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Hypothesis

Nicotinamide, a missing link in the early stress response in eukaryotic cells: a hypothesis with special reference to oxidative stress in plants

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Abstract

A hypothesis is presented suggesting that nicotinamide (NIC) is an initial signal substance in the response of eukaryotic cells to conditions which cause DNA-strand breakage, especially in connection with oxidative stress. In the stressed cell, NIC is released as a result of the activity of poly(ADP-ribose)polymerase (PADPRP). PADPRP is known to be activated by DNA-strand breakage, caused by e.g. oxidative stress or mutagens. NIC and its metabolite trigonelline (*N*-methylnicotinic acid) can induce defensive metabolism at the gene level. Connections between NIC and DNA-methylation are also considered. This hypothesis is discussed in the light of own observations and literature reports.

Key words: DNA methylation; Nicotinamide; Oxidative stress; Plant defense metabolism; Poly(ADP-ribose)polymerase; Trigonelline

1. Introduction and hypothesis

In eukaryotic cells the mechanism of the early stress response prior to the expression of defense-related genes is largely unknown. From studies with intact plants and plant tissue cultures, it is known that various types of biotic and abiotic stress cause increased biosynthesis of so-called secondary metabolites which may have a defensive function. There is also the induction of the defense systems involved in the inactivation of reactive oxygen species such as H_2O_2, $^{\cdot}OH$, O_2^- and various organic peroxides. In plants ascorbate peroxidase and glutathione reductase cooperate to build an important defense against H_2O_2, produced during various types of oxidative stress.

Some stress signals in plants have been suggested to function at the systemic level [1]. Although salicylic acid is suggested to act at the local level, it may act in connection with a systemic signal system [2]. Recently, a signal system involving the prostaglandin-like compound jasmonic acid in response to wounding or exposure to fungal cell wall fragments has been proposed [3,4]. This system probably involves some kind of plasma membrane receptor(s) and/or induction via lipase-mediated linolenic acid release. However, induction of defense systems in response to oxidative stress and/or DNA-strand breakage may involve another signal originating in the interior of the cell.

Here I propose that nicotinamide may function as an initial signal in the defensive response to DNA-strand breakage, caused by oxidative stress and/or by specific agents interacting with DNA (see Fig. 1). Data from the literature and own observations in support of this hypothesis are presented below.

2. Discussion

2.1. Poly(ADP-ribose)polymerase

The enzyme poly(ADP-ribose)polymerase (PADPRP) seems to be present in most eukaryotic cells, including plants, and is localized to the cell nucleus [5]. The role of PADPRP in cell metabolism is rather unclear, although many of its properties are known. A function of PADPRP in DNA repair has been suggested [6].

PADPRP is activated by various types of stress that cause strand breakage in DNA [5], e.g. oxidative stress [7] and exposure to various mutagens [8] or to UV light [6]. Activation of PADPRP does not occur at the gene level, but through interaction between PADPRP and damaged DNA [9].

PADPRP synthesizes polymers of ADP-ribose attached to various proteins associated with DNA [6]. The ADP-ribose used in this process comes from NAD, which is thus degraded with release of nicotinamide (NIC). This is a rapid process: when animal cells were exposed to H_2O_2 in vitro, about 80% of their NAD pool

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Abbreviations: CHS, chalcone synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; NIC, nicotinamide; PADPRP, poly(ADP-ribose)polymerase; PAL, phenylalanine ammonia-lyase; SAM, *S*-adenosyl-methionine; TRIG, trigonelline.

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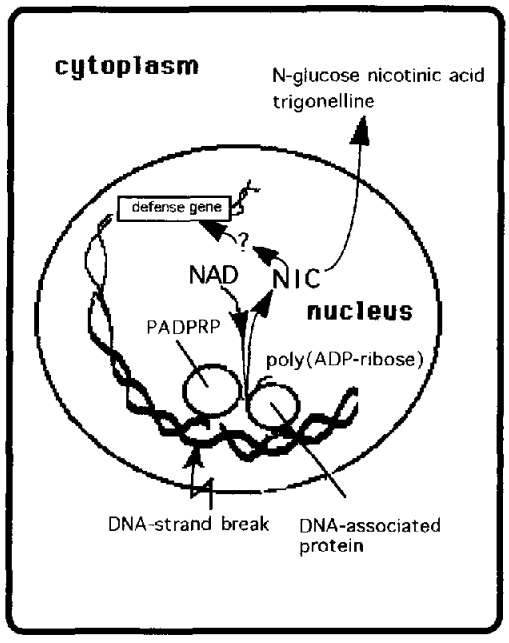

Fig. 1. A schematic figure showing cellular nicotinamide (NIC) release via poly(ADP-ribose)polymerase (PADPRP) activity induced by DNA-strand breakage.

was broken down within 20 minutes as a result of PADPRP activity [10].

