PHYTOSTEROL ALTERATIONS RESPONDING TO ROS INHIBITORS BY GC-MS IN ARABIDOPSIS

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Abstract

Phytosterols, essential components of cellular membrane integrity, play an important role in plant growth, development, and responses to various types of stressors. Emerging studies have proposed that phytosterols are a result of adaptation to the aerobic environment during evolution. We reasoned that phytosterols could dramatically respond to changes in external reactive oxygen species (ROS) levels. The levels of five phytosterols were measured by gas chromatography-mass spectrometry (GC-MS) after Arabidopsis plants were treated with the ROS-altering drugs 2'-3'-dideoxycytidine (DDC), H2O2, Catalase (CAT), diphenyleneiodonium (DPI) and salicylhydroxamic acid (SHAM) and auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and 1-N-naphthylphthalamic acid (NPA). We found that all redox-altering agents can dramatically reduce sterol levels, whereas CAT and NPA can partly increase them. Our results establish a link between redox balance and sterol level alteration.

Key words: ROS; ROS inhibitors; Sterol; Lipid rafts.

Introduction

Sterols, a type of lipid, are components of eukaryotic membranes, and have been suggested to maintain the domain structure of cell membranes by acting as membrane reinforcing agents (Dufourc, 2008). Together with sphingolipids (e.g., sphingomyelin and glycosphingolipids) and associated proteins, sterols are usually organized into liquid-ordered lipid domains, known as lipid rafts, which are thought to play an important role in fundamental biological processes, such as signal transduction, cellular trafficking, polarized secretion, membrane transport, and cytoskeleton reorganization (Simons & Ehehalt, 2002; Helms & Zurzolo, 2004; Bieberich, 2018). In terms of structure, cholesterol and ergosterol are the lipids enriched in animal and fungal cell membranes, respectively; however, their plant counterparts display a much greater structural variability. In addition to cholesterol, many cholesterol analogues in plants, such as campesterol, stigmastanol, and β-sitosterol, have been identified from detergent-resistant membranes, which are supposedly equivalent to lipid rafts (Mongrand et al., 2004; Borner et al., 2005). Plant species possess more than 250 different sterols (Akihisa et al., 1991), but campesterol, stigmastanol, and β-sitosterol are the dominant constituents of plant sterol profiles, implying that fluctuation in the levels of these major sterols can affect the physical function of lipid rafts.

The specific functions of each sterol are largely unknown. From the viewpoint of evolution, the advent of sterols is teleologically related to the increasing concentrations of oxygen that occurred around 2.5 billion years ago (Galea & Brown, 2009). Therefore, sterols may be considered to have been part of a primitive cellular defense system against oxygen and ROS (ROS). Indeed, lipid rafts that are composed of phytosterols work as an important redox signaling platform for the assembly of the NADPH oxidase complex and activation of downstream redox regulation of cell functions (Jin et al., 2011). In plants, lipid rafts also contain NADPH oxidase (Mongrand et al., 2004) and cytosolic NADH-ubiquinone oxido-reductases. The presence of these conserved components in lipid rafts strongly suggests that redox signaling is associated with the physical function of lipid rafts. The objective of the study was to examine the effects of several ROS-altering agents on the levels of five phytosterol compounds compared with the control samples.

Material and Methods

Plant materials and growth condition: Arabidopsis thaliana Col (Columbia wild type) was grown on a vertical plate containing 1X MS medium in a greenhouse for 7 days at 22°C under long days (15 h light and 9 h dark cycles) with white light illumination. Seven-day-old plants were transferred into a medium containing chemical compounds (DDC, H2O2, SHAM, DPI, CAT, TIBA, and NPA) for 7 days in a greenhouse under the same growing conditions. The dosages of these drugs are indicated in Table 1.

Phytosterol extraction and GC-MS analysis: Sterol extraction was performed according to the method described by Zhang et al. (2017) with some modifications. Freeze-dried samples (20 mg) were ground in liquid nitrogen and extracted with 4 mL of chloroform and methanol mixture (2:1, v/v), and then filtered through a rephiquik syringe filter (PVDF, 0.2 µm). The filtered mixture was further incubated at 70 °C for 1 h and dried using a vacuum freeze drier. 5α-cholestane (12.5 µL of 2 mg/mL) was added to the mixture as an internal standard. The samples were saponified with 2 mL 6% (w/v) KOH in methanol for 3 h at 90°C to release the sterol moiety of the sterol ester. Sterols were extracted three times with 2 mL of hexane and water (1:1) and dried using a vacuum freeze drier. The dried residues were derived with 100 µL BSTFA-TMCS (99:1) for 25 min at 75°C, and the volume
of the mixture was brought up to 500 µL with N-hexane. Sterol level was analyzed on DB-5 MS column (30 m × 0.25 mm × 0.25 μm, Agilent technologies) with helium as carrier gas. The temperature program was as follows: 100°C for 5 min., ramped to 280°C at 10°C/min., 300°C at 5°C/min., and held for 20 min (Zhang et al., 2017).

