Impact of Post-Anoxia Stress on Membrane Lipids of Anoxia-Pretreated Potato Cells. A Re-Appraisal[1]

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The importance of lipid peroxidation and its contributing pathways (via reactive oxygen species and lipoxygenase) during post-anoxia was evaluated with respect to the biphasic behavior of membrane lipids under anoxia (A. Rawyler, D. Pavelic, C. Gianinazzi, J. Oberson, R. Brändle [1999] Plant Physiol 120: 293–300), using potato (*Solanum tuberosum* cv Bintje) cell cultures. When anoxic cells in the pre-lytic phase were re-oxygenated for 2 h, superoxide anion was not detectable, the hydrogen peroxide (H_2O_2) level remained small and similar to that of controls, and cell viability was preserved. Lipids were intact and no lipid hydroperoxides were detected. However, small amounts of lipid hydroperoxides accumulated upon feeding anoxic cells with H_2O_2 and incubation for an additional 2 h under anoxia. When cells that entered the lytic phase of anoxia were re-oxygenated for 2 h, the H_2O_2 and superoxide anion levels were essentially unchanged. However, cell respiration decreased, reflecting the extensive lipid hydrolysis that had already started under anoxia and continued during post-anoxia. Simultaneous with the massive release of free polyunsaturated fatty acids, small amounts of lipid hydroperoxides were formed, reaching 1% to 2% of total fatty acids. Catalase and superoxide dismutase activities were not greatly affected, whereas the amount and activity of lipoxygenase tended to increase during anoxia. Lipid peroxidation in potato cells is therefore low during post-anoxia. It is mainly due to lipoxygenase, whereas the contribution of reactive oxygen species is negligible. But above all, it is a late event that occurs only when irreversible damage is already caused by the anoxia-triggered lipid hydrolysis.

Considerable evidence has been obtained that various stresses can induce the production of reactive oxygen species (ROS; such as the superoxide radical, $O_2^{\bullet -}$, the hydroxyl radical, $OH^{\bullet}$, and hydrogen peroxide, H_2O_2), which in turn generate severe peroxidative damage to proteins, nucleic acids, and lipids (Scandalios, 1993). The source of ROS has been primarily related to dysfunctions in electron transport chains and other membrane-associated processes (Elstner and Osswald, 1994).

A similar mechanism has been suggested to occur during the re-oxygenation stress following anoxic periods in animal and plant tissues and organs (Crawford et al., 1994). The increased peroxidation was ascribed to a higher ROS production and/or to weakened enzymatic (e.g. superoxide dismutase [SOD] and catalase [CAT]) or nonenzymatic (e.g. antioxidants) detoxification systems (Van Toai and Bolles, 1991; Ushimaru et al., 1992; Hurng and Kao, 1994; Drew, 1997). An increased resistance toward various stresses has been achieved in plants engineered to express higher SOD and CAT activities (Hérouart et al., 1993; Sen Gupta et al., 1993; Foyer et al., 1994). However, evidence for a similar adaptation upon post-anoxic stress is scarce (Yu and Rengel, 1999). A notable case is *Iris pseudacorus*, in which SOD was shown to be one of the anaerobic proteins (Monk et al., 1987b).

Membrane lipids are among the preferred targets of peroxidation processes in cells. This is the case, for instance, of anoxic plant tissues that released end products of lipid peroxidation such as malondialdehyde and ethane upon re-oxygenation (Hunter et al., 1983; Pfister-Sieber and Brändle, 1994). However, the mere occurrence of such end products of lipid peroxidation gives no clue as to the pathways by which they are formed.

Our aim is double. First, we want to know whether diacyl-lipid and free polyunsaturated fatty acids (PUFA) peroxidation is achieved by the chemical pathway (via ROS), which exhibits a broad attack spectrum (Halliwell, 1991; Foyer et al., 1994) and/or by the enzymatic pathway (via lipoxygenase [LOX]) that specifically attacks lipids (Hildebrand, 1989; Siedow, 1991; Rosahl, 1996). Second, we want to evaluate the relative importance of these two pathways in non-green cells. To this end we have studied the time dependence of these processes under anoxic and post-anoxic conditions, using cultivated potato (*Solanum tuberosum* cv Bintje) cells as a model system.

