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Lipid Peroxidation in Higher Plants[1]

THE ROLE OF GLUTATHIONE REDUCTASE

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ABSTRACT

To study the role of glutathione reductase in lipid peroxidation, bean leaves (*Phaseolus vulgaris*) cv Fori were treated with the herbicide acifluorfen-sodium (sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid). Acifluorfen is a potent inducer of lipid peroxidation. In beans, decrease of acid-soluble SH-compounds and lipid peroxidation, measured as ethane evolution, were the toxic events after treatment of leaves with acifluorfen. As a primary response to peroxidation, increased production of antioxidants, such as vitamin C and glutathione, was found. This was followed by elevation of glutathione reductase activity. Enhanced activity of the enzyme prevented both further decline of acid-soluble SH-compounds and lipid peroxidation. Increased production of antioxidants and elevated activity of antioxidative enzymes, like glutathione reductase, seem to be a general strategy to limit toxic peroxidation in plants.

The deleterious effect of many xenobiotics, including certain *p*-nitrodiphenyl ether herbicides, is believed to be strong oxidation of cell components, such as peroxidation of polyunsaturated fatty acids in biomembranes via free radical reactions (10, 13). Research to date has shown that peroxidation of lipids is responsible for damage of proteins, DNA, and pigments (11, 12, 24, 26). Protection against phytotoxic peroxidation is achieved by several antioxidants, such as the vitamins E and C or glutathione (3, 26). The lipophilic vitamin E, however, seems to be the most effective radical chain-breaking substance (2). It has to be reductively regenerated by water-soluble GSH[3] either directly or via a system consisting of GSH and the water-soluble vitamin C (18). To maintain a high level of active GSH, GSSG has to be rapidly reduced. This reaction is catalyzed by the enzyme GR (EC 1.6.4.2) in the presence of NADPH. GR has been isolated from a number of different organisms including higher plants and bacteria (9, 17).

Little is known, however, about the physiological role of GR in plants under peroxidative conditions. Halliwell and Foyer (9) proposed that the enzyme is involved in the ascorbate-glutathione cycle to reduce GSSG to GSH. Ascorbate is necessary to remove phytotoxic H_2O_2 in chloroplasts via the ascorbate/ascorbate peroxidase system (5, 14). A similar physiological function of GR has also been proposed by other researchers working with blue-green algae (25). Recently, Gillham and Dodge (7) found that GR is located in both the cytoplasm and the chloroplast.

This paper presents results showing some new aspects of the role of GR under peroxidative conditions in higher plants. For comparison, the enzyme GLO, which converts L-galactonolactone to vitamin C (16), was also investigated to differentiate between specific responses of enzymes either responsible for antioxidant recycling or antioxidant biosynthesis. Peroxidation was initiated in beans by the *p*-nitrodiphenyl ether acifluorfen. This compound has been shown to induce peroxidation of lipids (10). The peroxidation process was measured by ethane evolution, a decomposition product of ω-3-unsaturated fatty acid hydroperoxides (26).

MATERIALS AND METHODS

Materials. Acifluorfen-sodium was supplied by Rohm and Haas; L-galactono-1,4-lactone was purchased from Sigma.

Plant Cultivation. Seeds of beans (*Phaseolus vulgaris*) cv Fori were planted in moistened vermiculite. They remained in a darkened growth chamber with a constant temperature of 20°C and 80% RH. After 6 d, the etiolated seedlings were exposed for 7 d to a 12-h photoperiod (170 W/m^2 supplemental light) with d and night temperatures of 22 and 18°C, respectively.

Chemical Treatment of Plants. In the short-term experiment, primary bean leaves were treated 7 d after light exposure with 50 μl/leaf of an aqueous solution containing 0.5 mM acifluorfen. In the long-term experiment, primary leaves were treated 7 d after light exposure with 50 μl/leaf of an aqueous solution containing different herbicide concentrations. This procedure was repeated once after 2 d before leaves were excised for analysis after 4 d. The herbicide was applied on the surface of the leaves with a small brush.

Enzyme Assay. GR activity was assayed spectrophotometrically at 340 nm by oxidation of NADPH as described by Halliwell and Foyer (9). GLO activity was determined by floating disks of primary bean leaves on 10 ml incubation medium in the dark for 18 h. The incubation medium consists of 10 mM sodium phosphate buffer (pH 7.2) containing 20 mM galactonolactone. The activity of the enzyme was then determined by measuring the vitamin C content of the galactonolactone-incubated disks.

