Ascorbate and Dehydroascorbate Influence Cell Cycle Progression in a Tobacco Cell Suspension

Geert Potters*, Nele Horemans, Roland Julien Caubergs, and Han Asard
Department of Biology, University of Antwerp, Groenenborgerlaan 171, B–2020 Antwerp, Belgium

In addition to its well-known antioxidant properties, ascorbate (ASC) is capable of influencing normal cell cycle progression in plants. In this work we demonstrate that the oxidized molecule dehydroascorbate (DHA) is the active redox form in this respect. Our results indicate that the reduction of DHA might constitute the first step leading to this effect. During the last few years, evidence has already been accumulating, indicating the role of ASC in the regulation of cell division. For example, De Cabo et al. (1993) demonstrated a shortening of the G1 phase in dividing onion root meristem cells due to the action of monodehydroascorbate. Kerk and Feldman (1995) were able to establish a direct correlation between ASC redox status and cell proliferation rates in the maize (Zea mays) quiescent center. Also, during the growth cycle of a tobacco (Nicotiana tabacum L. cv Bright Yellow-2 [BY-2]) cell suspension, a significant decrease in the endogenous ASC level was shown, accompanied by an overall decrease in redox status (De Pinto et al., 1999; Kato and Esaka, 1999).

Kato and Esaka (1999) provided further evidence pointing at a possible ASC-mediated redox control of the cell cycle by demonstrating a transient peak in the DHA concentration, with a concomitant decrease in the ASC to DHA ratio during M phase. This increase also correlated with a temporary increase in ASC-oxidase expression. These results suggested the hypothesis that intracellular levels of DHA might be controlled by the cell cycle through the expression of ASC-oxidase. In addition the decrease in DHA could also constitute a necessary and positive signal for the cell to proceed into S phase (Kato and Esaka, 1999).

De Pinto et al. (1999) also suggested a role for DHA as a specific redox link between the apoplast and the cytoplasm. Because ASC is the major antioxidant in the apoplast, it is a likely candidate to play a role in the signaling from the apoplast to the cytoplasm related to the oxidative properties of a possibly stressful environment. The presence of a DHA transporter has recently been demonstrated at the plasma membrane of higher plant cells (Horemans et al., 2000). This transporter assists in the uptake into the cell of apoplastic DHA, resulting from the consumption of apoplastic ASC in a variety of different possible oxidative reactions; therefore, the transporter might relay the apoplastic redox status to the interior of the cell. The addition of external DHA is likely to affect the internal redox balances, possibly through its glutathione-dependent reduction. In this respect Reichheld et al. (1999) suggested the existence of an oxidative stress checkpoint pathway that controls cell cycle progression in environmental stress conditions and is seemingly responsive to one or more redox-sensing systems. For example, menadione (known to cause oxidative stress by generating oxygen radicals) has been shown to impair the G1/S phase transition in BY-2 cells (Reichheld et al., 1999). Thus, the ASC molecule is gradually considered not only an essential antioxidant, but it also plays a key role in plant cell signaling.

The literature discussed so far led to the hypothesis that induction of a shift in the intracellular ASC to DHA ratio should influence cell cycle progression. Here we describe the progression of the cell cycle after addition of ASC and DHA to a synchronized BY-2 cell suspension culture (Nagata et al., 1992). The results show an inhibition of cell cycle progression and provide a basis for the development of future experiments to uncover the links between oxidative stress, cellular redox status, and the control of cell division.

CELL CYCLE PROGRESSION IS SLOWED DOWN BY DHA AND ASC

To investigate the response of BY-2 cells with regard to cell cycle progression following the addition of DHA, we used a synchronized culture. Synchronization was performed according to Nagata et al. (1992). Thirteen milliliters of a stationary culture was transferred to 100 mL of fresh medium and supplemented with the S phase blocker aphidicolin (5 µg mL⁻¹). After 24 h cells were washed thoroughly and returned to the shaker; 3 h later propyzamide (6 µM) was added to block cells in prometaphase. After another 6 h cells were washed again. Mitotic index at each time point was determined microscopically with the fluorescent dye 4′,6-diamino-2-phenylindole, as described by Reichheld et al. (1999). A synchronization degree of ≥ 95% could be obtained (i.e.: the
mitotic index immediately after propyzamide release, data not shown). After one cycle the synchrony had fallen to a maximum of 15%. However, compared to the mitotic index in a non-synchronized culture (3%), or during the rest of the experiment (1%), this level of synchrony was still high enough to be used as a marker for cell cycle progression.