Cell cultures are well suited for studying such events because the restrictions in gas and solute diffusibility commonly encountered in whole tissues (e.g. roots, rhizomes, and tubers) are largely eliminated when working with isolated cells. Moreover, cell cultures allow for the close control of the physicochemical environment and the ability to work with a more homogenous biological material. This model system was recently employed by Rawyler et al. (1999) to show that membrane lipid integrity relies on a threshold of ATP production rate and by Ober-

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son et al. (1999) to emphasize the membrane-stabilizing effect of nitrate in anoxic potato cells.

The present article continues and extends our previous work (Rawyler et al., 1999). We show that post-anoxic peroxidation is negligible as long as membrane lipids are intact, and increases only when lipid hydrolysis takes place. The significance of these two peroxidation pathways upon re-oxygenation of anoxia-treated potato cells is assessed.

RESULTS

Potato cells lost gradually most of their respiration capacity after a 12 to 24 h of anoxic treatment followed by a 24-h post-anoxic period (Fig. 1). The post-anoxic recovery of cell respiration depended on the duration of the anoxic pretreatment. It was complete following the first 12 h of anoxia, partial between 12 and 18 h, and nonexistent for longer times (Fig. 1). This behavior was correlated with cell viability (Fig. 1, inset).

During the same time span of 12 to 24 h anoxia, lipids began to be hydrolyzed and high amounts of free fatty acids (mainly linoleic and linolenic acids) were released under anoxia (Rawyler et al., 1999). This hydrolysis pattern was essentially the same

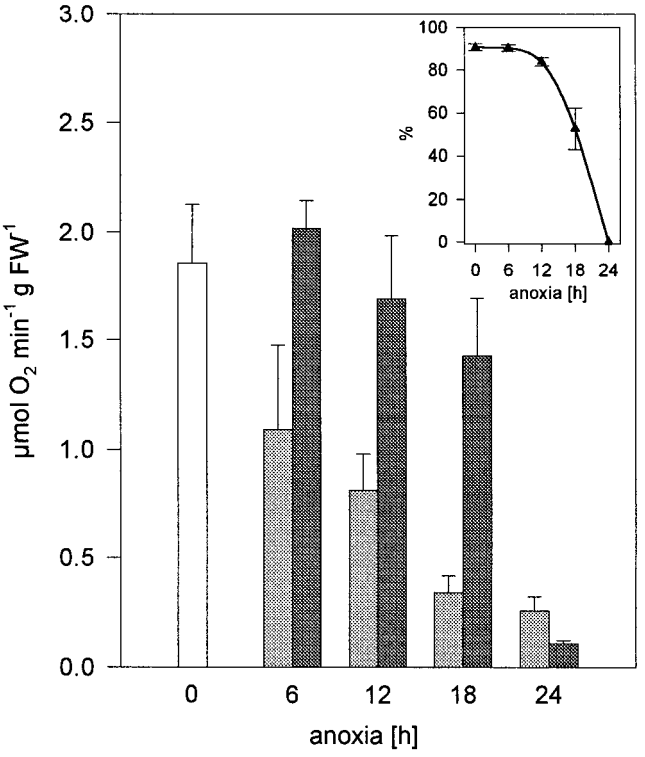

Figure 1. Oxygen uptake rate of potato cells measured immediately after various anoxia treatments and again after 24 h re-oxygenation (normoxia, white bars; directly after anoxia, light gray bars; after 24 h post-anoxia, dark gray bars). Inset, Percentage of living cells following anoxia (Trypan blue test). Values are means ± SD of three independent experiments.

