



NADPH oxidase activity in allergenic pollen grains of different plant species

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ABSTRACT

Pollen is an important trigger of allergic diseases. Recent studies have shown that ragweed pollen NAD(P)H oxidase generates reactive oxygen species (ROS) and plays a prominent role in the pathogenesis of allergies in mouse models. Here, we demonstrated that allergenic pollen grains showed NAD(P)H oxidase activity that differed in intensity and localization according to the plant families. The activity occurred at the surface or in the cytoplasm in pollen of grasses, birch, and ragweed; in subpollen particles released from ragweed pollen; and at the inner surface or in the cytoplasm but not on the outer wall, which was sloughed off after the rupture, of pollen of Japanese cedar and Japanese cypress. The activity was mostly concentrated within insoluble fractions, suggesting that it facilitates the exposure of tissues to ROS generated by this enzyme. The extent of exposure to pollen-generated ROS could differ among the plant families.

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Introduction

Pollen is an important trigger of seasonal rhinitis, conjunctivitis, and/or allergic asthma and an exacerbating factor in atopic dermatitis [1–6]. Pollen grains of trees of the *Cupressaceae*/*Taxodiaceae* family, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtuse*), *Cupressus* species, and *Juniperus* species, are relevant sources of allergens [1,2,4]. In Japan, a common seasonal allergic disease posing a major public health problem is caused by inhalation of the pollen of Japanese cedar and Japanese cypress, affecting around 20% of the total population [1,2]. *Cupressaceae* pollen has also been identified as a source of pollinosis in Mediterranean countries and the United States [2]. In Europe and North America, birch of *Betula* species is the most important allergenic tree [4]. Pollen grains of ragweed of *Ambrosia* species [5] and grasses of the *Poaceae* family, such as timothy (*Phleum pratense*), rye (*Lolium* species), Kentucky blue grass (*Poa*

pratensis), orchard grass (*Dactylis glomerata*), Bermuda grass (*Cynodon dactylon*), and so on [6], are also some of the most clinically relevant sources of allergens.

Molecules produced by allergen-producing organisms such as house dust mites and pollen have been suggested to be involved in the pathogenesis of allergic diseases through sensitization and/or exacerbation via IgE-independent mechanisms and the modification of IgE-dependent responses [7–23]. More information on the biochemical or biological activities of these molecules derived from allergen-producing organisms and their release or localization under physiological conditions is needed for understanding the mechanism of primary sensitization and exacerbation of the inflammation in allergic diseases. Pollen grains release substances, which have structural similarity with the inflammatory lipid mediators prostaglandin E₂ and leukotriene B₄ [24,25]. Lipid fractions from birch and grass pollen extracts induce chemotaxis and the activation of neutrophils [18] and eosinophils [19]. Those from birch pollen exhibit Th2-polarizing activity by inhibiting the production of IL-12 by dendritic cells [17]. Agea et al. reported that human CD1-restricted T cells and IgE in blood samples, which were obtained from allergic subjects during the pollinating season, recognized cypress pollen-derived phospholipids [23]. Very recently, we have analyzed the release of proteases, prostaglandin E₂- and leukotriene B₄-like substances, and proteins into soluble fractions, the former two of which might be involved in the pathogenesis of allergic diseases and the last of which are potentially allergenic, from relevant allergenic pollen grains of

Abbreviations: CM-H₂DCF-DA, 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, acetyl ester; DMSO, dimethyl sulphoxide; NADH, nicotinamide adenine dinucleotide (reduced); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NBT, nitroblue tetrazolium; PBS, Dulbecco's phosphate-buffered saline without calcium or magnesium; ROS, reactive oxygen species; RS, reactive species; SOD, superoxide dismutase.

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the *Cupressaceae/Taxodiaceae* family (Japanese cedar, Japanese cypress, and Rocky mountain juniper), white birch, short ragweed, and grasses (Kentucky blue and cultivated rye) [25,26]. We demonstrated the release of serine proteases or both serine and cysteine proteases from these pollen grains by using fluorogenic substrates, class-specific protease inhibitors, and zymography [25,26]. Runswick et al. [27] reported that diffusates from pollen of Kentucky blue grass, giant ragweed, white birch, and Easter lily degraded tight junctions *in vitro* and the diffusate of Kentucky blue grass pollen did so in a protease-dependent manner.