Addition of 1 mM DHA during G1 phase (2 h after release from the propyzamide block) resulted in a shift in the mitotic index (Fig. 1). DHA-treated cells reached the same maximal mitotic index as control cells (approximately 14%) only 4 h later. On the other hand, after addition of ASC (1 mM, during G1), an effect on the mitotic index could be observed, although in this case a drop in the percentage of dividing cells, rather than a time shift, was apparent. A significant ($P < 0.05$) difference exists between the 16-h time point mitotic index of ASC- and DHA-treated cells, providing additional proof for the different responses of the time curves after different treatments. Viability of the cell culture at the same time as the peak in mitotic index (13 h after propyzamide release) was not affected by the DHA, nor was it affected by the ASC (85% viable cells). These data demonstrate that exogenous DHA is able to induce a delay in cell cycle progression.

Oxidative stress induced by a variety of treatments including ozone (Luwe et al., 1993; Conklin et al., 1996), wounding (Takahama, 1993), or salt stress (Gossett et al., 1994), has been indicated to favor the aspartic acid of the plasmolysis to DHA. Francis (1998) suggested that cell cycle length differences due to stress factors are mainly attributable to changes in G1 length. Therefore, the effect of DHA on BY-2 cell cycle progression might be similar to the cells' response under oxidative stress. Redox signals in the cellular environment might therefore be interpreted as a signal to halt the cell cycle, before the cell decides to progress into another cell cycle round, until survival chances improve. This view is also supported by the observation of Kato and Esaka (1999) that the endogenous DHA content is increased during M phase. The resulting delay of the cell cycle, during which the superficial DHA is reduced, will grant the cells time to assess the environment.

**TIME KINETICS OF EXTERNAL AND INTERNAL DHA/ASC CONCENTRATIONS**

To investigate whether the addition of ASC and/or DHA effectively altered the internal ASC redox status, we monitored the levels of ASC and DHA in the medium and in the cells following the addition of ASC or DHA. In these experiments, non-synchronized BY-2 cells in full exponential growth phase were used. At intervals of 1 h, cells were collected on a Büchner filter, and aliquots (around 0.1 g fresh weight) were resuspended in 3% (w/v) m-phosphoric acid, 1% (w/v) polyvinylpyrrolidone, and snap-frozen in liquid nitrogen. ASC and DHA were subsequently extracted through three cycles of freezing and thawing; the homogenate was centrifuged at 50,000 g for 15 min at 4°C. The supernatant was used for ASC determination on reverse phase HPLC (RP type C-18 column, LiChroSpher, Alltech, Deerfield, IL; isocratic pump, 0.8 mL min$^{-1}$, LC-10ADVP, Shimadzu, Columbia, MD) coupled to an electrochemical detection system (reference potential 700 mV). Chromatogram analysis was performed with a Class VP software package (Shimadzu). Total ASC (ASC + DHA) was determined by reducing 100 μL of each sample with a 60-μL-solution consisting of 200 mM dithiothreitol and 400 mM Tris (pH 6). The DHA concentration was estimated as the difference between the reduced and total ASC concentration.

After addition of 1 mM DHA, the DHA disappeared rapidly from the medium (Fig. 2A), and after 4 h, DHA levels in the medium had fallen to control levels. Six hours after DHA addition, the ASC concentration in the medium seemed to rise (Fig. 2B). Internal ASC concentrations (Fig. 2C) started to rise immediately after the addition of DHA. Whereas the internal concentration of ASC was significantly increased following addition of DHA, the internal redox balance was not significantly affected (85% ASC).

When ASC (1 mM) was added to the medium, a rapid oxidation to DHA could be observed (Fig. 2, A and B). ASC disappeared from the medium only after oxidation (Fig. 2B). This disappearance was accompanied by an increase in the internal ASC concentration (Fig. 2C). A change in the internal redox status could not be observed after addition of ASC, suggesting a quick reduction of the DHA upon entering the cytoplasm. These results support the model outlined by Horemans et al. (1998) about the possible function of a DHA carrier at the plasma.

