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Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen

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ABSTRACT

Air pollution is frequently proposed as a cause of the increased incidence of allergy in industrialised countries. We investigated the impact of ozone (O_3) on reactive oxygen species (ROS) and allergen content of ragweed pollen (*Ambrosia artemisiifolia*). Pollen was exposed to acute O_3 fumigation, with analysis of pollen viability, ROS and nitric oxide (NO) content, activity of nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase, and expression of major allergens. There was decreased pollen viability after O_3 fumigation, which indicates damage to the pollen membrane system, although the ROS and NO contents were not changed or were only slightly induced, respectively. Ozone exposure induced a significant enhancement of the ROS-generating enzyme NAD(P)H oxidase. The expression of the allergen Amb a 1 was not affected by O_3, determined from the mRNA levels of the major allergens. We conclude that O_3 can increase ragweed pollen allergenicity through stimulation of ROS-generating NAD(P)H oxidase.

1. Introduction

The increase in respiratory diseases arising from allergies in industrialised countries in recent years is considered to be linked to changes in certain environmental factors. One such factor relates to the higher levels of atmospheric pollution and the greater presence and distribution of allergenic taxa. Climate change has brought about large increases in the concentrations of some airborne allergens, with the resulting higher incidence and/or severity of allergic illnesses (Gilmour et al., 2006). Significant increases in allergic responses are often reported, even in individuals who have never had symptoms previously, and this leads to the belief that these allergic responses might be caused by 'new' allergens that are released by plants used for ornamental purposes or reforestation, or by invasive plants.

In Europe the increases in pollinosis appear to be caused by pollen from birch, hornbeam and cypress trees, and above all, from ragweed, rather than by the classic allergenic pollens, such as grasses, pellitory, olive and mugwort (Corsico et al., 2000; Asero, 2004; Ridolo et al., 2006). *Ambrosia artemisiifolia* (short or common ragweed) is an annual, anemophilous, and extremely allergenic weed that can produce enormous amounts of pollen. As these pollen grains are small (18–22 μm), they can often be transported over long-distances (Mandrioli et al., 1998).

Ragweed started its expansion in Europe in the last decades of the 20th century, arriving from Hungary, the country where it was most abundant. Ragweed is now also found in Italy (D'Amato et al., 1998), and the two regions that have been most affected are Lombardia and Friuli Venezia-Giulia, which are located in the north-northeast of Italy (Mandrioli et al., 1998; Asero, 2002). Ragweed plants have been detected only sporadically in central-southern Italy (Pignatti, 1997). However, transboundary transport of ragweed pollen to central Italy has been demonstrated, which could have a clinical effect on atopic patients (Corsico et al., 2000; Cecchi et al., 2007). Ragweed pollen concentrations have been reported to have increased over the last decade (Oswalt and Marshall, 2008), mainly because ragweed is invasive in Europe and is an opportunistic and pioneer plant, invading field crops and open disturbed habitats or roadsides (Bassett and Crompton, 1975).

However, the increase in allergic disease might be attributed not only to greater concentrations of ragweed pollen in the atmosphere, but also to modifications to the allergenicity this pollen can promote. Although the role of atmospheric pollutants on the allergic sensitivity of airways is not yet completely clear, there is

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evidence to suggest that urbanisation increases allergic sensitization due to its high levels of exposure to ozone (O_3), nitric oxides (NO_x), sulphur oxides (SO_x) and particulate matter (PM_{10}) (D'Amato, 2002).

In addition to affecting the airways of allergic individuals, air pollutants can have direct effects on the aeroallergens in the atmosphere, which can result in changes in the antigenic characteristics of pollen. Air pollutants, and especially O_3 and respirable PM_{10}, can induce proinflammatory responses in the lung (Bernstein et al., 2004), and can have immunological adjuvant effects on IgE synthesis, as has been found with polyaromatic hydrocarbons in the particles of diesel exhaust (Nel et al., 1998).

Ozone is the main component in the so-called 'summer smog' comprised of photochemical oxidants and appears to account for up to 90% of the total oxidant levels in cities that have a mild sunny climate (Butkovic et al., 1990). Ozone is generated at ground level by photochemical reactions that involve ultraviolet radiation of atmospheric mixtures of NO_2 and hydrocarbons that can derive from vehicle emissions. These O_3 trends depend not only on the substrate supply (NO_2 emitted by cars), but also on the sunny weather, because of the transformation of NO_2 into O_3 during a photochemical smog.

Current safety standards for O_3 levels are exceeded frequently in most Mediterranean countries. Indeed, the 8 h average levels of O_3 for the period from 2000 to 2004 in different sites in Italy showed that the background O_3 pollution exceeded the European standards (Paoletti et al., 2007) that were fixed by the European Union at 0.060 ppm (Directive 2002/3/EC, 2004).

Ozone can affect animal and plant metabolism. Its toxicity is due to the generation of reactive oxygen species (ROS), such as the superoxide anion radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\bullet OH$), and singlet oxygen ($^1O_2$) (Mudd, 1997). The O_3 effect on pollen grains has been considered in particular in studies of plant germination *in vitro*. However, opposing effects have been reported: Feder (1968, 1981) observed a reduction in *Nicotiana tabacum* pollen tube elongation, and similar results were reported for corn pollen by Mumford et al. (1972). In contrast, Benoit et al. (1983) reported that the ability of the pollen of *Pinus strobus* to germinate was not significantly reduced by O_3 fumigation. It has also been reported that O_3 can influence allergen release from pollens grains, and Masuch et al. (1997) found that O_3 increases the content of group 5 allergenic proteins of *Lolium perenne*. When the pollen comes into contact with the airway mucosa, it releases not only allergenic proteins, but also lipid immunomodulators, such as pollen-associated lipid mediators (Traidl-Hoffmann et al., 2002), and nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidases (Boldogh et al., 2005). These latter are enzymes that have an oxidase activity that can produce the $\bullet O_2^-$ ion, which is converted into H_2O_2 through the action of the enzyme superoxide dismutase (SOD). Therefore the NAD(P)H oxidases have fundamental roles in inflammatory processes, as they can increase the levels of ROS in the epithelium of the respiratory apparatus, and promote the flow of neutrophils towards the respiratory apparatus (Boldogh et al., 2005).