In addition to pollen-derived lipids [17–19,23] and pollen proteases [25–27], a research group has suggested that ragweed pollen-derived NAD(P)H oxidase contributes to allergic inflammation [20,21]. They also reported that commercial pollen allergen extracts of weeds, trees, and grasses contain NAD(P)H oxidase activity [20] and that NAD(P)H oxidase is concentrated in the subpollen particles released from fresh pollen grains of ragweed and pigweed [22]. However, the location of NAD(P)H oxidase activity in grains of other allergenic pollens is unknown. Here, we investigated the activity and localization of NAD(P)H oxidase contained in allergenic pollen grains of Japanese cedar and Japanese cypress, which belong to the *Cupressaceae/Taxodiaceae* family, white birch, short ragweed, and two grasses of the *Poaceae* family, Kentucky blue and cultivated rye.

Materials and methods

Pollen. Pollen of Japanese cedar (*C. japonica*) was purchased from Wako (Osaka, Japan) or kindly provided by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan). Pollen of Japanese cypress (*Chamaecyparis obtusa*) was purchased from Wako. Pollen of white birch (*Betula alba*), water birch (*Betula fontinalis occidentalis*), Kentucky blue grass (*P. pratensis*), and cultivated rye grass (*Secale cereale*) were purchased from Sigma (St. Louis, MO). Pollen of short ragweed (*Ambrosia artemisiifolia*) was purchased from Polysciences (Warrington, PA).

Measurement of reactive species (RS). 3-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCF-DA) (Invitrogen, Carlsbad, CA) was used to detect the presence of reactive species (RS) [28]. CM-H₂DCF-DA was dissolved in dimethyl sulfoxide (DMSO) as a 50 mM stock solution. Pollen grains (10 µg/ml) were hydrated in Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS) on ice for about 30 min. The reaction was started with the addition of 20 µM CM-H₂DCF-DA and

conducted at 37 °C (200 µl/well) [20–22]. Upon oxidation via interaction with RS, CM-H₂DCF-DA forms a fluorescent compound. The fluorescence intensity was measured at 488 nm excitation and 530 nm emission on a fluorometer, SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, CA).

Nitroblue tetrazolium (NBT) assay. Pollen grains (1 mg/assay) were hydrated in pure water on ice for about 30 min and mixed with 2 mM nitroblue tetrazolium (NBT) (Sigma) with or without 1 mM nicotinamide adenine dinucleotide phosphate (reduced) (NADH) (Sigma) or 1 mM nicotinamide adenine dinucleotide (reduced) (NADPH) (Sigma) resolved to pure water. The reaction was started with the addition of NBT and conducted at 37 °C in PBS (500 µl/test) [20–22]. During the reaction, the suspensions were gently rotated. After 30 min of the reaction, the suspensions were centrifuged for 3 min at 7300g. Residual NBT was removed by washing with 1 ml of pure water twice, and the formazan sediment was dissolved in 400 µl of methanol. After extraction with methanol by rotation for 20 min at room temperature and centrifugation, the absorbance of supernatants was measured at 530 nm (200 µl/well).

In parallel experiments, after the reaction had lasted 15 min and 60 min, the suspensions were moved to ice and pollen grains and formazan deposits were immediately visualized under a microscope.

Inhibition of pollen-induced NBT reduction by superoxide dismutase. Pollen grains (20 mg/ml) were mechanically disrupted by shaking with beads of tungsten and zirconium for 20 min at 1500 rpm in ShakeMasterNeo (Bio Medical Science, Tokyo, Japan). The suspensions of the disrupted pollen grains (0.2 mg/assay) were mixed with NBT with or without NADH. The reaction was conducted with or without superoxide dismutase (SOD) from bovine erythrocytes (Sigma) (50 U/ml) at 37 °C in PBS (500 µl/test). After 30 min to 3 h of the reaction, the suspensions were centrifuged for 25 min at 20,000g. Residual NBT was removed by washing with 0.5 ml of pure water once, and the formazan sediment was dissolved in 750 µl of DMSO. The absorbance of supernatants after extraction with DMSO by sonication (As One, Osaka, Japan) for 2 min and centrifugation was measured at 530 nm (150 µl/well).

