

Arrest of mitochondrial biogenesis in copper-treated sycamore cells

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Abstract Sycamore suspension cells (*Acer pseudoplatanus* L.) were grown in the presence of sublethal concentrations of copper (50 μ M). During the first 5–6 days of treatment, growth was not affected, but cell respiration (coupled and uncoupled) declined to ~60% of its normal value. This decline of respiration was attributed to a progressive diminution of the number of mitochondria in copper-treated cells, based on the demonstration of the concomitant decline of (1) cardiolipin (diphosphatidylglycerol) and cytochrome *aa*₃ (cytochrome oxidase), two specific markers of mitochondrial inner membrane, and (2) fumarase activity, a specific marker of mitochondrial matrix space. In addition, the mitochondria extracted from copper-treated cells presented the same properties as those from control cells, concerning substrate oxidation, cardiolipin and cytochrome *aa*₃ contents, and fumarase activity. These results strongly suggest that copper triggered an arrest of mitochondrial biogenesis, which preceded cell division arrest.

Key words: Mitochondria; Respiration; Copper; Stress; *Acer pseudoplatanus*

1. Introduction

Copper is an essential microelement for plants [1], involved in various metalloproteins with key roles in metabolism such as superoxide dismutase, polyphenol oxidase, cytochrome oxidase and plastocyanin. However, this cation becomes strongly phytotoxic at high concentrations. Phytotoxic amounts of copper most frequently originate from urban, industrial (smelters) and agricultural (cupric fungicide treatments) activities. At the level of whole plants, excess copper is known to inhibit growth and photosynthesis, the thresholds of toxicity being dependent on the plant species. The effects of copper on photosynthetic electron transport and photosystems have been extensively characterized using thylacoids or photosystem particles (for review, see [2,3]). Excess copper is also known (1) to inhibit a large number of enzymes, notably enzymes involved in the Benson-Calvin cycle, chlorophyll synthesis, and fatty acid metabolism, (2) to trigger oxidative damage and alteration of cell membrane properties, and (3) to induce thiol-rich polypeptides and enzymes, mostly peroxidases and esterases (for reviews, see [4–7]). However, little is known about the effects of copper on non-photosynthetic metabolism, notably on cell respiration. A partial inhibition of respiration by copper has been reported in the aquatic angiosperm *Vallisneria spiralis* [8]. Induction of the activity of malate and isocitrate dehydrogenases has also been reported in the roots of *Phaseolus vulgaris* grown in excess copper conditions [9].

In the present work, we used sycamore cell suspensions as a model permitting the control of culture conditions, in particular copper concentrations. We report a decrease of respiration in cells treated with sublethal copper concentrations, and we present evidence for a diminution of the number of their mitochondria, suggesting that copper, directly or indirectly, triggered an arrest of mitochondrial biogenesis that preceded growth arrest.

2. Materials and methods

2.1. Materials

Sycamore cells (*Acer pseudoplatanus* L.) were grown at 20°C as a suspension in liquid nutrient medium containing sucrose [10]. The culture medium was kept at a volume of 0.3 l and stirred continuously at 60 rpm. The cell suspensions were maintained in exponential growth by subculturing every 7 days. Control cells were grown with traces of copper (0.2 μ M). In order to maintain a constant supply of copper, the culture medium was renewed every 48 h. Under the wet weight doubling time for control cells was 3.5–4 days and the maximum density of sycamore cells was attained after 13–15 days. The cell wet weight was measured after straining culture aliquots onto a glass fiber filter. The cell number was determined as described by Pascal and Douce [11].

2.2. Isolation and purification of mitochondria

Washed cells (50 g, wet weight) were suspended in their culture medium containing 0.5 M mannitol, 1% (w/v) cellulase and 0.1% pectolyase Y-23 (both enzymes from Seishin Pharmaceutical Co., Nishinomiya, Japan) adjusted to pH 5.7. The cells were incubated with constant shaking (20 cycles/min) at 30°C. This high temperature considerably increases the yield of intact protoplasts. After digestion for 1 h, the suspension was filtered through one layer of Miracloth (Krantex, Alfortville, France) which retained any undigested aggregates. The protoplasts were then collected by centrifugation (150 × *g* for 10 min) and washed twice with 150 ml of suspension medium containing 0.5 M mannitol, 5 mM phosphate buffer (pH 7.5), 10 mM KCl, 5 mM MgCl₂, 1% PVP (*M_r* ~25 000, Serva) (w/v) and 0.1% BSA (w/v).

