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Ozone-induced caspase-like activities are dependent on early ion channel regulations and ROS generation in *Arabidopsis thaliana* cells

Daniel Tran,[1,2] Marika Rossi,[3] Bernadette Biligui,[1,2] Tomonori Kawano,[3-6] Stefano Mancuso[3,5,6] and François Bouteau[1,2,3,5,*]

[1]Univ Paris Diderot, Sorbonne Paris Cité; Institut des Energies de Demain (IED, FRE 3597); Paris, France; [2]Institut de Biologie des Plantes; Orsay, France; [3]LINV-Department of Plant Soil & Environmental Science; University of Florence; Florence, Italy; [4]Graduate School of Environmental Engineering; University of Kitakyushu 1-1; Kitakyushu, Japan; [5]University of Florence LINV Kitakyushu Research Center; Kitakyushu, Japan; [6]Univ Paris Diderot, Sorbonne Paris Cité; Paris Interdisciplinary Energy Research Institute (PIERI); Paris, France

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Using *A. thaliana* cultured cells; we recently reported new insights regarding the effect of acute O_3 exposure.[1-3] This consist in an oxidative dependent controlled cell death process involving cell shrinkage due to an early activation of anion channel[1] and a delayed activation of K^+ outward currents,[2] but also to early events like Ca^{2+} influx or singlet oxygen production possibly linked to mitochondrial dysfunction.[1] Here we provide evidence that most of these early events act downstream of caspase-like activities as recently demonstrated for K^+ channel activation.[2]

Programmed cell death (PCD) is a fundamental process remarkably conserved in eukaryotes. In plants as well as animals or simple eukaryote, oxidative stress conditions were shown to induce such PCD.[4] Although apoptosis, the best-defined form of animal PCD, cannot occur in plant cells, plants and animals do share some common characteristics of PCD.[5,6] It is well accepted that caspases play a major role in the PCD process within animal systems. Currently, no true caspases have been found within plant systems, but evidence suggests that caspase-like proteins participate in plant PCD.[4,7] We demonstrated that O_3, a major secondary air pollutant, induced an acute cell death in suspension cells of *Arabidopsis thaliana*.[1-3] The development of this cell death needs an active metabolism and induces large cell shrinkage,[1] hallmarks of PCD. It further requires caspase-like activities.[2,8] Cell shrinkage has been proven to be a critical initial molecular event that involves dysregulation of the cellular ionic status and the subsequent progression of apoptosis, e.g., caspase and nuclease activation.[9,10] We effectively showed that the caspase-like activities and cell death induced by O_3 were dependent on a delayed, ROS sensitive, activation of K^+ outward rectifying currents through GORK channels.[2] Here, we further show that caspase-like activities induced by acute O_3 exposure are also dependent on different early events participating to development of O_3-induced PCD, the activation of anion and Ca^{2+}channels and the generation of singlet oxygen.[1]

Ozone Induces a Ca^{2+} and Anion Channel-Dependent Activation of Caspase-Like Activities

Arabidopsis thaliana L. cell suspensions were grown in Gamborg culture medium under continuous shaking (120 rpm) at 22 ± 2°C.[2] Experiments were performed using log-phase cells 4 d after sub-culture. Ozone exposure of the cell suspension (50% packed cell volume, PCV) have been conducted using 0.1 L/min; 10 mg O_3/h (35 ppm) which allow reaching 80% of cell death in 2 h.[1,2] The experiment was conducted in vivo by incubating the cells with a pan-caspase fluorescent inhibitor (zVADfmk) (CaspAce™, Promega, Ex = 490 nm, Em = 528 nm, Promega) known to interact with activated caspase-like in plant cells.[11,12] Briefly, cells were incubated with 10 μM FITC-VAD-fmk during 15 min before treatment. All samples were placed between slide and coverslip and imaged using an oil immersion objective (40×, N.A. 1.25) and observations were performed on a laser scanning confocal microscope (Leica SP2 AOBS AOTF). Images were analyzed using Leica Confocal Software.[2] No significant fluorescence was observed in control cells (**Fig. 1A**), whereas green fluorescent patches appeared progressively in O_3-treated cells suggesting that caspase-like proteins were activated (**Fig. 1A–C**). The caspase-like activity was observed mostly in the nucleus and just feebly in the cytoplasm (**Fig. 1M**). Pre-treatment of the cell with the Ca^{2+} channel La^{3+} before the O_3 pulse significantly

