $ONOO^-$ can be considered beneficial when the entire organism is considered, due to its cytotoxic effects on invading pathogens [13] and tumor cells [30]. In plants, we have shown that the ROI and NO produced during the onset

of a pathogen-induced hypersensitive reaction cooperate to trigger hypersensitive cell death [9]. We therefore investigated whether this trigger occurs through mechanisms similar to those described in animals, by analyzing the effect of exogenous $ONOO^-$ on soybean suspension cultures. Whereas exposure of animal cells to $ONOO^-$ in the range 1–1000 μM causes a concentration-dependent cell death [7,16,30], exposure of soybean cell suspensions to $ONOO^-$ did not cause cell death at concentrations up to 1 mM [10].

Since at neutral pH the half-life of $ONOO^-$ is ~2 s, we investigated the effects of $ONOO^-$ when cell suspensions were exposed for a prolonged period. SIN-1 is a NO donor which provides a convenient source of $ONOO^-$ as it gradually decomposes to yield equimolar amounts of NO and O_2^-. Treatments of soybean cell suspensions with SIN-1 failed to reduce cell viability at concentrations up to 5 mM. This is in contrast to mammalian cells, where this compound has been shown to induce concentration-dependent cell death [3,16,30]. It is worth noting that SIN-1 induces accumulation of a transcript encoding PR-1 in tobacco leaves [12], while $ONOO^-$ induces protein nitration in soybean cell suspensions, leading to changes in the redox state of the cell [10]. $ONOO^-$ is thus expected to have physiological functions in plant. Obviously, these findings were unexpected if compared with the key role that $ONOO^-$ plays in the mammalian immune system.

The observation that hypersensitive cell death is activated following interaction of NO with H_2O_2 rather than O_2^- has also been reported in other systems. Indeed, although in animals the reaction of NO with H_2O_2 does not appear to be directly involved in killing, 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 [14,15]. However, the molecular mechanisms of this interaction are not clearly understood. In vitro studies have suggested that a reaction between gaseous NO and H_2O_2 produces singlet oxygen or hydroxyl radicals [33]. Alternatively, the toxicity of NO/H_2O_2 may be due to the production of a potent oxidant formed via a trace metal-, H_2O_2- and NO-dependent process [14]. The iron liberated from ferritin by NO may in fact significantly contribute to elevate oxidative stress caused by H_2O_2 [32].

4. SOD function in the induction of hypersensitive cell death

The lack of NO/O_2^- synergism to trigger NO-mediated cell death in plants prompted us to investigate the role of H_2O_2 by monitoring NO killing in the absence of H_2O_2 formation [10]. O_2^- appears to be an important initial product of the pathogen-induced oxidative burst in plant cells [28]. H_2O_2 can then be formed non-enzymatically by dismutation of O_2^- [17] or enzymatically by the action of SOD, an enzyme family with disparate regulation and protein localization [4]. The balance between O_2^- and H_2O_2 depends on the rate of production of O_2^- and efficiency of its dismutation. It is worth noting that the rate of reaction of NO with O_2^- is approximately three times faster than the reaction of O_2^- with SOD [21]. Submicromolar concentrations of NO can compete with endogenous SOD for O_2^- by a rapid reaction that generates $ONOO^-$. This may obviously occur when O_2^- and NO are produced simultaneously [32]. SOD, in order to be effective, must be at a concentration comparable to or higher than NO and close to the site of NO/O_2^- generation [8].

Cu,Zn-SOD is among the key cellular enzymes by which neurons and other cells protect themselves from NO-mediated damage [38]. In macrophages undergoing apoptotic cell death, Cu,Zn-SOD is down-regulated both at the transcriptional and the translational level [5] indicating that the cell death program can be switched on or off by modulating SOD activity. Indeed, down-regulation of Cu,Zn-SOD in PC12 cells leads to their death via the $ONOO^-$ pathway [38], whereas its overexpression protects RAW 264.7 macrophages against NO cytotoxicity [5]. The Cu,Zn-SOD inhibitor sodium diethyldithiocarbamate (DDC) has been shown to block H_2O_2 accumulation in *Arabidopsis thaliana* cells undergoing an oxidative burst following harpin treatment. This leads to an increased concentration of O_2^- and abolishes hypersensitive cell death [1, 11]. The addition of DDC to soybean cell suspensions agitated at high speed (100 rpm) blocked NO-induced, ROI-dependent cell death and abrogated both H_2O_2 accumulation and cell death in soybean suspension cells challenged with avirulent *Pseudomonas syringae* [10]. This cell death could be rescued by the addition of sublethal amounts of H_2O_2. However, the augmentation of the endogenous oxidative burst with salicylic acid and cantharidin had a very limited effect, which is consistent with the ineffectiveness of O_2^- in triggering NO-induced cell death. Thus, it can be concluded that in soybean cells, the H_2O_2 accumulation detected during the pathogen-induced oxidative burst derives from the SOD-catalyzed dismutation of O_2^-. Moreover, this demonstrates that H_2O_2, and not O_2^-, is the key effector in ROI-mediated cell death.