Since sycamore protoplasts have an average diameter of 20–30 μ m, a rapid and effective procedure for the gentle rupture of intact protoplasts (i.e. for stripping the cell membrane) was to pass them through a fine nylon mesh (Nybolt PA, 20 μ m) affixed to the cut end of a 100-ml disposable syringe [12]. The lysate was then homogenized and centrifuged for 20 min at 15 000 × *g* (SS-34 rotor, Sorvall) and the mitochondrial pellet was resuspended in ~4 ml of suspension medium. The suspension thus obtained (washed mitochondria) was purified by centrifugation in self-generated density Percoll gradients (Pharmacia Fine Chemicals) [13]. The washed mitochondria were layered on Percoll medium (24% Percoll (v/v), 0.5 M mannitol, 10 mM phosphate buffer (pH 7.5), 1 mM EDTA, 1% PVP (*M_r* ~25 000, Serva) (w/v) and 0.1% BSA (w/v)) in Sorvall SS-34 tubes. After 35 min centrifugation at 40 000 × *g* the purified mitochondria were collected and washed twice with the same medium devoid of Percoll. This procedure resulted in mitochondria with better than 90% intact outer membranes as judged by their impermeability to cytochrome *c* [14]. On average, from 50 g of intact cells, which contained approx. 36 mg of mitochondrial protein, the yield of mitochondria was approximately 7.5 mg of protein (20%).

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2.3. O₂ uptake measurements

Oxygen uptake by either cells or mitochondria was monitored polarographically with a Clark-type electrode system (Hansatech Ltd., King's Lynn, Norfolk, UK). A value of 276 nmol of O₂/ml was used for the solubility of O₂ at 20°C. Sycamore cell respiration was measured at 20°C in the culture medium at pH 6.5. For mitochondrial respiration, the reaction medium contained 0.5 M mannitol, 10 mM KCl, 5 mM MgCl₂, 5 mM phosphate buffer (pH 7.2), 0.1% BSA (w/v). Mitochondrial O₂ uptake was measured in the presence of a mixture of substrates and appropriate cofactors.

2.4. Crude extract preparation and determination of fumarase activity

Cells (250 mg wet weight) were suspended in 2 ml of a medium containing 100 mM Pi (pH 7.5), 5 mM MgCl₂, 2 mM DTT, 1 mM EDTA, 0.025% (v/v) Triton X-100, 0.5 mM PMSF and were then disrupted by sonication for 30 s (20 kHz, 80 W; Sonimass 250 T). After stirring in a Potter-Elvehjem homogenizer, samples were centrifuged at 48 000 × g for 30 min. The supernatant comprised the crude extract. All procedures were carried out at 4°C under argon. Fumarase (EC 4.2.1.2) activity was assayed on cell and mitochondrial crude extracts, by recording spectrophotometrically (at 240 nm) the formation of fumarate in the presence of 50 mM L-malate [15].

2.5. Cytochrome oxidase measurements

Cytochrome *aa*₃ measurements were carried out in whole cell homogenates obtained by sonication and in isolated mitochondria, according to the procedure described by Bligny and Douce [16]. Cytochrome *aa*₃ was measured from the difference spectra between dithionite-reduced minus oxidized preparations. Spectra were made at liquid nitrogen temperature (77 K) using an Aminco DW-2 spectrophotometer. The wavelengths selected for measurements were those given by Chance and Williams [17]. The magnitude of low temperature enhancement was measured according to Lance and Bonner [18].

2.6. Lipid measurements

Lipids were extracted from sycamore cells and mitochondria, and analyzed according to Douce et al. [19]. Polar lipids were resolved by two-dimensional TLC [19]. Cardiolipin was identified by the chemical methods described previously [12,20].