Results

Generation of RS by allergenic pollen grains

Pollen grains of the different plant species tested oxidized CM-H₂DCF-DA to a fluorescent compound, indicating the genera-

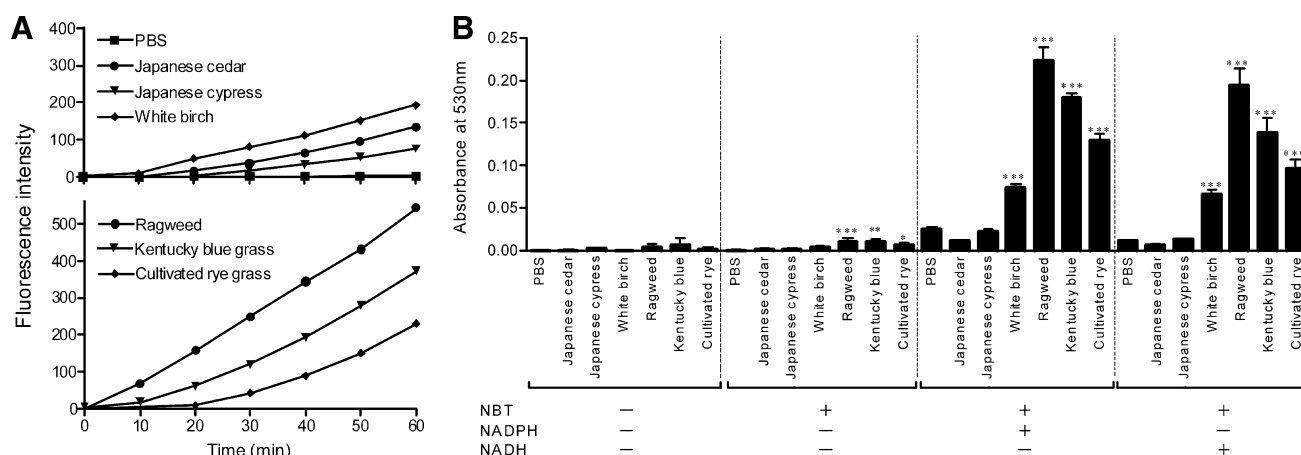


Fig. 1. Generation of RS and reduction of NBT by allergenic pollen grains. (A) Generation of RS. Time-dependent increases in fluorescence on the addition of pollen grains are shown. Data are representative of two independent experiments with similar results. (B) Reduction of NBT. After a reaction for 30 min, the soluble NBT was removed by washing with pure water. Methanol was used to dissolve the formazan sediment. However, the sediment was not completely dissolved. After centrifugation, the absorbance of the supernatant was measured. Means $\pm$ SDs for three wells are shown. The data were analyzed by one-way ANOVA and Tukey's post hoc test. A p value <0.05 was regarded as statistically significant. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Data are representative of three independent experiments with similar results.

tion of RS, and ranked in activity as follows: ragweed > the *Poaceae* family (grasses: Kentucky blue > cultivated rye) > birch > the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) (Fig. 1A).

Reduction of NBT by allergenic pollen grains: I. Absorbance of formazan sediment extracted with methanol

The activity of pollen grains to reduce NBT to formazan with or without NADPH or NADH as an electron donor was evaluated first by measuring the absorbance of the colored formazan sediment

extracted with methanol (Fig. 1B) and then by microscopy (Figs. 2 and 3). Without NADPH or NADH, NBT's reduction to formazan by pollen grains of ragweed and the *Poaceae* family (Kentucky blue grass and cultivated rye grass) was detectable in the first assay (Fig. 1B). On the addition of NADPH or NADH, the activity was much enhanced and birch also exhibited significant activity, suggesting the RS-generating activity of ragweed, grasses, and birch to be due to pollen NAD(P)H oxidase. Pollen grains of the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) did not show increased absorbance