It has been extensively demonstrated that pathogens induce changes in the antioxidant status of plant cells [39]. The amount of SOD enzymes increases in tobacco infected with TMV during the expression of the hypersensitivity [31] and in *Phaseolus vulgaris* a Cu,Zn-SOD activity increases in the hypersensitive response of resistant leaves [6]. Soybean suspension cultured cells possess an elevated SOD activity (Fig. 1), which may serve to limit the oxidative stress caused by the continuous agitation [41]. A partial purification of these enzymes, causing the inactivation of the Fe- and Mn-SOD isoforms, however demonstrated that Cu,Zn-SOD could account for most of this activity. Moreover, the dramatic reduction of total SOD activity in DDC-treated cells reflected the complete inhibition of the Cu,Zn-SOD isoform. As O_2^- from the oxidative burst is believed to be extracellular, we expected that extracellular

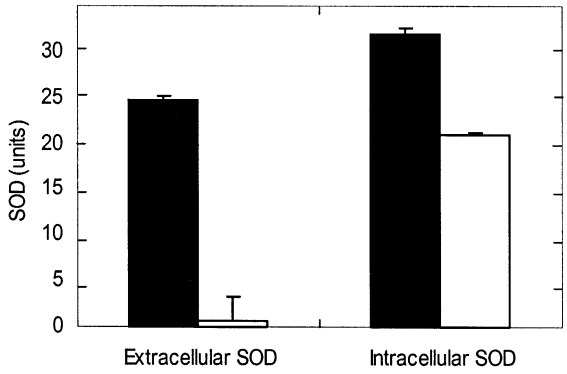

Fig. 1. SOD activity in soybean suspension cultured cells. Black bars, total SOD; white bars, Cu,Zn-SOD. Extracellular SOD activity is expressed as SOD units ml^{-1} of soybean growing liquid medium 3 d after subculture. Intracellular SOD activity is expressed as SOD units mg^{-1} total proteins. SOD activity was measured as described in [10].

Cu,Zn-SOD would be involved in the accumulation of H_2O_2. Analysis of SOD activity in soybean media indicated the presence of at least one extracellular form of SOD, which however is not a Cu,Zn-SOD isoform (Fig. 1). Although total and Cu,Zn-SOD activities remained constant during the hypersensitive response, analysis of Cu,Zn-SOD transcript accumulation showed a strong and sustained induction within 1 h of the addition of the avirulent pathogen. This suggests that protein turnover might play a role in the response of Cu,Zn-SOD to activated oxygen or nitrogen species [40].

The recent finding that LSD1, a negative regulator of cell death, regulates salicylic acid induction of Cu,Zn-SOD [25] by monitoring an O_2^- dependent signal [22], further supports the proposed role for SOD as a modulator of O_2^- function. Indeed, the spreading lesion phenotype of lsd1 mutants is correlated with a lack of up-regulation of Cu,Zn-SOD that appears to be responsible for detoxification of accumulating O_2^- before lsd1 can trigger cell death [25].

5. Balance model

Herein, we propose a model (Fig. 2) in which if the NO/O_2^- balance is in favor of O_2^-, NO is scavenged before it can react with H_2O_2. If the balance is in favor of NO, O_2^- is scavenged before it is dismutated to H_2O_2. Scavenging NO with O_2^- or scavenging O_2^- with NO leads to the formation of $ONOO^-$, which is not an essential intermediate of NO-mediated cell death. The observation that cell death during the HR is under control of the ratio of NO/ROI, and is not dictated by changes in the concentration of one of the two components is physiologically relevant. The formation of ROI is an inevitable event in normal cell metabolism [35] and an excess of ROI accumulate during exposure to various stresses [26]. Nitric oxide concentration is also subject to significant variations. The emission of NO from plants occurs under stress situations, such as herbicide treatment or pathogen attack, as well as under normal growth conditions. In most cases, NO production in plant tissues has been linked to the accumulation of NO_2 [24]. Nitric oxide can be produced from NO_2 either non-enzymatically through light-mediated conversion by carotenoids or enzymatically through NADPH nitrate reductases [24]. Recent studies have shown that plants produce NO from L-arginine in a reaction catalyzed by NO synthases [2]. In fact, there is increasing evidence that NOS-dependent NO production is involved in diverse physiological plant processes like growth and development, in addition to the hypersensitive disease resistance response [29]. Based on these observations, it may be hypothesized that $ONOO^-$