Thus, during various types of stress causing an increased frequency of strand breaks in DNA, there is rapid release of NIC inside the cell. This probably occurs in connection with most types of oxidative stress. Although NIC release by this process has been known for a long time, the possibility that NIC is an important signal-mediating molecule has not been considered. When NIC has been released from NAD, it may be metabolized and excreted from the cell. In animal cells NIC may be methylated to *N*-methyl nicotinamide, while in plant cells *N*-methyl-nicotinic acid (trigonelline) and *N*-glucose nicotinic acid may be the major metabolites [11].

A relationship between PADPRP activity and cytochrome P-450 genes has been indicated, since inhibition of PADPRP activity caused decreased cytochrome P-450 gene expression after dioxin treatment of cultured murine cells [12]. Treatment of pigment-producing red beet (*Beta vulgaris*) cells with the PADPRP inhibitor 3-methoxybenzamide results in perturbed betalain synthesis [13], which indicates a connection between PADPRP and secondary metabolism in plants. However, the possibility that PADPRP inhibitors also inhibit steps in the metabolization of NIC to TRIG cannot be excluded.

Several compounds with the potential to intercalate into DNA are able to induce PAL activity and isoflavonoid accumulation in pea [14], speculatively via DNA-strand breakage. The occurrence of strand breaks will then activate PADPRP, which results in NIC release from NAD. NIC may then induce an array of defense genes, such as the genes for phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS).

2.2. *Other sources of nicotinamide release*

In addition to nuclear DNA-associated PADPRP, there may also be other sources of NIC release from NAD, localized outside the nucleus. NIC can be released by mono-ADP ribosylation of proteins in mitochondria in response to oxidative stress [15] or by cytoplasmic cyclic ADP-ribose (cADPR) synthase [16]. These activities also contribute to increased intracellular free Ca^{2+} levels by mobilizing Ca^{2+} from intracellular stores [15,16].

2.3. *Effects of exposure to NIC*

In rat liver NIC has been shown to activate defense-related enzymes, such as cytochromes P-450 and b_5, NADPH-cytochrome *c*-reductase and glutathione transferase [17], as well as to increase the level of glutathione [18,19]) and of glutathione reductase [18]. Other observations also point to an effect of NIC on gene expression, e.g. an increased rate of glutathione transferase gene transcription in hepatocytes [20], increased transcription of membrane-bound alkaline phosphodiesterase in human tumor-derived fibroblasts [21], increased expression of the growth hormone (GH) gene and an increase in its response to triiodothyronine (T3) in human pituitary cells [22] and Friend erythroleukemia cell differentiation [23]. A compilation of NIC effects in animal tissues is shown in Table 1.

We have observed that NIC can induce secondary metabolite accumulation and defense gene expression in

Table 1
A compilation showing the effects of nicotinamide exposure in animal and plant tissues

Effects of NIC exposure in animal tissues
↑ cyt P-450, glutathione transferase (GT) (rat liver) [17]
↑ glutathione (GSH), glutathione reductase (GR) (rat liver) [18]
↑ GT gene transcription (hepatocyte) [20]
↑ membrane bound alkaline phosphodiesterase (human fibroblasts) [21]
↑ growth hormone (GH) gene expression (human pituitary cells) [22]
↑ cell differentiation (Friend erythroleukemia cells) [23]
Effects of NIC exposure in plant tissues
↑ chalcone synthase (CHS) gene expression [25]
↑ glutathione reductase (GR) gene expression [25]
↑ phenylalanine ammonia-lyase (PAL) activity*
↑ indole alkaloid accumulation*
↑ anthocyanin accumulation [24]
↑ levels of reduced (GSH) and oxidized (GSSG) glutathione [24,25]
↓ chlorophyll *a/b*-binding protein (CAB) gene expression [25]

↑ = increase; ↓ = decrease;
* T. Berglund et al., unpublished.

plants. These observations involve disparate biosynthetic pathways and different plant species. NIC induces increased anthocyanin [24] and alkaloid (Berglund et al. unpublished) accumulation in *Catharanthus roseus*. In *Pisum sativum* this compound induces transcription of the CHS and glutathione reductase (GR) genes [25]. CHS is a key enzyme in flavonoid biosynthesis, which gives rise to a broad spectrum of substances which defend against UV-light and microorganisms. A compilation of NIC effects in plant tissues is shown in Table 1.