2.7. [³¹P]NMR analysis

Perchloric extracts were prepared from 9 g wet weight, and phosphocholine and phosphoethanolamine were quantified from [³¹P]NMR spectra according to Aubert et al. [21].

2.8. Protein determination

Mitochondrial proteins were prepared in the presence of deoxycholate 0.04% (w/v), and determined according to the method of Lowry et al. [22] using BSA as a standard.

3. Results

3.1. Effects of copper treatment on cell growth

Fig. 1A shows the increase in wet weight during the growth of sycamore cells in liquid medium containing copper concentrations of 0.2 (controls), 5, 20, 50 and 100 µM. In order to maintain a constant supply of copper, the growth medium was renewed every 48 h, for all concentrations. Cells grown with 0.2 or 5 µM copper exhibited an exponential growth similar to that observed in standard sycamore cell cultures,

usually grown with 1 µM copper [11,23]. The doubling time was 3.5–4 days and the maximum density of cells was attained after 13–15 days of growth (maximal cell density is usually obtained after 7–8 days when the culture medium is not renewed). Comparison of the increase in wet weight between control cultures and cultures containing high concentrations of copper indicates that an excess of copper (up to 50 µM) (1) did not decrease the initial growth rates of the cells and (2) limited the maximum density of the cells. At 50 µM copper, cells nevertheless remained viable for more than 12 days and growth could be reinitiated after removing copper from the culture medium (by extensive washing), after a 48 h lag phase (Fig. 1A). In contrast, growth stopped immediately in cells treated with 100 µM copper, and cell death occurred owing very likely to deleterious effects on enzyme activities [4] and/or membrane properties [24].

3.2. Effects of copper treatment on cell respiration

Fig. 1B illustrates the time course of evolution of cell O₂ uptake by control (0.2 µM) and copper-treated sycamore cells. In cells grown with 0.2 µM (control) and 5 µM copper, respiration was not significantly modified during the 12 days of culture. In contrast, a steady decline of respiration was observed in cells treated with 20 µM and 50 µM copper. In cells grown with 50 µM copper, respiration attained ~60% of its original value after 5–6 days. A careful comparison between Figs. 1A and 1B indicates that respiration then stabilized, in correlation with the growth arrest. The rates of uncoupled respiration (in the presence of 2 µM *p*-trifluoromethoxyphenylhydrazine) exhibited similar patterns of evolution with a ~80–90% stimulation of O₂ uptake in control and copper-treated cells (data not shown). On the other hand, removing copper from the culture medium was accompanied by an increase of cell respiration (Fig. 1B, arrow) to normal values. On a cell weight basis 2 days were required to recover the initial rates of respiration (control cells). In contrast, the respiration of cells treated with 100 µM copper declined rapidly (Fig. 1B), reflecting cell death triggered by this lethal concentration.

The decline of respiration at sublethal copper concentrations (50 µM) could reflect a direct effect of this cation on mitochondrial oxidative properties. Mitochondria from control cells and cells treated with 50 µM copper for 7 days were therefore isolated and purified according to the procedure described in Section 2. In fact, no significant differences in their oxidative properties were detected. For example, the rates of O₂ uptake in state 3 that are dependent on succinate, malate, pyruvate, oxoglutarate and NADH were about the same for mitochondria extracted from normal and copper-treated cells (results not shown, see [12]). As a whole, these results strongly suggest that the decline of respiration in cells

Table 1

Cytochrome oxidase, cardiolipin content and fumarase activity of sycamore cells (control cells and cells treated with 50 µM copper for 7 days) and the corresponding mitochondria

	Cytochrome <i>aa</i> ₃ (nmol)		Cardiolipin (µg)		Fumarase activity (µmol)	
	Control	Copper-treated	Control	Copper-treated	Control	Copper-treated
Cells (g wet weight ⁻¹)	0.38	0.21	21.0	12.7	2.2	1.2
Mitochondria (mg protein ⁻¹)	0.40	0.39	29	27	4.7	4.8

Cytochrome oxidase, cardiolipin content and fumarase activity in cells and mitochondria were measured as described in Section 2. Control cells were grown with 0.2 µM copper, and treated cells were grown with 50 µM copper for 7 days. Data are mean values for three independent experiments with S.D. of less than 10%.

