PRESENCE OF NITRIC OXIDE SYNTHASE ACTIVITY UNDER NaCl-INDUCED STRESS IN COTTON CALLUS

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Abstract

The presence of nitric oxide synthase (NOS) in cotton callus Coker 312 was studied. The immunoblot analysis of cytosolic extracts with a polyclonal antibody against the C-terminus region of murine iNOS revealed an immunoreactive protein of 130kDa. The evidence presented by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline assay indicates the production of nitric oxide as an adaptive mechanism of plants under NaCl induced physiological stress.

Introduction

Nitric Oxide (NO), an inorganic free radical, is synthesized from the biological conversion of L-arginine to L-citrulline by Nitric Oxide Synthase (NOS). Once produced, NO can be converted into nitrate and nitrite in the presence of oxygen and water. It is highly soluble in lipids and in water and therefore capable of diffusing freely in the cell (Ninnemann *et al*, 1996). NOS comprises of a family of several constitutive or inducible isoenzymes and their particular localization has been well characterized to be tissue specific in mammalian systems, *e.g.* mammalian NOS occurs in both constitutive (neuronal [nNOS} and endothelial [eNOS]) and inducible (iNOS) isoforms. iNOS has been shown to play a central role in the animal immune system; its transcriptional level and activity are induced during the activation of the immune response (Snyder, 1992). The production of NO is known as a potent signaling molecule with multiple biological functions, *i.e.* vascular regulator in brain neuromodulation (Koprowski *et al*, 1993), and activation of immune cells (Mitsuhata et al., 1994), however the role of NOS has not been well defined in plant systems.

Until recently, research on the effects of NO in plants was focused on atmospheric pollution by the oxides of nitrogen, NO and NO_2 (Nitrogen dioxide). NO uptake into foliage as well as its subsequent metabolism and phytotoxicity, are well documented (Durner J., Klessig D.F. 1999). It was subsequently demonstrated that plants not only respond to substantial amounts of NO, but also produce substantial amounts of NO. Mounting evidence also suggests that NO is a novel effector in plant growth, development, and defense responses (Cueto M., Hernandez-Perera *et al* 1996).

Studies in oxidative stress are considered as the causative agent in many situations alter the oxygen status of the plant cell and lead to the formation of the most reduced forms: H_2O_2, O^-_2, and a more powerful oxidant, the hydroxyl radical (OH^-). When present in relative low amounts, ROS and especially H_2O_2 can act as signals for the activation of defense responses against stress. However, higher amounts produced by uncontrolled ROS generation can cause severe injury like chlorophyll breakdown, DNA fragmentation, ion leakage, lipid peroxidation, and finally, cell death. Cell ion leakage to intercellular compartments occurs as an early step, leading to a special kind of programmed cell death. In potatoes, NO has been shown to decrease the extent of ion leakage originated by diquat, providing strong evidence in favor of NO as a potent antioxidant.(Beligni M.V., Lamattina L. 1999.)

This study focuses on; (1) the detection of the inducible Nitric Oxide Synthase (iNOS) enzyme in cotton callus Coker 312 and (2) its subsequent activity when subjected to induced NaCl stress.

Methods and Materials

Sample Preparation

Five grams of cotton callus Coker 312 (salt sensitive and salt Tolerant) tissue was transferred to culture tubes containing 25 ml of media containing either 0 NaCl (salt-sensitive) or 150 mM NaCl (salt-tolerant). Each culture tube was connected to an aerator for constant aeration. At time point zero, the tubes were either left untreated as controls or treated with either 100mM NaCl (salt-sensitive), 250mM NaCl (salt-tolerant), or 0.2 µM Paraquat. Samples were harvested at 0minutes, 15minutes, 30minutes, 60minutes, and 120minutes respectively. After incubation cotton callus was recovered by removing media. Samples were stored at -70°C upon isolation.

Protein Extract Preparation

Frozen samples were thawed and homogenized at 4°C to extract intracellular proteins in 5ml extraction buffer containing 100mM Tris; 10mM isoascorbic acid; 20g/L PVP -10: 1.5g insoluble PVP; 0.1mM EDTA and 2ml TritonX-100 (Rohm and Haas Co., Philadelphia, PA). Finally, PMSF (10uM) was added and the samples were stored at-70°C.