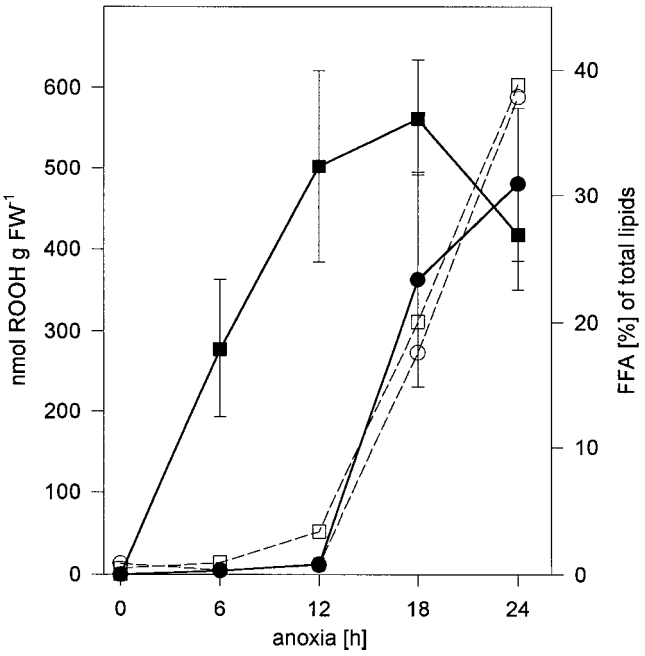

Figure 2. Comparison of the effects of exogenous H_2O_2 addition under anoxia (mimicking ROS production under re-oxygenation) and of post-anoxia on lipid hydroperoxide production (black symbols) and lipid hydrolysis (white symbols) in potato cells. Cells were pre-incubated under anoxia for the specified time periods. Then in a first treatment they received a single addition of 1 mM H_2O_2 followed by a further 2-h period under anoxia (■, □). In a second treatment, cells were re-aerated for 2 h (●, ○). When 1 mM H_2O_2 was given to potato cells under anoxia, it was destroyed within 30 min and the cells survived without any sign of damage (data not shown). Lipid hydroperoxide data are the mean of three to four measurements ± SD The data for lipid hydrolysis are taken here from one representative experiment, since they are in perfect agreement with previously published data (Rawyler et al., 1999). The amount of total cell fatty acids taken as 100% was 25.6 ± 2.4 µmol g fresh weight^{-1} (n = 10).

when the anoxic treatment was continued in the presence of 1 mM H_2O_2 (to mimic ROS production under re-oxygenation) or followed by a 2-h re-oxygenation period (Fig. 2). Small and comparable amounts of lipid hydroperoxides were detected in both treatments. When H_2O_2 was added under anoxia, the peroxidation process started immediately. Because most free fatty acids found between 0 and 12 h have saturated acyl chains (Rawyler et al., 1999), this peroxidative process is likely to attack native membrane lipids to yield phospholipid hydroperoxides. It is surprising, however, that no lipid hydroperoxides were formed when H_2O_2 was added to cells that were not submitted to any anoxic pretreatment (time 0 in Fig. 2). On the other hand, no lipid hydroperoxides were formed after up to 12-h anoxia followed by re-oxygenation, whereas their production occurred in parallel to the release of free fatty acids (Fig. 2). These findings suggest that ROS detoxification was still very efficient and that lipid peroxidation by endogenously formed ROS (under post-anoxia) was quantitatively of minor importance. Nevertheless,

the formation of these early lipid peroxidation products (Fig. 2) essentially mirrored the changes in post-anoxic respiration capacity (Fig. 1) and the time-course of lipid hydrolysis (Fig. 2).

Detectable ROS were produced in very low amounts after 1 h of post-anoxic treatment (Table I). Chemiluminescence-based methods (Warm and Laties, 1982; Auh and Murphy, 1995) yielded only very weak signals. More reliable results were obtained for superoxide anion and hydrogen peroxide with spectrophotometry (Schneider and Schlegel, 1981; Patterson et al., 1984). Whereas the cellular levels of H_2O_2 of re-oxygenated cell suspensions never exceeded those of normoxic suspensions, superoxide anion formation showed a small, but distinct peak after 18 h of anoxia and then decreased to very low levels. These data suggest that anoxia-treated potato cells were intrinsically unable to build up significant amounts of ROS upon re-oxygenation or that the detoxification capacities of potato cells were efficient enough to prevent the accumulation of higher ROS amounts.

The low apparent ROS production (Table I) might be due to efficient enzymatic detoxification reactions. Two obvious candidates are SOD and CAT. To get an insight into the detoxification capacity of these enzymes, a treatment of 18 h of anoxia followed by 3 h of post-anoxia was applied. The anoxic treatment of 18 h was selected because it represented the latest point at which cells still recovered most of their initial respiration rate upon re-oxygenation (Fig. 1). The post-anoxic period of 3 h allowed us to obtain a conservative estimation of the SOD and CAT amounts. At this point more than 60% of the initial SOD specific activity remained, whereas that of CAT was slightly enhanced (Table I). We have presently no simple explanation for the decrease in SOD activity. At any rate, the residual amounts of these two enzymes appear sufficient to contribute to ROS detoxification under these conditions.