NIC treatment of plant tissue cultures also causes induction of PAL (Berglund et al., unpublished), which is the link between primary metabolism and the phenylpropanoid/flavonoid pathway. Furthermore, such treatment induces a strong and long-lasting increase in the levels of reduced (GSH) and oxidized (GSSG) glutathione in *C. roseus* [24] and *P. sativum* [25]. Thus, it can be concluded that NIC is a potent inducer of defensive metabolism including glutathione metabolism, as well as of the accumulation of various defensive substances, so-called secondary metabolites.

NIC also induces an early increase in CHS gene transcription in light-, as well as dark-grown *P. sativum* cultures. This increase in CHS gene transcription precedes the rise in GSH level seen in the light, while in dark-grown cultures there is no early rise in GSSG. These observations eliminate the possibility that NIC induces CHS gene transcription via an increase in GSH or GSSG. Furthermore, the absence of an initial increase in the GSSG level in dark-grown cultures argues against a role for NIC itself as a general inducer of oxidative stress. However, this finding does not exclude the possibility that GSH can selectively induce the later expression of defensive genes, such as that coding for plant cytosolic copper/zinc superoxide dismutase [26].

Plant cells seem to be active in the metabolism of NIC to N-methylated and glucosylated compounds [11]. One reason for this could be that NIC is a potent regulatory substance, whose level is also controlled by the rate of degradation. On the other hand we cannot exclude the possibility that some metabolite(s) of NIC, e.g. trigonelline, is a further link in NIC-induced defense gene activation (see below). This possibility is presently under investigation.

2.4. *Relationships between defensive metabolism, oxidative stress and PADPRP activity*

There is a considerable overlap between defensive metabolism and secondary metabolism in plants. Many types of biotic and abiotic stress in plants cause the induction of secondary metabolism and accumulation of secondary metabolites. The plant stressors O_3, UV and fungal elicitor induce secondary metabolism (stilbene production) in conifers [27,28]. This induction of secondary metabolism is associated with the induction of key enzymes of the phenylpropanoid/flavonoid pathway, such as PAL and CHS. Secondary metabolism is also induced by heavy metal ions (e.g. Hg^{2+}, Cu^{2+}), chloroform, 2,4-dinitrophenol and various thiol reagents, as reviewed by Wolters and Eilert [29].

Most of these stressors, e.g. decreased temperature [30], O_3 [31], UV-light [32], fungal elicitor [33], Cu^{2+} [34] and SO_2 [35] cause oxidative stress, which is reflected in increased levels of reduced and oxidized glutathione, as well as in a rise in the activity of glutathione reductase. An increase in the GSH level is often preceded by an increase in the level of GSSG. The effects of stressors on glutathione metabolism have been thoroughly reviewed by Alscher [36] and Smith et al. [37]. As discussed earlier, treatments which give rise to oxidative stress and to elevated levels of reactive oxygen species, such as H_2O_2, O_2^- and $^{\cdot}OH$, also cause DNA-strand breakage and induction of PADPRP activity.

Thus, there is a common factor in the induction of PADPRP activity, induction of secondary metabolism and changes in the levels of glutathione, namely, oxidative stress.

2.5. *Different signal systems depending on the type of stressor?*

We have observed that both nicotinamide and trigonelline contents increase in leaf tissue of intact plants of *P. sativum* after UV-B exposure and in plant tissue cultures of *C. roseus* following treatment with free radical-generating substances (Kalbin, Ohlsson, Strid, Rydström and Berglund, unpublished results). This also indicates that these treatments may induce DNA-strand breaks in plant tissues. In human skin, DNA-strand breaks constitute a considerable portion of the lesions caused by UV-B and UV-A exposure [38]. However, yeast elicitor did not cause increased levels of the nicotinamide metabolites nicotinic acid-*N*-glucoside and/or trigonelline in cell suspensions cultures of *N. tabacum* [39].

Although oxidative stress has been observed in response to treatment of plant tissue with fungal elicitor, the response may depend on the nature and purity of the preparation and the amount of elicitor applied. Thus, one may speculate that fungal elicitors, which are proposed to act via membrane receptors and/or the jasmonate-based signal system proposed by Farmer and Ryan [3], do not generally induce the release of nicotinamide or a nicotinamide-derived signal. A differential response of plant cells to various threats with regards to nicotinamide and/or trigonelline may reflect a basic difference in the signal pathways employed by internal and external sensors.

In contrast to a receptor/sensor stress signal system involving the plasma membrane, the present hypothesis involves a mechanism initiated in the interior of the cell and in threats against DNA from substances and physical agents which penetrate membranes and escape recog-

nition at that level. These two strategies are complementary and may be connected to one another. Of course one cannot exclude the possibility that various stressors can activate several signal systems. Oxidative stress may, for example, influence lipase activity and jasmonate biosynthesis as well as increase the level of cytoplasmic free calcium ions and release nicotinamide from NAD.