Measurement of Inducible Nitric Oxide Synthase (iNOS) Activity

Nitric Oxide Synthase (NOS) activity was measured using the NOS *detect* assay system (Stratagene, La Jolla, CA) by monitoring the conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline from cytosolic cellular extracts. Each reaction was carried out under the following conditions: 50mM HEPES buffer pH 7.4, 1mM EDTA, 0.1mM dithiotreitol, 1.250mM CaCl2, 1mM NADPH, and 2.6uM L-[^{14}C]arginine. Each reaction was incubated at 25°C for 2 hours. Reactions were terminated by the addition of stop buffer (20mM HEPES containing 10mM EDTA and 10mM EGTA). AG 50W-X8 resin (400µl) was added to bind unreacted L-[^{14}C]arginine and added to spin cups and cup holders. Separation of neutrally charged citrulline from positively charged arginine was achieved by centrifuging spin cups and collecting the eluate. L-[^{14}C]citrulline was quantified using a liquid scintillation counter. In an effort to define the potential similarities in this enzymatic system and those described in higher order organisms the inhibitory effect of L-NMMA, an L-arginine analog was used as a control for every sample. It is well known that L-NMMA is an inhibitor of constitutive and inducible nitric oxide Synthase in animal cells.

Antibodies

A polyclonal antibody against the peptide PT387 (Ac-Cys residues 1131 to 1144) from the C-terminus of the deduced amino acid sequence of murine iNOS was used for immuno-detection.

Western Blot Analysis

SDS-PAGE was carried out in 7.5% acryl amide slab gels. Samples were prepared in 100mMTris extraction buffer, and were heated at 95°C for 5 minutes. For immunoblot analyses, polypeptides were transferred onto polyvinylidene difluoride membranes using(Immobilon P. Millipore Corp., Bedford, MA) using a Semi Dry transfer (Bio-Rad) with 10%SDS,10%Methanol, pH 11.0 at 1.5mA for 8hrs. For immunodetections, the polyclonal antibody against iNOS was enhanced with an alkaline phosphatase mix (SIGMA).

Results and Discussion

The analyses by immunoblot of cotton callus extracts with a polyclonal antibody against the peptide PT387 from the C-terminus of the murine iNOS revealed an immunoreactive polypeptide of about 130kDa (Figure 1). Immunoreactive bands with similar mobility were obtained with the hepatic iNOS lipopolysacharide-induced rat and murine lysates (Transduction Laboratories, Lexington, KY) used as positive controls. iNOS was detected in both the NaCl-tolerant and the NaCl-sensitive callus tissue at about the same concentration.

Incubation of cotton callus cytosol extracts with radiolabeled arginine resulted in the conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline. Formation of L-[^{14}C]citrulline also increased proportionally to the amount of extract in the reaction mixture(data not shown). Prior to treatment with NaCl, iNOS activity was essentially zero in both cell lines (Figure 2). Addition of NaCl to the samples increased iNOS activity in both cell lines (Figure 2); however, there was a 3-4-fold increase in the NaCl-sensitive cell line, while iNOS increased 12-fold in the NaCl-tolerant cell line. Paraquat treatment also resulted in significant increases in iNOS activity in both cell lines, and again the increase in the NaCl-tolerant callus was significantly higher than the increase observed in the NaCl-sensitive callus (Figure 3).

It is interesting to note that there appears to be a biphasic mode for iNOS induction. In the control tissue prior to the addition of NaCl, iNOS activity was non-detectable; however, at time point 0, which actually represents 1-2 minutes after the addition of NaCl, there was a dramatic increase in iNOS activity. At the 15 minute time point, iNOS activity had returned to near base line levels and then increased dramatically again within 30 minutes. This suggests that perhaps there is a large pool of iNOS that is constitutively expressed. Once this pool is depleted, a second phase of iNOS induction occurs.

To our knowledge this is the first documented report providing evidence for a NOS-like enzyme in cotton, suggesting the conservation of NOS function across animal and plant kingdoms. Nitric oxide and L-[^{14}C]citrulline were synthesized in NaCl-tolerant and NaCl-sensitive cotton callus derived from *cv* Coker 312, but the NOS activity was 2-3 times greater in the NaCl-tolerant callus. This study provides evidence of an upregulation mechanism under NaCl induced physiological stress and a possible adaptive mechanism developed by NaCl tolerant Coker 312.