It is known that H_2O_2 can trigger the production of acetaldehyde from ethanol, probably in a CAT-dependent back-reaction (Monk et al., 1987a; Zuckermann et al., 1997). In anoxic potato cells, acetaldehyde formation was indeed stimulated by exogenous H_2O_2 and this effect could be partly reversed by the CAT inhibitor aminotriazole (Fig. 3). This is further evidence for the potentially hazardous impact of exogenous H_2O_2 on potato cells and points again to an active participation of CAT in H_2O_2 removal.

As LOX can also contribute to lipid peroxidation, we measured the activity of this enzyme in cell extracts prepared from anoxia-treated cells. Table II shows that the specific LOX activity increased slightly during anoxia with a maximum at 18 h, then decreased again. These values were mirrored in the LOX behavior as revealed by western blotting (Fig. 4). Data from Figure 4 and Table II indicate that the potato cell LOX is neither very active nor very abundant when compared for instance with the LOX of potato tubers (Galliard and Matthew, 1973).

Table I. *Formation rates of superoxide anion, cellular levels of H_2O_2, and SOD and CAT activities of potato cells incubated up to 24 h under anoxia and subsequently re-oxygenated for 0, 1, or 3 h*

Values are averages ± SD, and the number of replicates is given in brackets. A dash means that data were not determined for that time point.

Incubation Time under		Superoxide Anion		H_2O_2		SOD	CAT
Anoxia	Post-anoxia	Method I[a]	Method IIa	Method III[a]	Method IV[a]		
h		*μmol g fresh wt^{-1} h^{-1}*		*nmol/g fresh wt*		*units[c] min^{-1} mg protein^{-1}*	*μmol min^{-1} mg protein^{-1}*
0	0	tr[b]	0 ± 0 (3)	tr[b]	86 ± 46 (5)	133 ± 20 (12)	71 ± 13 (12)
12	0	tr[b]	0 ± 0 (3)	tr[b]	89 ± 35 (3)	–	–
12	1	tr[b]	0 ± 0 (3)	tr[b]	83 ± 23 (4)	–	–
18	0	tr[b]	55 ± 9 (3)	tr[b]	81 ± 18 (4)	109 ± 15 (3)	70 ± 6 (3)
18	1	tr[b]	104 ± 21 (3)	tr[b]	71 ± 28 (4)	–	–
18	3	–	–	–	–	83 ± 6 (3)	85 ± 2 (3)
24	0	tr[b]	0 ± 0 (3)	tr[b]	71 ± 76 (3)	–	–
24	1	tr[b]	9 ± 8 (3)	tr[b]	93 ± 61 (4)	–	–

[a]Method I, Lucigenin chemiluminescence (Auh and Murphy, 1995); II, NH_2OH-dependent NO_2^- formation (Schneider and Schlegel, 1981); III, luminol chemiluminescence (Warm and Laties, 1982); IV, $TiCl_4$-pyridylazoresorcinol (Patterson et al., 1984). [b]tr, Trace amounts only (signals were never significantly higher than background noise). [c]One SOD-525 unit is defined as the enzyme amount that doubles the autooxidation rate in the specified assay (see "Material and Methods").

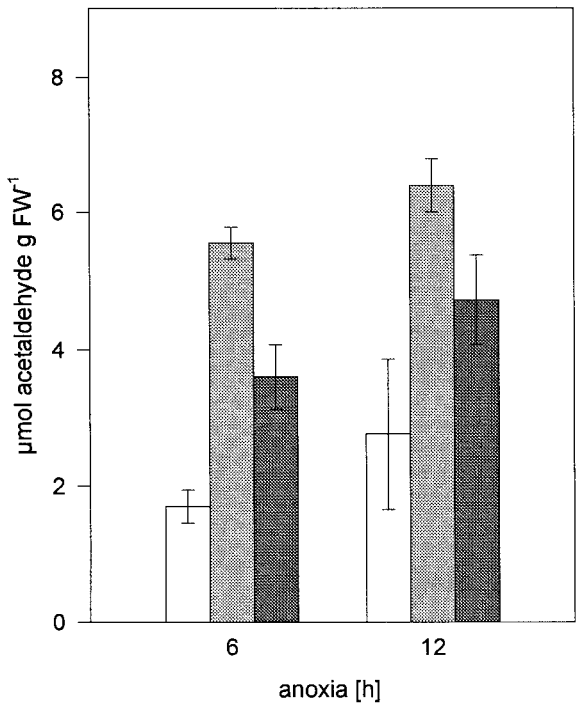

Figure 3. Acetaldehyde production during the last hour of anoxia periods of 6 and 12 h (white bars) and after the application of 1 mM H_2O_2 for an additional 15 min period under anoxia in the absence (light gray bars) or the presence (dark gray bars) of 10 mM aminotriazole. Data are given as averages ± SD, with $n = 6$.