Analysis. Total glutathione content was determined according to the method of Law *et al.* (14). The vitamin E and vitamin C content of primary leaves were determined by HPLC technique and dye titration with 2,6-dichlorophenolindophenol, respectively, as described by Finckh and Kunert (3).

To determine herbicide-induced ethane evolution, disks of acifluorfen-pretreated primary leaves (0.2 g) were placed into 10 ml vials and sealed with rubber septa. Incubation followed for 18 h at 20°C under slight shaking and continuous light of 40 W/m^2. The vials contained 2.5 ml medium consisting of 10 mM

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[3] Abbreviations: GSH, reduced glutathione; GLO, galactonolactone oxidase; GR, glutathione reductase.

sodium phosphate buffer (pH 7.2). The hydrocarbon gas produced was analyzed according to the method of Kunert and Böger (11).

Statistical Analysis. All estimates of sample variability are given in terms of the SE. The significance of differences was determined by the Student's two-tailed t test. P values ≤ 0.05 were considered significant.

RESULTS

Treatment of bean leaves with different concentrations of the herbicide acifluorfen in the long-term experiment increased the amount of total glutathione (GSH + GSSG) and the activity of GR in leaves (Fig. 1). No visible damage of plants was found with the herbicide concentrations used. Acifluorfen concentrations above 0.5 mM, however, produced necrotic spots on leaves 4 d after herbicide treatment. Both the amount and the activity were significantly higher ($P < 0.01$) after treatment with 0.5 mM of the herbicide than with 0.05 mM. When GR activity increased, a decline of SH-compounds was prevented. Further, ethane production, an index of *in vivo* lipid peroxidation, was significantly lower ($P < 0.05$) in leaves treated with 0.5 mM of the herbicide than with 0.1 mM.

In the short-term experiment, treatment of bean leaves with 0.5 mM acifluorfen increased total glutathione production. After 4 h, the total glutathione content was significantly 2.1-fold higher ($P < 0.05$) in herbicide-treated leaves than in untreated leaves at the beginning of the experiment (Fig. 2). However, the amount of acid-soluble SH-compounds significantly declined ($P < 0.05$) in acifluorfen-treated leaves compared to untreated control leaves. A minimal amount of SH-compounds was found 5 h after herbicide treatment. When the content of total glutathione in acifluorfen-treated leaves was at maximum, activity of GR increased. Over a time period of 3 h, elevation in activity was 38% above the untreated control ($P < 0.05$). Higher activity of GR was accompanied by both decline of the total glutathione content and regeneration of acid-soluble SH-compounds.

Vitamin C production increased in bean leaves after treatment with acifluorfen (Fig. 3). After 5 h, herbicide-treated leaves had a 2.5-fold higher ($P < 0.05$) vitamin C content than untreated leaves at the beginning of the experiment. Concurrently to the elevation of the vitamin C content, the activity of GLO was 52% higher in acifluorfen-treated leaves than in untreated control leaves.

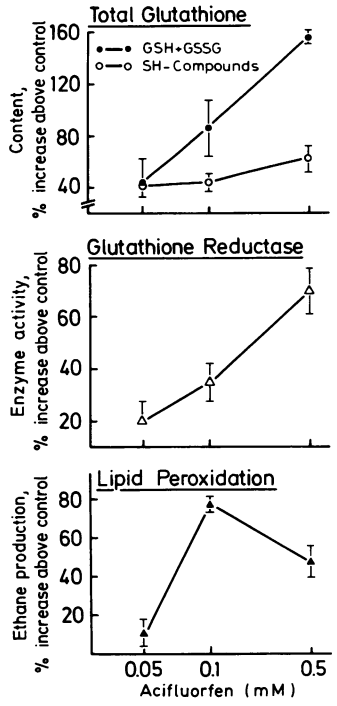

FIG. 1. Increase of total glutathione (GSH + GSSG), acid-soluble SH-compounds, GR activity, and evolution of ethane above control in bean leaves after long-term treatment with different concentrations of acifluorfen. In untreated leaves, total glutathione was 5.7 ± 0.5 μmol/g dry weight, acid-soluble SH-compounds were 4.9 ± 0.8 μmol/g dry weight, GR activity was 2.0 ± 0.3 μmol NADPH oxidized/min·g dry weight, and ethane evolution was 10 ± 1.1 pmol/h·g dry weight. Data shown represent the means ± SE of six different samples.