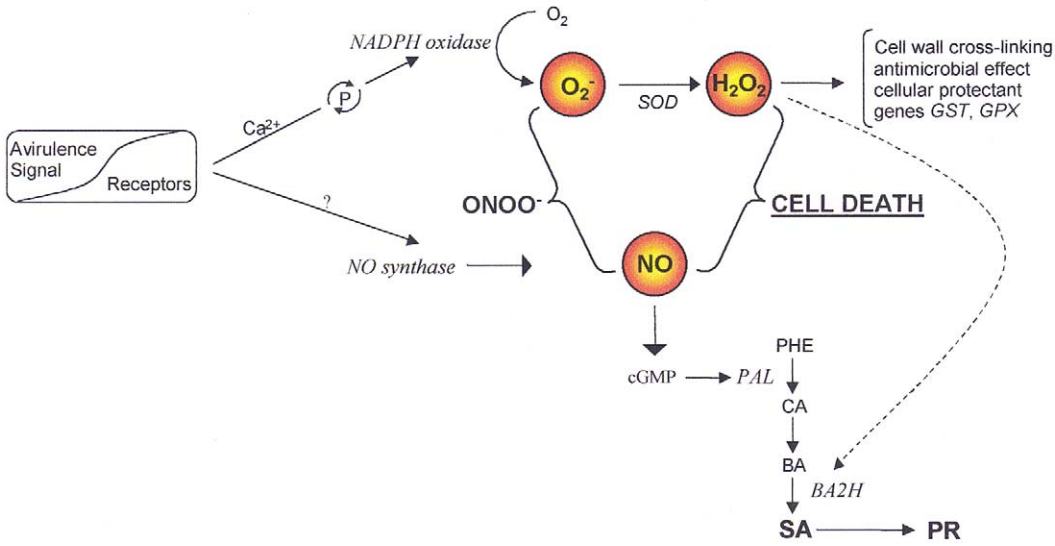

Fig. 2. Model of action of NO and H_2O_2 in the hypersensitive response. Ca^{2+}, Ca^{2+} influx; P, phosphorylation dependent step; H_2O_2, hydrogen peroxide; NO, nitric oxide; $ONOO^-$, peroxynitrite; GST, glutathione S-transferases; GPX, glutathione peroxidases; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; BA2H, benzoic acid 2-hydroxylase; PHE, phenylalanine; CA, cinnamic acid; BA, benzoic acid; SA, salicylic acid; PR, pathogenesis-related proteins.

is continuously formed in healthy cells. Consequently, plant cells may have developed specific mechanisms to overcome the toxicity of $ONOO^-$, and may have adopted different NO/ROI signals for triggering cell death during the hypersensitive response.

6. Conclusions

The HR appears to be tightly regulated by the rapid accumulation of ROI and NO, processes that closely resemble the antimicrobial activities of macrophages during the immune response. Several lines of evidence suggest that death of host cells during the HR results from the activation of suicide processes, encoded by the plant genome and activated by a fine modulation of O_2^-, H_2O_2 and NO levels. SOD-mediated dismutation of O_2^- to H_2O_2 during the HR is required for the cell death, which is induced only in the presence of NO and H_2O_2, and not by $ONOO^-$. Although the mechanisms by which the NO/ROI balance triggers cell death appear to diverge between the animal and plant kingdoms, the central hypothesis in which the balance between NO and O_2^- concentrations, modulated by their rate of production and by rate of O_2^- dismutation, represents a critical determinant in the aetiology of many human diseases [8] was extended to and now verified in plants [10].

This findings now open new ways to analyse the plant disease defense mechanisms, based on redox signals, which are involved in the hypersensitive cell death and in the establishment of SAR. Focusing exclusively on the NO/ROI biochemical pathways will enable identification and selection of improved traits that can be introduced or selected into a variety of crops by either conventional breeding or through biotechnological applications.

Acknowledgements

This work was supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica.

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