DISCUSSION

Membrane lipids can be affected under the dual aspects of oxygen stress, namely oxygen deprivation (anoxia) and the subsequent re-oxygenation (post-anoxia). It is generally considered that the former has only a very limited impact on cell lipids, as shown for instance by the arrest of the desaturation of membrane lipids (Brown and Beevers, 1987). By contrast, the latter is regarded as the cause of the hazardous lipid peroxidation, which is most frequently attributed to the action of ROS (Crawford, 1992; Blokhina et al., 1999). However, ROS and LOX can promote lipid peroxidation. It is thus surprising that the respective contribution of both pathways to the formation of post-anoxic peroxidation products and to tissue damage has never been considered in plant systems. Such a differentiation between ROS and LOX is not restricted to post-anoxia, but has also been suggested for membrane disorders generated by other factors (Shewfelt et al., 1994).

In our laboratory we study the behavior of membrane lipids under these two conditions using potato cell suspension cultures. In a previous article we addressed this behavior under anoxia and showed that it was characterized by an extensive degradation of membrane lipids caused by a lipolytic acyl hydrolase, leading to the release of free PUFA (Rawyler et al., 1999). During a pre-lytic phase, cells coped with the reduced energy supply and their membranes remained intact. Lipid hydrolysis started only after about 12 h of anoxia, reaching 50% to 60% after 24 h (Oberson et al., 1999; Rawyler et al., 1999; see also Fig. 2).

Here we have addressed the impact of post-anoxic treatment on cell lipids, while still keeping in mind the contribution of anoxia. Instead of measuring late products of lipid peroxidation such as malondialdehyde and ethane (e.g. Hunter et al., 1983; Pfister-Sieber and Brändle, 1994), we thought it more dependable to determine the formation of lipid hydroperoxides because they reflect more specifically the lipid peroxidation process (Mihaljevic et al., 1996).

The role of ROS will be considered first. A crucial observation, which was independently provided by several methods (Table I), is that the ROS (H_2O_2 and $O_2^{\bullet -}$) levels detected in potato cells upon post-anoxia are very low. It is worth recalling that the ROS level actually measured by a given method always reflects the balance between ROS-generating and ROS-consuming processes. Thus low ROS levels can be due either to a low intrinsic production rate or to efficient scavenging by antioxidants and detoxifying enzymes such as CAT and SOD, or still to a high reactivity toward potential targets such as lipids. This latter possibility can be excluded since only small lipid hydroperoxide amounts (<2% of total cell fatty acids) could be detected (Fig. 2) and the overall acyl composition of total lipids, more particularly the proportion of PUFA (data not shown; see also Rawyler et al., 1999), was unchanged during all treatments. On the other hand, the persistence of CAT and SOD activities suggests that the detoxifying potential of potato cells toward ROS is still present after 18 h of anoxia plus 3 h of re-oxygenation (Table I). The efficiency of this ROS detoxification potential is illustrated by the property of potato cell CAT that re-

Table II. *Specific activity of LOX in potato cells incubated under anoxia*

Values are given as averages ± SD (n = 3–4).

Incubation Time under Anoxia	Specific LOX Activity
h	*nmol O_2 min^{-1} mg $protein^{-1}$*
0 (Normoxia control)	1.5 ± 0.6
6	2.2 ± 0.5
12	2.3 ± 0.1
18	3.6 ± 0.4
24	2.2 ± 1.2

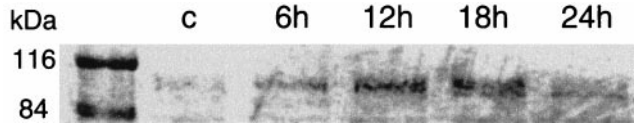

Figure 4. Western-blot analysis of lipoxygenase in potato cells incubated up to 24 h of anoxia (c, normoxic control). Each lane was loaded with a volume equivalent to 6.4 mg fresh weight (mean protein content was 4.7 ± 0.2 mg protein g fresh weight $^{-1}$, $n = 5$).

moves H_2O_2 not only by classical dismutation, but also in a back-reaction from ethanol to acetaldehyde (Fig. 3), as proposed earlier (Monk et al., 1987a; Zuckermann et al., 1997). All these data consistently reflect the fact that cell viability, as estimated by the capability to restore cell respiration, was fully preserved at least up to 12 h of anoxic treatment (Fig. 1).