Acknowledgements

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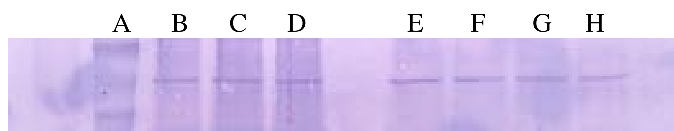

Figure 1. Western blot analysis of the 130 kDa band of iNOS from rat iNOS (positive control) (lane B), NaCl-tolerant cotton callus (lanes C and D), and NaCl-sensitive cotton callus at different concentrations (lanes E,F,G, and H)

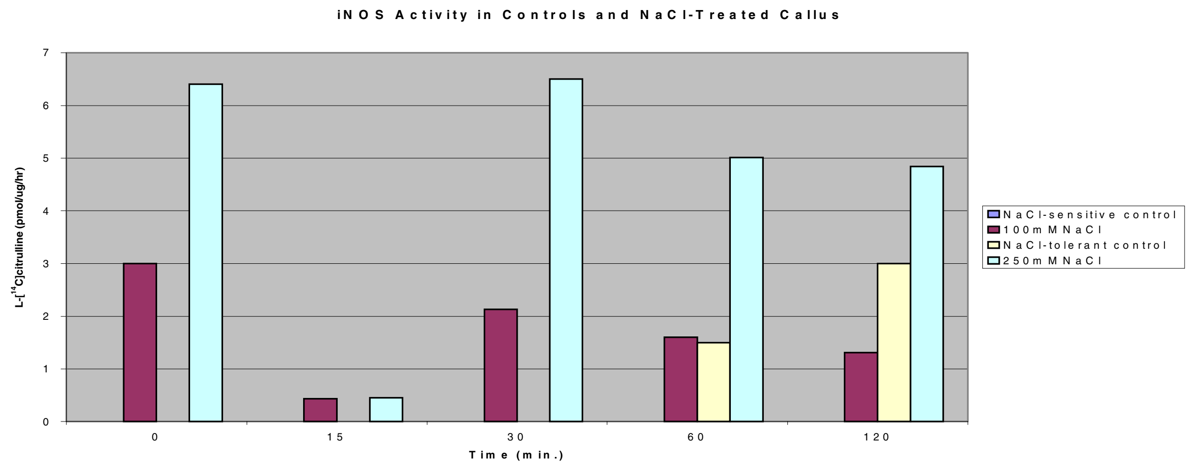

Figure 2. iNOS Activity in Control and NaCl-treated NaCl-Tolerant and NaCl-Sensitive Callus Tissue.

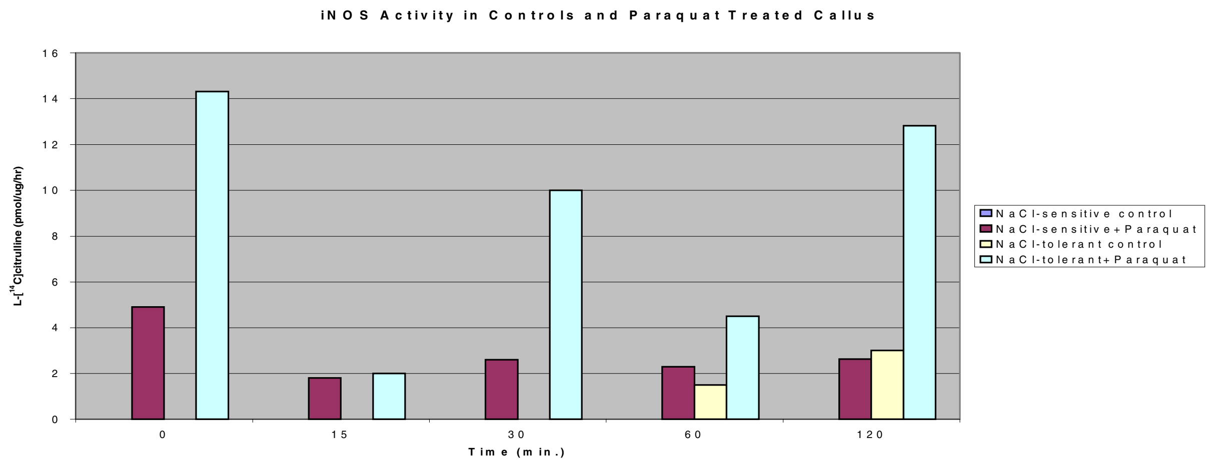

Figure 3. iNOS activity in control and paraquat treated NaCl-tolerant and NaCl-sensitive callus tissue.