Furthermore, from studies in animal cells it is known that defense-linked transcription factors like NFkB may be activated independently of nuclear derived signals and DNA damage [40]. NFkB is known to be activated by reactive oxygen species [41]. Thus, several parallel systems may be active in signalling and sensing oxidative stress. One may speculate that the state of the cell, whether it is growing or ultimately differentiated, as well as the degree of oxidative stress may influence the signals used to induce defensive metabolism. However, NIC may speculatively also be involved in nuclear independent activation of defense signals, because it is released from NAD also in extranuclear compartments in response to oxidative stress, as mentioned earlier in this text.

2.6. *Nicotinamide, trigonelline and DNA-methylation*

In plant tissues NIC can be metabolized to trigonelline (TRIG) via amidohydrolase and methyltransferase activities [42,43]. The presence of the quarternary ammonium compounds trigonelline, betaine or choline is associated with resistance against fungal infection (necrotic lesions) in plants [44]. These compounds can also confer leaf resistance to fungal infections in barley [45,46].

It has been suggested that the physiological effects of TRIG and certain other quarternary ammonium compounds in plants could occur at the level of DNA-methylation. DNA-methylation probably influences the accessibility of DNA for transcription [47]. Involvement of DNA-methylation in the regulation of plant gene expression in response to environmental stimuli has been suggested by Galaud et al. [48]. TRIG, betaine and choline show a hypomethylating effect in plants [45,46]. Accordingly, it is suggested that DNA hypomethylating agents, such as 5-azacytidine or ethionine, can induce resistance to fungal infections in intact plants [46]. It has also been shown that 5-azacytidine can promote secondary metabolite accumulation in plant tissue cultures [49].

From plant tissue culture studies it has been suggested that NIC treatment can cause a decrease in the level of DNA-methylation (hypomethylation) [50]. Although not explored, one may speculate that this effect of NIC could at least partially be mediated via TRIG. The formation of trigonelline from NIC may influence DNA-methylation in two ways: (1) Deamidation of NIC to nicotinic acid followed by methylation of nicotinic acid to TRIG consumes *S*-adenosyl-methionine (SAM), which is the methyl donor employed when DNA is methylated. In this way the size of the SAM pool may be decreased and less will be available for DNA-metylation. When animals were treated with excess nicotinamide in the food, there was a drain of the SAM pool in the liver [51]. (2) TRIG per se seems to influence the degree of DNA-methylation [45,46]. The mechanism underlying this phenomenon is not yet known, but could involve inhibition of DNA-methyltransferase activity and/or activation of a postulated [48] DNA-demethylase.

Thus, a number of observations indicate a possible connection between NIC metabolism and defensive metabolism via DNA-demethylation. Although not further discussed here, it is also possible that *N*-methyl nicotinamide in animal systems may be a functional analogue to TRIG in plant systems.

However, stress-induced DNA-strand breakage may cause cell-cycle arrest [52]. TRIG can cause cell-cycle arrest as well [53]. Thus, because DNA-methylation is generally linked to DNA replication, the DNA-demethylating effect of TRIG may occur through the action of demethylase activity. It must be noted that NIC and TRIG may act also in other cells than in those where they were released or synthesiszed.

2.7. *Conclusion and future perspectives*

In conclusion, I propose that nicotinamide is a link between oxidative stress, as well as other conditions causing an increased frequency of DNA-strand breakage, and defense gene activation. This does not exclude the possibility that oxidative stress, e.g. via changes in glutathione levels, can induce defensive metabolism independently of the occurrence of DNA-strand breaks, but this may be a slower process, and oxidative stress-induced activation of transcription factors independent of nuclear derived signals may also occur. Still, we cannot rule out the possibility that metabolites of NIC, e.g. N-methylated NIC compounds, can constitute a further link in the chain between oxidative stress and defense gene expression. This possibility is under investigation.

There is also a possibility that NIC/NIC-metabolite release is a signal from a collapsing cell in an apoptotic/necrotic area, serving as a signal to surrounding and maybe distant tissues to mobilize their defense lines.

The exploration of the present hypothesis could lead to the identification of a new class of plant protective substances derived from NIC, with a wide range of applications. From a biotechnological point of view, NIC may also be a strong tool for induction of stress-respondive cellular functions, of use in plant, as well as in animal cell technology. We have already shown that in plant cell cultures, NIC is a potent inducer of secondary metabolism. One property of NIC that is of paramount importance, when considering its potential use in biotechnological systems, is its low toxicity.

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