**Figure 1.** Effect of addition of ASC and DHA on the mitotic index of a synchronized BY-2 cell culture. Control: ○, full line; 1 mM DHA: ▲, dashed line; 1 mM ASC: ■, dashed-dotted line. Values are given with se; n = 3. Abscissa presents the time after propyzamide release.
membrane of BY-2 cells. The fact that the cytoplasmic ASC to DHA ratio does not change agrees with the findings of Takahama (1994), who observed a shift in the apoplastic, but not in the cytoplasmic, ASC redox status under different growth conditions. Our results are also consistent with those of De Pinto et al. (1999). These authors demonstrated a 10-fold rise of the internal ASC concentration inside BY-2 cells (after addition of DHA) and a rise in the internal DHA content.

The question remains about the molecular mechanism by which DHA affects the cell cycle. After uptake, DHA is clearly reduced inside the cell, possibly through the ASC glutathione pathway, thereby breaking into the cell’s GSH and NADPH reserves. A number of redox sensitive proteins have been suggested to be involved in this reduction. It has been discussed (Morell et al., 1997; Foyer and Mullineaux, 1997) that there might not be a specific DHA reductase, but that different proteins in the plant cell might fulfill this task. Glutathione is also known to be able to reduce DHA directly. According to De Pinto et al. (1999), addition of DHA did affect the ratios of reduced glutathione to oxidized glutathione and of NADH to NAD$^+$. However, although their data considered proliferation and showed effects over a longer time period (up to 7 d), our data show an immediate DHA effect within hours (Fig. 1). Our results strongly suggest that the effect of DHA and ASC on cell proliferation is directly linked to a DHA influence on cell cycle control. An accurate description of the reduced glutathione to oxidized glutathione and NAD(P)H to NAD(P)$^+$ ratios immediately after DHA addition should be very interesting. Such experiments might provide a quantitative image of fluxes of “reductive potential” to ASC during the DHA reduction. In addition to the ASC glutathione pathway, De Pinto et al. (1999) suggest that DHA might influence ribonucleotide reductase activity, and thereby DNA replication, through the thioredoxin system. Thioredoxin possesses DHA reductase activity (Morell et al., 1997) and might therefore constitute a worthy alternative for the ASC glutathione cycle.

In conclusion, our results demonstrate that, upon exogenous addition, ASC is oxidized to DHA (Fig. 2, A and B), subsequently taken up, and intracellularly re-reduced to ASC (Fig. 2C). Furthermore, DHA exerts an effect upon the cell cycle regulating pathways (Fig. 1). However, to date no evidence is available on the molecular mechanism behind this effect. Since the ASC redox status is apparently not affected, the influence on the cell cycle might result from a transient destabilization of other internal redox balances. The fact that the length of the delay (4 h, Fig. 1) corresponds to the time needed for DHA disappearance from the medium (Fig. 2A) indirectly suggests that the cell cycle delay might be correlated to DHA reduction. However, no information is available yet about possible DHA reduction capacity changes during cell cycle progression or as a result of the synchronization treatment. The observed DHA reduction in non-synchronized cells (Fig. 2) might not occur in synchronized cells. Further experiments are planned to investigate the DHA reduction capacity under these conditions. On the whole, our work shows a link between consumption of apoplastic ASC and physiological consequences for the cell metabolism.

As the front line shielding the internal cell compartments from a possibly hostile environment, the apoplast can both modulate the environment with its defensive systems and relay information about the

Figure 2. A, DHA concentration. B, ASC concentration in the growth medium of a BY-2 cell suspension. C, ASC concentration in the cells in control situation (●, full line), after addition of 1 mM DHA (▲, dashed line) or 1 mM ASC (■, dashed-dotted line). Values are given with SE; n = 2.
environment to the inside of the cell. Redox signaling has been implied in cell growth regulation (González-Reyes et al., 1998; Sánchez-Fernández et al., 1997), and apart from a possible direct effect of an active oxygen species like H$_2$O$_2$, one major redox-based signal might come from the ASC redox status. ASC and its redox forms, occupying a central role in plant defense, might provide the plant cell with an excellent system to sense the environment and react appropriately by contributing to the stress-related control over cell cycle progression on a molecular level and thereby cell proliferation.

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LITERATURE CITED