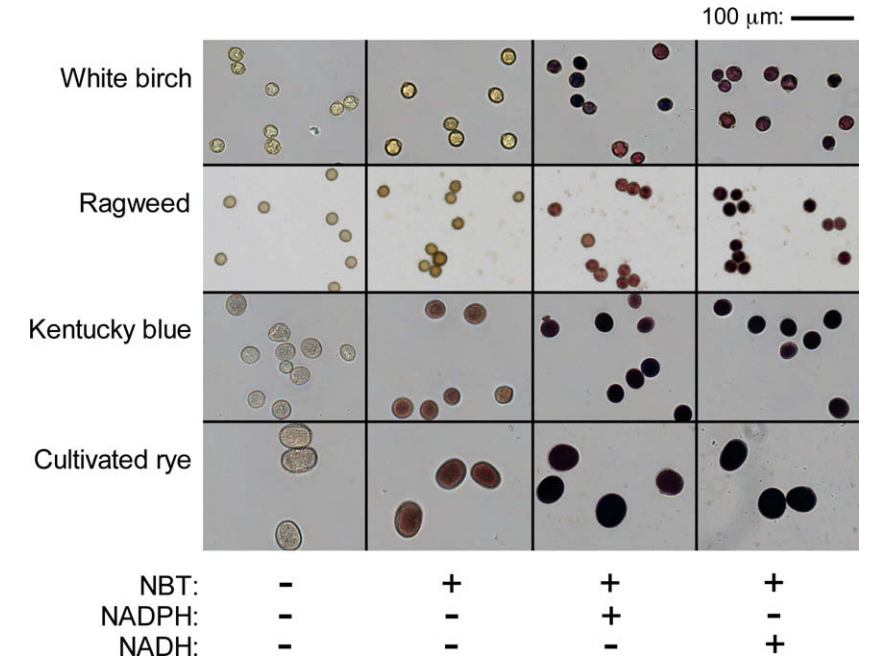


Fig. 2. Visualization of formazan deposits in pollen grains of birch, ragweed, and the grasses Kentucky blue and cultivated rye. Formazan deposits produced by the reduction of NBT were visualized in purple. Images were captured under a microscope at a magnification of 100 after a reaction for 60 min. Results are representative of three independent experiments. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

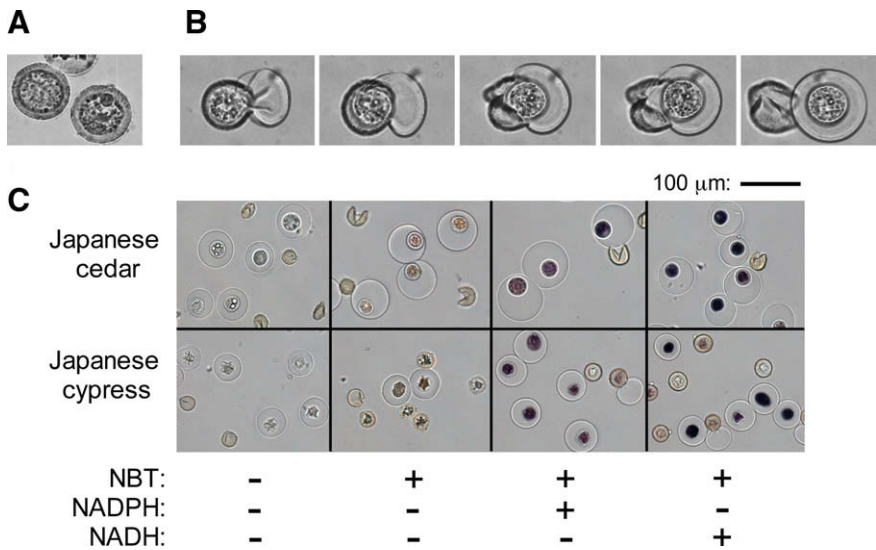


Fig. 3. Visualization of formazan deposits in pollen grains of Japanese cedar and Japanese cypress, which belong to the *Cupressaceae/Taxodiaceae* family. (A,B) Pollen grains of the *Cupressaceae/Taxodiaceae* family rupture and slough off the pollen outer wall after being suspended in PBS (B, from left to right) but not in pure water (A). Results for Japanese cypress pollen were shown in (A) and (B). (C) Formazan deposits produced by the reduction of NBT were visualized in purple. Images were captured under a microscope at a magnification of 100 after 60 min (C). Results are representative of three independent experiments.

in the first assay (Fig. 1B). However, pollen grains after the extraction with methanol, particularly in the reaction with NADPH or NADH, still appeared colored (data not shown), suggesting methanol could extract only a small proportion of the total formazan deposit; therefore, next we analyzed the pollen grains under a microscope after the reaction (Figs. 2 and 3).