The sudden addition of 1 mM H_2O_2 to cell suspensions would correspond to an almost "infinite" formation rate of endogenous H_2O_2 by these cells. In this situation the above mentioned detoxification reactions catalyzed by CAT may be temporarily overwhelmed, and this would explain why lipid peroxidation can be observed (though to a low level) under anoxia in the presence of exogenous H_2O_2 (Fig. 2). This process might occur via a Fenton reaction of Fe^{2+} with H_2O_2 to form hydroxyl radicals that promote hydrogen abstraction from acyl chains; the resulting acyl radicals react in turn with the oxygen produced by the CAT action on H_2O_2 to yield lipid hydroperoxides (Halliwell and Gutteridge, 1990). The peroxidation that occurs under these conditions must take place at the level of diacyl-lipids, since no free fatty acids are available during the pre-lytic phase (Fig. 2). However, the absence of lipid hydroperoxides after the addition of H_2O_2 to normoxic cells (time 0 in Fig. 2) is difficult to explain now. It might be that Fe^{3+} is the major form of iron in normally aerated cells and as such cannot efficiently sustain the Fenton-type reactions with H_2O_2 as described above. On the other hand, the level of endogenous H_2O_2 (Table I) is much lower (as should also be its formation rate). The absence of lipid hydroperoxides during the pre-lytic phase of anoxic/post-anoxic treatments (Fig. 2) is therefore not surprising and would be expected from the cell viability (Fig. 1). Thus we conclude that post-anoxic damage to membrane lipids is negligible in cells pre-incubated up to 12 h under anoxia (pre-lytic phase), though becoming visible afterward, and the role of ROS in the post-anoxic peroxidation of potato cell lipids is at best marginal.

The largely unspecific character of the chemical peroxidation processes involving ROS (Halliwell and Gutteridge, 1990) cannot account for the remarkable parallelism between the massive release of free fatty acids (mainly PUFA) during the lytic phase and the accumulation of lipid hydroperoxides (Fig. 2). This specific requirement for free PUFA strongly suggests that LOX, which is known to exhibit a high preference toward free PUFA (Hildebrand, 1989; Grechkin, 1998), is the main initiator of post-anoxic lipid peroxidation in potato cells. This view is supported by the persistence of LOX activity (Table II) and of its protein content (Fig. 4) in cell extracts throughout all treatments. Furthermore, the increase in LOX activity up to 18 h of anoxia suggests that LOX could be another yet unrecognized anaerobic protein (Sachs et al., 1996).

Therefore, it is mainly through the LOX pathway—and not via ROS—that free PUFA are peroxidized during post-anoxic treatment of potato cells. Thus lipid peroxidation relies primarily on the availability of free PUFA, in agreement with the results of Todd et al. (1990). This implies that the release of free fatty acids from membrane lipids, which can already occur under anoxia (Henzi and Braendle, 1993; Rawyler et al., 1999), is a prerequisite for their post-anoxic peroxidation. This mechanism, which involves the sequential participation of one or more lipolytic enzymes and of LOX, resembles those proposed by Slusarenko et al. (1991) and by Farmer and Ryan (1992) to describe the response of some plants to pathogen attack. It is nevertheless striking to observe that a single mechanism can be used on a small scale to achieve the limited and highly controlled changes required by signaling processes (Farmer et al., 1998), and on a large scale, leading to the general degradation of most cell lipids in autophagic (Aubert et al., 1996) and necrotic processes (this paper).

We conclude that in potato cells the re-oxygenation stress per se was not the key factor for cell death. The biological relevance of post-anoxic peroxidation processes in systems where an early lipolysis occurs should therefore be considered with great caution.