Results

Seven-day-old Arabidopsis thaliana (Col) seedlings were treated with the ROS-altering drugs 2′-3′-dideoxycytidine (DDC), H₂O₂, CAT, diphenyleneiodonium (DPI), and salicylhydroxamic acid (SHAM), along with auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and 1-N-naphthylphthalamic acid (Table 1), and the growth phenotype was usually obvious after 7 days (Fig. 1). We first tested the effect of H₂O₂ on sterol levels, given that it is a strong oxidant that is capable of oxidizing lipids and impacting membrane permeability (Floyd & Lewis, 1983; Bienert et al., 2007; Ouchi et al., 2019). We found that it significantly reduced campesterol and stigmasterol levels by 41% and 47%, respectively, relative to their levels in untreated plants, but slightly increased cholesterol level (Fig. 2A). These results prompted us to investigate whether the external reduction of H₂O₂ could increase sterol levels. A significant increase in cholesterol and campesterol levels when CAT was added to the growth medium (Fig. 2). Interestingly, the brassicasterol level was also greatly increased (more than three-fold) compared to that in untreated plants. Therefore, the presence of CAT, an antioxidant enzyme that scavenges H₂O₂ by dismutating it into molecular oxygen and water (Nicholls, 2012), can improve sterol levels.

We then sought to examine the effects of the superoxide dismutase inhibitor DDC (Maître et al., 1993), a peroxidase inhibitor SHAM (Kukavica et al., 2012), and a plasma membrane NADPH oxidase inhibitor, DPI (Bolwell et al., 1998) on sterol level alterations. Plants treated with DDC had much less campesterol, stigmasterol, and β-sitosterol, but there was no obvious effect on cholesterol and brassicasterol levels (Fig. 3). SHAM strongly reduced the sterol content; in particular, cholesterol and brassicasterol were decreased to approximately 44% and 15% of basal levels, respectively. DPI also reduced all sterol levels except those of brassicasterol, with the greatest impact on cholesterol levels (decreased by 71%).

Fig. 1. The phenotypic changes in Arabidopsis thaliana (Col) treated with different drugs. A, Arabidopsis plants on drug-free medium. B, 14-day old Arabidopsis plants treated with DDC. C, 14-day old Arabidopsis plants treated with H₂O₂. D, 14-day old Arabidopsis plants treated with CAT. E, 14-day old Arabidopsis plants treated with SHAM. F, 14-day old Arabidopsis plants treated with DPI. G, 14-day old Arabidopsis plants treated with TIBA. H, 14-day old Arabidopsis plants treated with NPA.
PHYTOSTEROL RESPONSE TO ROS INHIBITORS

Table 1. Various inhibitors used in this study.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Final concentration</th>
<th>Effect</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
<td>3 mM</td>
<td>Strong oxidant</td>
<td>(Floyd &amp; Lewis, 1983; Bienert et al., 2007; Ouchi et al., 2019)</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
<td>100 μg/ml</td>
<td>Antioxidant enzyme to scavenge H$_2$O$_2$</td>
<td>(Nicholls, 2012)</td>
</tr>
<tr>
<td>DDC</td>
<td>Diethyldithiocarbamic acid</td>
<td>10 mM</td>
<td>Superoxide dismutase inhibitor</td>
<td>(Maître et al., 1993)</td>
</tr>
<tr>
<td>SHAM</td>
<td>Salicylhydroxamic acid</td>
<td>100 μM</td>
<td>Peroxidase inhibitor</td>
<td>(Kukavica et al., 2012)</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
<td>1 μM</td>
<td>NADPH oxidase inhibitor</td>
<td>(Bolwell et al., 1998)</td>
</tr>
<tr>
<td>NPA</td>
<td>N-1-naphthylphthalamic acid</td>
<td>100 μM</td>
<td>Polar auxin transport inhibitor</td>
<td>(Kliimbt, 1992)</td>
</tr>
<tr>
<td>TIBA</td>
<td>2,3,5-triiodobenzoic acid</td>
<td>50 μM</td>
<td>Polar auxin transport inhibitor</td>
<td>(Choi et al., 1997)</td>
</tr>
</tbody>
</table>

Fig. 2. Measurement of phytosterol levels after treatment with H$_2$O$_2$ and CAT. Col refers to the untreated control group. The content of cholesterol (A), brassicasterol (B), campesterol (C), stigmasterol (D), β-sitosterol (E) was measured under H$_2$O$_2$ and CAT treatment conditions. Error bars represent standard deviation over three independent experiments; the data were evaluated by Student's t-test to determine statistical significance. *: p<0.05; **: p<0.01.