Reduction of NBT by allergenic pollen grains: II. Visualization of pollen grain-structural change and formazan deposits by microscopy

Ragweed pollen released small subpollen particles visualized in the presence of NBT with or without NADPH or NADH (Fig. 2, Ragweed). Pollen grains of the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) ruptured and shed their outer wall in PBS and each of the reaction solutions (Fig. 3B and C) but not in pure water or saline (Fig. 3A and data not shown). After the outer wall had sloughed away, the inner cell wall was covered with a thick transparent layer, which immediately swelled (Fig. 3B).

On the addition of NADPH or NADH, formazan deposits were clearly visible in the pollen grains of all the plant species after 15 min (data not shown) and 60 min (Figs. 2 and 3C) even in the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) (Fig. 3C), which did not show increased absorbance in Fig. 1B. Without the addition of NADPH or NADH, formazan deposits were observed in pollen of the *Poaceae* family (grasses: Kentucky blue and cultivated rye) and ragweed after 15 min (data not shown) and 60 min (Fig. 2) and pollen of birch (Fig. 2) and the *Cupressaceae/Taxodiaceae* family after 60 min (Fig. 3C), although to less of an extent than with NADPH or NADH.

The NBT-reducing activity, which was enhanced by the addition of NADPH or NADH (Figs. 2 and 3C) and therefore was suggested to be due to the generation of reactive oxygen species (ROS) by pollen NAD(P)H oxidase, occurred at the surface (plasma membrane or surface wall) or in the cytoplasm in pollen of the *Poaceae* family (grasses: Kentucky blue and cultivated rye), birch, and ragweed, in subpollen particles released from ragweed pollen (Fig. 2), and at the inner sur-

face (plasma membrane or inner cell wall) or in the cytoplasm but not on the outer wall, which was shed after the rupture, of pollen of the *Cupressaceae/Taxodiaceae* family (Fig. 3).

SOD inhibited the pollen-induced NBT reduction

Mechanical disruption of pollen grains enabled the detection of the NBT-reducing activity in the presence of NADH even in the *Cupressaceae/Taxodiaceae* family (Fig. 4, Japanese cedar), although the formazan sediment was not completely dissolved with the solvent used, DMSO. SOD inhibited the NBT reduction induced by the suspensions of the mechanically disrupted pollen grains (Fig. 4), which has been reported using ragweed pollen by Bacsı et al. [21]. The result indicates that superoxide radical, a species of ROS [28], significantly contributes to the NBT reduction and supports the presence of NAD(P)H oxidase in the pollen grains of different plant species.

Discussion

In the present study, we investigated the activity and localization of NAD(P)H oxidase contained in allergenic pollen grains of different plant species. All the pollen tested showed NAD(P)H oxidase activity, but the intensity and location of the activity differed according to the plant families (Figs. 1–3). A difference among plant families was also observed in the structural change of pollen grains *i.e.* rupture, shedding of the pollen outer wall, and swelling of the thick transparent layer covering the inner cell wall in the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) (Fig. 3) and the release of NAD(P)H oxidase-positive subpollen particles from ragweed pollen (Fig. 2, Ragweed), the latter of which has been reported by Bacsı et al. [22]. Pollen NAD(P)H oxidase was located at the surface (plasma membrane or surface wall) or in cytoplasm in pollen of the *Poaceae* family (grasses: Kentucky blue and cultivated rye), birch, and ragweed, in subpollen particles released from ragweed pollen (Fig. 2), and at the inner surface (plasma membrane or inner cell wall) or in cytoplasm but not on the outer wall, which sloughed off after the rupture, of pollen of the *Cupressaceae/Taxodiaceae* family (Fig. 3). Inhibition of pollen NBT-reducing activity by addition of SOD indicates the generation of superoxide radical by pollen NAD(P)H oxidase (Fig. 4).