*Correspondence to: François Bouteau; Email: francois.bouteau@univ-paris-diderot.fr
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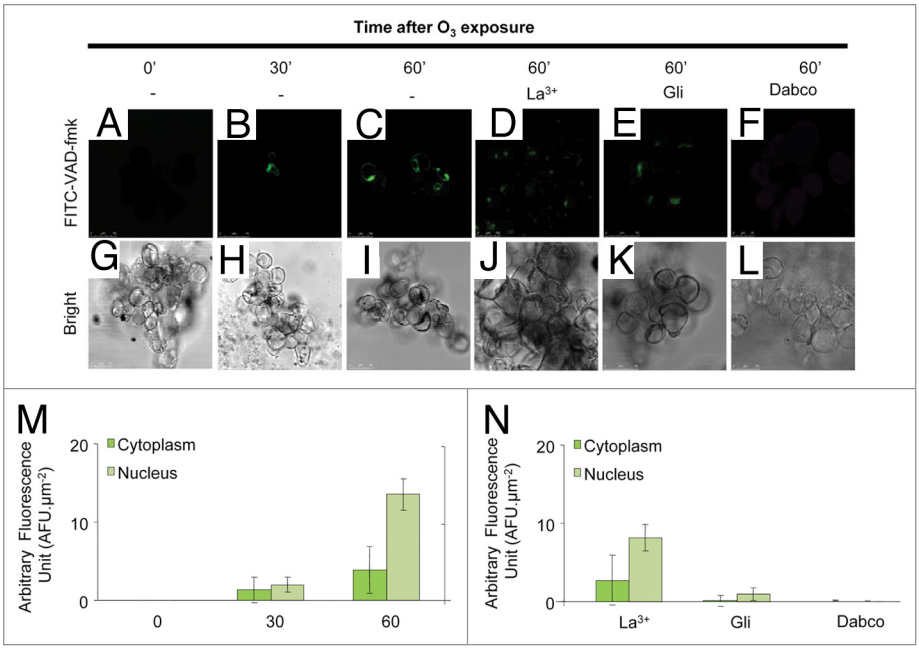

Figure 1. Time-dependent O_3-induced caspase-like activity and its modulation by ionic channels and singlet oxygen. O_3-induced cell death in *A. thaliana* cells was achieved by exposing cell suspensions to a 10 min pulse of ozonized air.[1] Observation of caspase-like activities induced by O_3 treatment visualized with the fluorescent caspase inhibitor FITC-VAD-fmk (green fluorescence in cells) was time dependent (**A–C**). Cells pre-treated with (**D**) a Ca^{2+} channel blocker La^{3+} (500 µM), (**E**) an anion channel blocker Glibenclamide (gli 200 µM) or (**F**) a scavenger of singlet oxygen Dabco (5 mM) 15 min prior to O_3 treatment. Corresponding bright field images (**G–L**). Scale bar = 50 µm. Each image is representative of symptoms observed in three independent experiments. (**M and N**) Quantification of the fluorescence according to the treatment and the cellular compartment.

reduced the labeling in nucleus (**Fig. 1D and N**). Pre-treatment of the cell with the anion channel blocker glibenclamide before the O_3 pulse drastically reduced the labeling in the cytoplasm and nucleus (**Fig. 1E and N**). These data indicates that the activation of a plasma membrane anion and Ca^{2+} channels occurs upstream of capase-like activity stimulation by O_3. These data could be correlated with the anion channel dependent increase in transcripts of the vacuolar processing enzyme gene (*VPE*γ) by O_3,[1] the enzyme presenting capsase-1 activity.[13]

Ozone Induces a Singlet Oxygen-Dependent Activation of Caspase-Like Activities

Several different pathways leading to cell death seemed to be triggered in response to O_3,[1] among them a mitochondrial pathway.[14] Since in our model DABCO, a scavenger of singlet oxygen, was also found to drastically reduce the O_3-induced cell death and mitochondrial depolarization,[1] a classic hallmark of cell undergoing PCD and a prerequisite to release of caspase from mitochondria in animal,[15] we further test its impact on caspase-like activities. As observed for ion channel blockers, DABCO drastically reduced the caspase-like activities since no green fluorescent patches could be detected in O_3-treated cells after DABCO pre-treatment (**Fig. 1F–N**).

Our data suggest that in addition to the delayed outward rectifying K^+ channel activation,[2] the activation of anion channel[1] and in a minor way the Ca^{2+} influx,[1] as a production of singlet oxygen possibly linked to mitochondrial dysfunction could also participate to the activation of caspase-like activities. Further studies are thus needed to understand the nature, role and regulation of the caspase-like proteins in O_3-induced cell death.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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