The difficulty of improving resistance to peroxidation in plants by increasing a single enzyme of the ROS detoxification arsenal (Foyer et al., 1994) could be explained simply by the fact that lethal damages other than lipid peroxidation might also have occurred. Overexpression of a particular enzyme such as SOD (Yu and Rengel, 1999) would be of no use in a system already weakened or dying because of anoxia-induced lipid hydrolysis. We would like to point out that the first task in improving the resistance of a given plant to oxygen stress is to clearly identify which of the two successive stress components (anoxia and re-oxygenation) contains the critical step.

MATERIALS AND METHODS

Chemicals

Except where indicated, chemicals were high purity products of Fluka AG and Sigma AG (Buchs, Switzerland).

Cells

Potato (*Solanum tuberosum* cv Bintje) cells were cultivated in Murashige-Skoog medium (Rawyler et al., 1999). Cells (4 d old) were harvested in their mid-log phase.

Anaerobic Conditions

Cells were incubated and harvested in an anaerobic workbench (Forma Scientific, Type 1029, Marietta, OH), exactly as previously described by Rawyler et al. (1999). The O_2 level, monitored with a Toray LF-700 oxygen ana-

lyzer (Lippke, Neuwied, Germany), never exceeded the detection limit of 0.001%.

Cell Treatments

Sterile vessels containing cell suspensions (about 15 mg cell fresh weight/mL) were closed with special aluminum caps or with compressed cellulose plugs that allowed gas exchange with the anaerobic atmosphere of the workbench while maintaining aseptic conditions. The vessels were placed on a rotary shaker and incubated for up to 24 h under anoxia. Next, vessels were withdrawn from the workbench and further incubated in normal air for a reoxygenation period of up to 24 h. Incubations were carried out in darkness at 24°C to 26°C. Under these conditions, cell suspensions became anoxic or normoxic 10 to 15 min after the atmosphere modification. In addition, regular microscopic controls carried out at the end of incubations (see also below) showed no detectable bacterial contamination. Cells were collected by filtration under reduced pressure (and under anaerobic conditions whenever required), immediately frozen in liquid N_2, and stored at −80°C until use. Additional experimental details are given in the legends to figures and tables.

Control of Cell Viability

Cell viability after the desired incubation time was microscopically assessed by the capacity of cells to exclude Trypan Blue when suspended in a medium containing 0.2% (w/v) dye.

Protein Determination

The protein concentration of cell extracts was measured by a dye-binding microassay (Bio-Rad Laboratories AG, Glattbrugg, Switzerland), using bovine serum albumin as a standard.

Determination of Respiratory Activity of Cells

O_2 uptake was measured at 25°C using a Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). The glass cuvette contained 5 mL of air-saturated, well-stirred Murashige-Skoog medium supplemented with 90 mM Suc. Reaction was started by the addition of cells (40–80 mg fresh weight).

Lipid Analysis

Total cell lipids and free fatty acids were determined by a combination of thin-layer and gas chromatography as described by Rawyler et al. (1999).

Determination of Lipid Hydroperoxides

Total lipids were extracted by placing 25 to 50 mg of cell fresh weight in 2.5-mL screw-cap glass tubes containing 2 mL of de-oxygenated chloroform:methanol (2:1, v/v). After shaking and sonicating (30 s), the homogenate was filtered using a 2-mL plastic syringe sequentially through a filter paper (type LS 14, Schleicher and Schuell, Feldbach, Switzerland) and a microfilter (Chromafil Einmalfilter, type O-20/3, 0.2-μm pore diameter, Macherey-Nagel, Oensingen, Switzerland). The filtered extracts were dried under N_2 and their hydroperoxide content measured according to Mihaljevic et al. (1996) using 13-hydroperoxy-octadecadienoic acid as a standard. This method measures early lipid hydroperoxides, namely the hydroperoxy groups of any acyl chain, irrespective of their occurrence as free fatty acid or esterified acyl chain.

Determination of ROS

The level of superoxide was measured by the superoxide-induced oxidation of hydroxylamine to nitrite (Schneider and Schlegel, 1981) and by the chemiluminescence of lucigenin (Auh and Murphy, 1995). Hydrogen peroxide was assayed by monitoring the chemiluminescence intensity of luminol in the presence of ferricyanide (Warm and Laties, 1982) and by its reaction with the Ti(IV) Cl_4-pyridylazoresorcinol complex (Patterson et al., 1984). Chemiluminescence was measured in a Celltester M-1060 luminometer (Lumac, Landgraaf, The Netherlands). When H_2O_2 was added to the cells, its concentration was estimated by combining the Merckoquant peroxide test (Merck AG, Dietikon, Switzerland) with a densitometric analysis at 650 nm (model CD60, Desaga, Heidelberg).