The structural change of pollen grains during hydration in the buffer solutions (Figs. 2 and 3), strength of the ROS-generating activity (Figs. 1–3), and location of pollen NAD(P)H activity after the hydration (Figs. 2 and 3) were not the same among the pollen of the different plant families tested. Pollen of the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress) ruptured and shed their outer wall in PBS and each of the reaction solutions (Fig. 3). With the sloughing away of the outer wall, the inner wall was covered with a thick transparent layer, which immediately swelled (Fig. 3B). Although Bacsı et al. [22] reported that commercial ragweed and pigweed pollen released few subpollen particles compared with fresh weed pollen, the commercial ragweed pollen grains tested in the present study released subpollen particles visible in the presence of NBT (Fig. 2, ragweed). The difference in pollen structure or change during the hydration and generation of ROS could relate to the difference in the strength of ROS-generating activity (Fig. 1–3) and to the difference in the pattern of release, strength, and protease class of pollen protease activity [25,26] among the plant families.

Most of the activity was detected within insoluble fractions (Figs. 2 and 3 and data not shown). The pollen extracts prepared by mild agitation in PBS and then centrifuged and filtered, exhibited no activity or much less activity (data not shown) than the pollen grains (Fig. 1B–3). Mechanical disruption of pollen grains achieved the successful detection of the activity even in Japanese cedar pollen and the

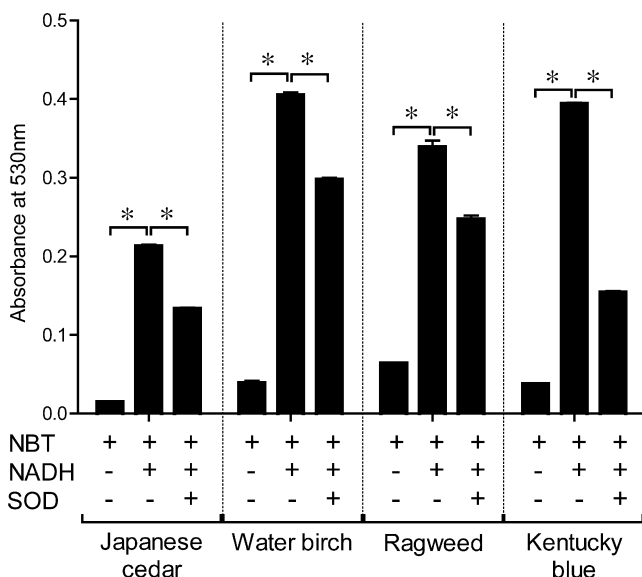


Fig. 4. SOD inhibits pollen-induced NBT reduction. Suspensions of mechanically disrupted pollen grains were used for the assay. After a reaction for 3 h, the soluble NBT was removed by washing with pure water. DMSO was used to dissolve the formazan sediment. However, the sediment was not completely dissolved. After centrifugation, the absorbance of the supernatant was measured. Means $\pm$ SDs for three wells are shown. The data were analyzed by one-way ANOVA and Tukey's post hoc test. A p value < 0.05 was regarded as statistically significant. * $p < 0.001$. Data are representative of two independent experiments with similar results.

significant inhibition by SOD (Fig. 4). Previous studies indicated the presence of NADPH-dependent ROS-generating activity in the plasma membrane of plant cells, similar to the presence of cell-membrane components of human NAD(P)H oxidase [29,30]. Taken together, pollen NAD(P)H oxidase activity may be possibly located in the plasma membrane and/or intracellular organelle membrane of pollens. The presence of pollen NAD(P)H oxidase in insoluble fractions, possibly as membrane-associated forms, could avoid loss of local activity at sites on the epithelia where pollen grains have attached, and facilitate the exposure of tissues to ROS generated by this enzyme, contributing to the sensitization process and pathogenesis of allergic inflammation.

In summary, we demonstrated that allergenic pollen of the *Cupressaceae/Taxodiaceae* family (Japanese cedar and Japanese cypress), birch, ragweed, and the *Poaceae* family (grasses: Kentucky blue and cultivated rye) showed NAD(P)H oxidase activity which differed in intensity and localization according to the plant families. Pollen NAD(P)H oxidase activity was concentrated in insoluble fractions when the pollen grains were hydrated under mild conditions, which could facilitate the exposure of tissues to ROS generated by this enzyme. The extent of this exposure could differ among the plant families according to where in pollen grains the NAD(P)H oxidase resides and pollen structural changes during hydration in the mucosa. This information would be useful for exploring the contribution of pollen NADPH oxidase and other pollen-derived substances among plant species to the sensitization process and pathogenesis of allergies.

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