On the basis of the concomitance of episodes of high O_3 levels (Gabusi and Volta, 2005) and the high concentrations of ragweed pollen in the atmosphere during the summer months (Mandrioli et al., 1998), the aim of the present study was to determine whether O_3 affects mature ragweed pollen grains after their dispersal. In particular, we determine first whether O_3 can increase the allergy potency of ragweed pollen by stimulating allergen and NAD(P)H oxidase release, and secondly whether O_3 can induce physiological alterations that affect pollen viability, which is the ability of the pollen to complete post-pollination events and to achieve fertilisation.

For this purpose, under laboratory conditions, we exposed ragweed pollen to a high O_3 concentration (100 nL L^{-1}) during the day. This concentration corresponds to peak O_3 levels measured during the summer in central Italy (http//www.arpa.umbria.it). We specifically evaluated: (i) pollen ROS and NO content, as NO is considered to be a mediator of inflammatory responses (Moilanen and Vapaatal, 1995); (ii) activity of the NAD(P)H oxidase, which can generate ROS; (iii) expression of the major ragweed pollen allergens; and (iv) pollen viability.

2. Materials and methods

2.1. Pollen

Ragweed (*Ambrosia artemisiifolia*) pollen was purchased from Greer Laboratories (Lenoir, NC, USA). The pollen was aliquoted into 2 mL, sterile microcentrifuge tubes, and stored dry at ≤ 4 °C until use.

2.2. Ozone treatment

The O_3 treatment was performed by exposure of 0.5 g pollen in Petri dishes to 100 nL L^{-1} O_3 for 5 h (08:00 h to 13:00 h) per day for 7 consecutive days in a plexiglass chamber (0.32 m^3) under light with a photosynthetic photon fluence of 120 μmol m^{-2} s^{-1}, as previously described (Pasqualini et al., 2009). A non-fumigated ($-O_3$) pollen sample was maintained in a filtered-air plexiglass chamber under the same experimental conditions. After each daily O_3 fumigation, the pollen samples were all left in a growth chamber under controlled conditions (14 h photoperiod, photosynthetic photon fluence rate of 120 μmol m^{-2} s^{-1}, day/night air temperature 25 °C/20 °C, and relative humidity 60%–75%) until the next treatment. After the 7 days of this O_3 fumigation, the pollen was either immediately analysed for viability and ROS, H_2O_2 and NO content, or frozen under liquid N_2 and stored at −80 °C for protein quantification, NAD(P)H oxidase activity assay, and RNA analysis. The O_3 treatment was replicated three times.

2.3. Pollen viability

Pollen viability was estimated using the fluorescein diacetate stain (FDA; Sigma–Aldrich, St. Louis, MO, USA), as reported by Heslop-Harrison and Heslop-Harrison (1970). Pollen grains (2 mg) were hydrated for 30 min in 2 mL 0.4 M sucrose. Then 20 μl of these suspensions were placed on a microscope slide and stained with 4 μM FDA. The total number of pollen grains was visually counted using bright-field microscopy, while the fluorescent pollen grains in the same field of view were counted using a UV epifluorescence microscope (DMLB; Leica, Leica Microsystems, Wetzlar, Germany) with a 450 nm excitation filter and a 535 nm emission filter. Pollen viability was determined as the percentage of fluorescing pollens relative to the total pollen grains. For each sample (control and O_3 treated), ten slides were prepared, and for each slide, at least 100 pollen grains were counted.

2.4. Determination of intracellular nitric oxide

Intracellular NO was visualised as according to the method of Bright et al. (2009) with slight modifications using the fluorescent NO indicator dye 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; Molecular Probes, Invitrogen, Carlsbad, CA, USA) (Kojima et al., 1998). The pollen (1 mg) was first incubated for 30 min at 4 °C in 1 mL MES–KCl buffer containing 8% sucrose, 10 mM MES, 5 mM KCl, 50 mM $CaCl_2$ (pH 6.8), in the absence and presence of 200 μM of the NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma–Aldrich). DAF-FM diacetate (10 μM) was then added, and the samples were incubated in the dark for 20 min, to allow the dye to enter the pollen. The pollen suspensions were then centrifuged at 6500 × *g* for 1 min, and the resulting supernatant was discarded. Fresh MES–KCl buffer was then added to the pellets, which were resuspended and left at room temperature for 20 min. Finally, 50 μl pollen suspension were placed onto a glass slide and covered with a glass coverslip before being examined with a UV epifluorescence microscope with a 450 nm excitation filter and a 535 nm emission filter. Intracellular NO was determined as the percentage of fluorescing pollens relative to the total pollen grains. For each sample (control and O_3 treated), six slides were prepared, and for each slide, at least 100 pollen grains were counted.

2.5. Detection of the reactive oxygen species

ROS detection was performed using the fluorescent ROS indicator dye 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Molecular Probes) (Setsukinai et al., 2003). Pollen grains (2 mg aliquots) were hydrated at 4 °C in 2 mL 100 mM phosphate-buffered saline (PBS) for 30 min, and then transferred to 2 mL microcentrifuge tubes and centrifuged at 2000 × *g* for 30 s. After centrifugation, the supernatant was discarded and fresh PBS containing 2.5 μM $DCFH_2$-DA was added to