Determination of Acetaldehyde Production

Cells were incubated for 6 and 12 h under anoxia as described above, except that for the last hour 10-mL aliquots were anaerobically transferred in flasks (63 mL total capacity) that were tightly closed with butyl septa maintained with aluminum crimp-on caps and further incubated. H_2O_2 (1 mM final concentration) was injected through the septum with a syringe so as to mimic post-anoxia. Where indicated, aminotriazole (10 mM final concentration) was injected 5 min before the time zero of measurement to inhibit catalase. Acetaldehyde was measured after 0 and 15 min by headspace analysis using a Sigma 300 gas chromatograph (Perkin-Elmer AG, Rotkreuz, Switzerland). One milliliter of gaseous atmosphere was withdrawn from the flask with a gas-tight syringe and injected onto a 1.8-m × 1/8-inch metal column packed with Porapak Q30 (Macherey and Nagel, Oensingen, Switzerland) isothermally maintained at 180°C. Compounds were eluted with N_2 and detected by flame ionization. Acetaldehyde produced during the last hour (time zero) and after a further 15 min of the above treatments (H_2O_2 ± aminotriazole) under anoxia corresponded to the total acetaldehyde content of the flask (gas + liquid phases), which was itself calculated from the gaseous concentration of acetaldehyde and its partition coefficient (Kimmerer and MacDonald, 1987).

Determination of SOD and CAT Activities

Potato cells (100 mg fresh weight) were homogenized at 4°C for 30 s in 1 mL of an extraction medium made up of 5 mg polyvinylpolypyrrolidone in 50 mM sodium phosphate buffer (pH 7) plus 0.1 mM EDTA-Na_2, using a Polytron PT-1200 homogenizer (Kinematica AG, Littau, Switzerland). The homogenates were filtered and centrifuged at 10,000*g* for 15 min. The clear supernatants were used to determine enzymatic activities. The SOD activity was measured with a spectrophotometric assay kit ("SOD-525," BIOXYTECH S.A., Bonneuil, Marne, France). The CAT activity was measured according to Bergmeyer (1974) using the H_2O_2 extinction coefficient given by Klapheck et al. (1990).

SDS-PAGE and Immunoblotting

Potato cells were homogenized in a 1:5 (w/v) ratio with extraction buffer (20 mM sodium phosphate, pH 7.5, 1% [w/v] polyvinylpolypyrrolidone, and 0.1% [v/v] β-mercaptoethanol) at 4°C for 30 s with a Polytron PT-1200 homogenizer (Kinematica AG). Homogenates were centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was mixed with one-fourth volume of sample buffer (60 mM Tris-HCl, pH 6.8, 25% [w/v] glycerol, 2% [w/v] SDS, 14.4 mM β-mercaptoethanol, and 0.1% [w/v] bromphenol blue} and boiled for 5 min. SDS-gel electrophoresis was carried out with a Mini Protean II Dual Slab Cell (Bio-Rad, Glattbrugg, Switzerland) according to Laemmli (1970), using 1-mm-thick gels (12% [w/v] acrylamide in resolving gel) with an acryl- to bisacrylamide ratio of 37.5 to 1. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 to detect the protein pattern or blotted onto nitrocellulose membranes (0.45 μm; Bio-Rad). Immunoblotting was performed as described by Mitsuhashi and Feller (1992) except that nitrocellulose membranes were incubated overnight at 4°C in the presence of the primary antibody against LOX-H3 protein from potato (Royo et al., 1999).

Determination of LOX Activity

Potato cells (about 400 mg fresh weight) were homogenized at 4°C for 1 min in 2 mL of 50 mM potassium phosphate buffer (pH 7.5) with a Polytron homogenizer (see above). Homogenates were centrifuged at 40,000*g* for 15 min at 4°C. The clear supernatants were used to determine enzyme activity essentially as described by Galliard (1977). LOX activity was followed with a Clark oxygen electrode in a thermostated (25°C) cuvette of a total volume of 5 mL containing 0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]/KOH buffer, pH 7.7 (air-saturated), 0.66% [w/v] carbonyl- and peroxide-free Triton X-100, and 1 mL of enzymic extract. Reaction was started by the addition of 2 mM linolenic acid from a 500 mM stock solution in ethanol.

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