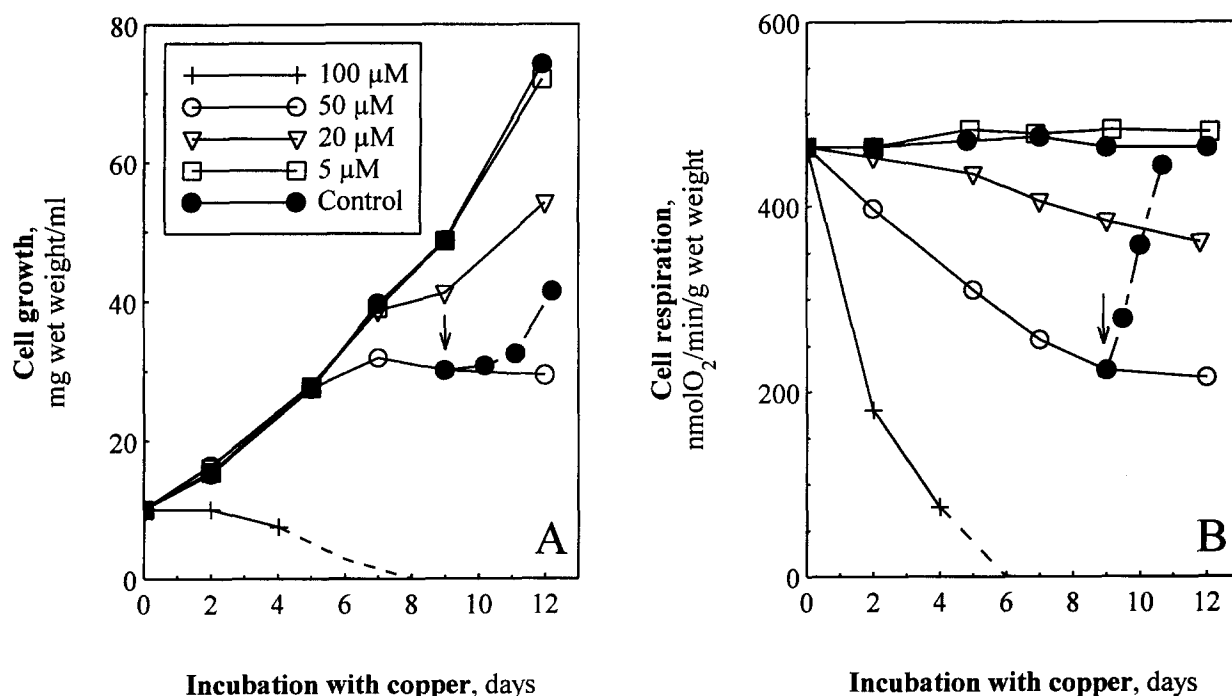


Fig. 1. Effect of copper on growth (A) and respiration (B) in sycamore cells. At different times following subculturing, cells were harvested, counted and respiration rates were measured as described in Section 2 in control cells (0.2 μ M copper; solid symbols) and cells treated with increasing concentrations of copper (5, 20, 50, 100 μ M; open symbols). Arrows indicate the time when copper was removed (by extensive washing) from the culture medium. A: Evolution of cell wet weight (mg/ml) for control (solid symbols) and copper-treated (open symbols) cells. B: Evolution of cell respiration in control (solid symbols) and copper-treated (open symbols) cells. Symbols are as in A.

treated with sublethal copper concentrations could result from a diminution of the number of their mitochondria. We therefore analyzed the evolution of mitochondrial markers during the course of incubation of cells with 50 μ M copper.