Fig. 3. Phytosterol level under DPI, SHAM, and DDC treatment. Col refers to the untreated control group. The contents of cholesterol (A), brassicasterol (B), campesterol (C), stigmasterol (D), and β-sitosterol (E) were measured under DPI, SHAM, and DDC treatment conditions. Error bars represent standard deviation over three independent experiments; the data were evaluated by Student's t-test to determine statistical significance. *: p<0.05; **: p<0.01.
Since sterols are essential components of lipid rafts and a previous study has shown that auxin efflux proteins are also associated with sterol- and sphingolipid-enriched lipid rafts (Yang et al., 2013), we sought to test the effect of the auxin efflux inhibitors NPA (Kliimt, 1992) and TIBA (Choi et al., 1997) on sterol levels. As shown in Fig. 1, 100 µM NPA can strongly reduce plant size and inhibit lateral root formation after a 7-day treatment, suggesting an inhibitory effect. Interestingly, NPA treatment led to a significant increase in campesterol, brassicasterol, and β-sitosterol levels, but a decrease in stigmasterol. However, TIBA, reduced all sterol levels except those of cholesterol, suggesting that TIBA and NPA have different modes of regulation on sterol levels.

**Discussion**

Galea & Brown (2009) hypothesized the relationship between ROS and sterol formation more than 10 years ago. They reported that sterols might have evolved as an adaptive mechanism for protection against oxygen and/or ROS in eukaryotes. The synthesis of sterols, such as ergosterol in fungi, cholesterol in animals, and β-sitosterol in plants, is an oxygen-intensive process; 12, 11, and 11 molecules of O₂ are required for ergosterol, cholesterol, and β-sitosterol synthesis, respectively (Summons et al., 2006). These sterols are susceptible to ROS oxidation, and the depletion of sterols increases susceptibility to cellular ROS attack (Iuliano, 2011). More specifically, the 5,6-double bond and the allylic C7 carbon in the B ring of cholesterol confer susceptibility to ROS and other radicals, such as peroxyl radicals (Murphy & Johnson, 2008). Since phytosterols contain the same ring skeleton as cholesterol, phytosterols would presumable be subjected to ROS oxidation in a manner similar to that of cholesterol in animals, resulting in oxidized derivatives, such as hydroxy-, keto-, epoxy-, and triol-phytosterol (Murphy & Johnson, 2008). Overall, our study documented an altered level of many sterols upon introduction of various ROS interfering agents, supporting the view that sterols work as a cornerstone for oxygen adaption and also a barrier to redox attack (Dotson et al., 2017; Zuniga-Hertz & Patel, 2019).

Because DPI can induce mitochondrial superoxide production by inhibiting NAD(P)H oxidase (Li et al., 2003) and DDC can cause superoxide accumulation by inhibiting superoxide dismutase (Biagini et al., 1995), sterol depletion could be associated with increased superoxide. However, caution is needed when interpreting these data. That is, the sterol decrease by DPI and DDC may not be directly attributed to the superoxide oxidizing attack on sterols, given that there was no reaction between superoxide and cholesterol in an *in vitro* experiment (Lee et al., 1997). However, superoxide and H₂O₂ together led to the production of oxidized cholesterol molecules, such as keto-, hydroxy-, and hydro-cholesterol (Lee et al., 1997), and the simultaneous production of superoxide and H₂O₂ could generate a strong inhibitory effect. Indeed, the application of SHAM, a peroxidase inhibitor that can promote ROS production (Brouwer et al., 1986; Tsukagoshi et al., 2010; Samuilov & Kiselevsky, 2016), led to a reduction in the contents of all measured sterols, suggesting that the strong inhibitory effect conferred by SHAM might be associated with the generation of multiple reactive species.

Compared with superoxide, H₂O₂ is much more stable and displays high oxidative activity, and an *In vitro* experiment showed that it did not oxidize cholesterol directly (Morzycki & Sobkowiak, 2015). In contrast, animal cells accumulated cholesterol after a short period of treatment with H₂O₂ (Ryu et al., 2016), which is in agreement with previous findings showing that DHCR24 catalyzes the conversion of desmosterol to cholesterol by scavenging H₂O₂ (Waterham et al., 2001; Lu et al., 2008).
Similarly, the slightly elevated level of cholesterol by H$_2$O$_2$ was also detected in Arabidopsis in this study, implying a conserved response in both animal and plant cell. Interestingly, the addition of CAT to the plant growth medium increased the contents of almost all sterols, strongly suggesting that the depletion of environmental H$_2$O$_2$ could boost endogenous sterol levels. Since H$_2$O$_2$ can function as a signaling molecule and is particularly involved in establishing intercellular tunnels (Liang, 2018), the current results do not clarify the target sterol of H$_2$O$_2$ oxidation, and further study is needed to elucidate the role of H$_2$O$_2$ signaling in sterol metabolism.

Lipid rafts play a significant role as signaling hubs (Simons & Toomre, 2000). Lipids rafts have been shown to be associated with one of the most important signaling pathways in plants, the auxin signaling pathway (Yang et al., 2013). Our data showed that TIBA can lower the contents of all measured sterols, suggesting a link between auxin efflux and sterol-enriched lipid rafts. However, NPA, another auxin efflux inhibitor, exerted different effects on sterol content. In contrast to TIBA, NPA increased brassicasterol, campesterol, and β-sitosterol content. This difference could be due to the fact that TIBA and NPA belong to different classes of transporter inhibitors, and have the differing working mechanisms (Teale & Palme, 2017). Further studies are needed to clarify the role of these inhibitors in sterol level alteration.

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**References**


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