3.3. Effects of copper treatment on mitochondrial markers

Table 1 shows the contents of control and copper-treated sycamore cells of three mitochondrial markers: cardiolipin (diphosphatidylglycerol) and cytochrome *aa₃* (cytochrome oxidase), two specific markers of mitochondrial inner membrane, and fumarase activity, a specific marker of mitochondrial matrix space. Incubation of the cells with 50 μ M copper resulted in a steady decline of the three markers. Interestingly, after 7 days the decline was comparable for the three markers, when expressed as a percentage of the values in control cells (~40–45% decline for the three markers). Moreover, we verified that the mitochondrial content in the three markers did not vary significantly between the mitochondria extracted from control and copper-treated cells (Table 1). The time course of evolution of these markers is represented in Fig. 2, as well as the decline of cell respiration. All the values are expressed as percentages of control. Fig. 2 reveals that the decline of mitochondrial markers closely matched the decrease of cell respiration, suggesting that copper exposure resulted in a diminution of the number of mitochondria in sycamore cells. On the other hand, removing copper from the culture medium resulted in an increase of the mitochondrial markers to control values (not shown), in correlation with the recovery of control respiration. Finally, we observed that phosphocholine and phosphoethanolamine, two markers of autophagy in carbon-starved cells [21], did not accumulate in 50 μ M-treated cells (Table 2). This suggested that the diminution of mitochondria in copper-treated cells was due not

to an autophagic process, but rather to an arrest of mitochondrial biogenesis. However, cells exposed to lethal concentrations of copper (> 100 μ M) accumulated phosphocholine and phosphoethanolamine (Table 2), suggesting that an autophagic process could occur, as reported by Ouzounidou et al. [25] in maize plants.

4. Discussion

Incubation of sycamore cells with 50 μ M copper for 5–6 days did not interfere with growth, but resulted in a marked decline of respiration, which was attributed to a diminution of the number of their mitochondria. Indeed, we observed that this decline of respiration was paralleled by a decline of three specific mitochondrial markers. Bligny and Douce [20] have demonstrated that in plants, cardiolipin (diphosphatidylglycerol) is localized specifically within the inner mitochondrial membrane. This distribution has been confirmed for a variety of tissues (see [26]) and thus cardiolipin has been used as a marker to estimate the evolution of mitochondria/cell in sycamore cells subjected to carbon deprivation [12] or in postmeristematic cells of young wheat leaves grown under elevated CO₂ concentrations [27]. Cytochrome *aa₃* (cytochrome oxidase) was also used as a specific marker of the mitochondrial inner membrane. We observed that the decline of respiration was strictly correlated with a decline of the two markers, cardiolipin and cytochrome oxidase, which strongly suggests that a diminution of inner membrane surface and its components of electron transport chain occurred in copper-treated cells. Fumarase activity was also used as a specific marker of the mitochondrial matrix. The decrease of its activity in cells matched the decline of respiration and of inner mitochondrial membrane markers. Parenthetically, it appeared that fumarase

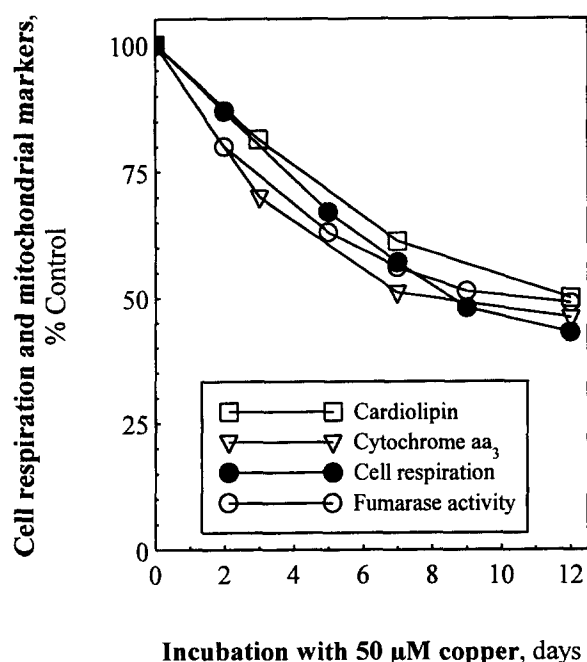


Fig. 2. Effect of copper on mitochondrial markers in sycamore cells treated with 50 µM copper. At various times during copper exposure, cells were harvested and cardiolipin (□), cytochrome oxidase (▽) contents and fumarase activity (○) were measured according to the procedures described in Section 2. Cell respiration is also presented (●). All values are expressed as percentage of the control values.

activity was not stimulated in copper-treated cells, when malate and isocitrate dehydrogenases have been shown to be induced during copper exposure [9]. On the basis of mitochondrial protein, fumarase specific activity was not affected by copper treatment. In addition, on the basis of mitochondrial protein, we observed no variation (1) in cardiolipin and cytochrome oxidase contents or (2) in substrates' oxidative properties. Furthermore, preliminary experiments carried out using electron microscopy have not revealed significant structural modifications of mitochondria in copper-treated cells including their size, cristae, or the electron density of the matrix space (results not shown). Taken together, these results strongly suggest that the number of mitochondria was reduced in copper-treated cells, while the functional and structural characteristics of mitochondria were not affected.

A progressive diminution of the number of mitochondria/cell has been reported in sycamore cells during the course of carbon deprivation, which accounted for the decrease in respiration [12]. In this case, carbohydrate starvation triggered growth arrest and an autophagic process characterized by the apparition of autophagic vesicles leading to the regression of

cytoplasm including mitochondria [21]. Phosphocholine accumulation resulting from phosphatidylcholine degradation has been shown to be a marker of the autophagic degradation of membrane systems [21,28]. In copper-treated sycamore cells, the regression of the mitochondrial system is unlikely to result from an autophagic process since phosphocholine did not accumulate and growth was not arrested. In addition, electron microscopy did not show any evidence of autophagic processes in copper-treated cells (data not shown). However, phosphocholine and phosphoethanolamine accumulated in cells treated with lethal concentrations of copper (> 100 µM), indicating that an autophagic process could occur, as has been suggested in maize plants exposed to excess copper [25].

The diminution of the number of mitochondria in cells exposed to sublethal copper concentrations likely originated from a marked reduction of mitochondrial biogenesis, thus leading to a dilution of these organelles in the cell population. Indeed, within 5–6 days of exposure to 50 µM copper, a 3-fold increase of wet weight was observed (Fig. 1A), while the mitochondrial system (assessed from cell respiration and mitochondrial markers) was reduced 2-fold. Further experiments will be necessary to determine whether the inhibition of mitochondrial biogenesis resulted from a direct action of copper, and/or from a secondary consequence of copper exposure. This inhibition of mitochondrial biogenesis could account for the decline of respiration reported in *Vallisneria spiralis* exposed to excess copper [8]. The results presented here also show that cell growth stopped after 6–7 days of exposure to 50 µM copper. This is consistent with the stabilization of the mitochondrial system (assessed from the arrest of the decrease of cell respiration and mitochondrial markers). The arrest of mitochondrial biogenesis and the subsequent reduction of the number of mitochondria in copper-treated cells could be responsible for the growth arrest. Growth inhibition has been reported in the roots of lettuce [29], *Agrostis gigantea* [30] and maize [25] exposed to excess copper. Experiments are now being carried out to study the evolution of the mitochondrial system in roots of maize plants exposed to excess copper.

Little is known about mitochondrial biogenesis in plants. Cucumber cotyledons during early seedling growth have been used as a model to investigate mitochondrial function and biogenesis [31]. Robertson et al. [27] have reported that elevated CO₂ triggered a 3-fold increase of mitochondria in post-meristematic cells of young wheat leaves. Our results indicate that cell suspensions treated with copper represent a model to study mitochondrial biogenesis. Indeed, removing copper from the culture medium of copper-treated cells resulted in an increase of cell respiration and mitochondrial markers to normal values, preceding the reinitiation of cell growth. These observations indicate that mitochondrial proliferation was restored. It will thus be possible to investigate mitochondrial

Table 2
Phosphocholine and phosphoethanolamine contents (µmol/g wet weight) in sycamore cells

	Control	Sucrose starved (2 days) ^a	50 µM copper (5 days)	100 µM copper (2 days)
Phosphocholine	nd ^b	0.95	nd	0.35
Phosphoethanolamine	nd	0.26	nd	0.11

Perchloric extracts were prepared from sycamore cells, and phosphocholine and phosphoethanolamine were quantified from [³¹P]NMR analysis, according to Aubert et al. [21]. The data are from a representative experiment.

^aFrom Aubert et al. [21].

^bNot detected (< 0.2 µmol/g wet weight).

biogenesis in a model where culture conditions can be easily controlled.

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