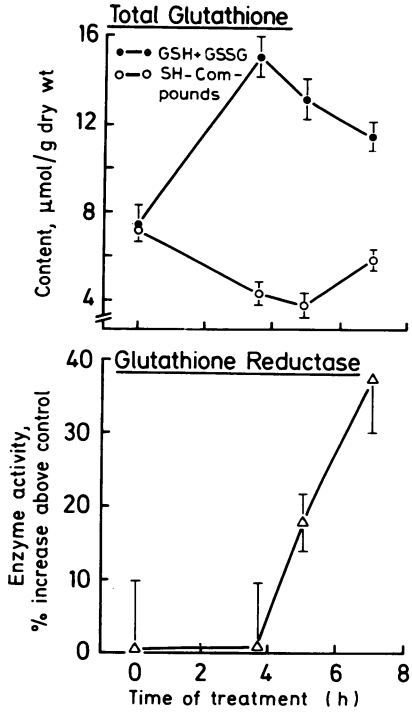

FIG. 2. Total content of glutathione (GSH + GSSG), acid-soluble SH-compounds, and increase of GR activity above control in bean leaves after short-term treatment with 0.5 mM acifluorfen. Activity of GR in untreated control leaves was 4.2 ± 0.5 μmol NADPH oxidized/min·g dry weight. Data shown represent the means ± SE of four different samples.

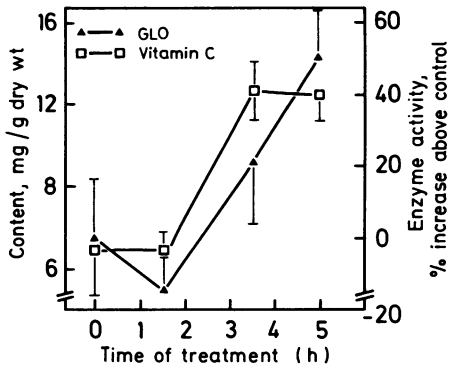

FIG. 3. Vitamin C content and increase of GLO activity above control in bean leaves after treatment with 0.5 mM acifluorfen. Activity of GLO in untreated control leaves was 0.44 ± 0.08 mg vitamin C produced/h·g dry weight. Data shown represent the means ± SE of four different samples.

DISCUSSION

Cells have different lines of defense to prevent toxic peroxidation. One line of defense is the presence of antioxidative enzymes, such as GR. We have strong evidence from work with a mutant of *E. coli* that lack of this enzyme almost totally inhibited cell growth under peroxidative conditions (21). In beans, peroxidation induced by the herbicide acifluorfen resulted in a significant increase of GR activity that prevented both oxidation of SH-compounds, mainly GSH (8), and peroxidation of lipids.

Our results with total glutathione and GR activity are in contrast to the results reported by Kenyon and Duke (10). They only found a strong decrease of both glutathione and GR activity after treatment of cucumber disks with acifluorfen in the light. However, sensitivity of plants seems to be dependent on different factors, such as the species used, the sufficient response of the plant to peroxidative conditions (21), and metabolism (6). In our experiments with beans both enhanced GR activity and higher production of antioxidants, like glutathione and vitamin C, seem to play an important role to limit peroxidation. Increased production of antioxidants that was directly related to elevated activity of biosynthesis enzymes, like GLO, was a primary event followed by increase of activity of antioxidant recycling enzymes, such as GR. A similar response of GR in maize seedlings was found by Foster and Hess (4) after exposure of seedlings to an O_2-enriched atmosphere. Higher total glutathione synthesis is connected to GSH-dependent feed back inhibition of the enzyme γ-glutamylcysteine synthetase (19). Depletion of GSH under peroxidative conditions can stimulate activity of the synthetase resulting in enhanced production of total glutathione. Reduction of GSSG by increased GR activity can, however, reactivate the feedback inhibition of the synthetase. Finally, both inhibition of the enzyme and catabolic processes decrease the amount of total glutathione in the cell. We have no evidence by now whether elevation of GR activity in plants depends on higher enzyme synthesis or activation by GSSG. From work with microorganisms it is known that the enzyme activity is determined by the concentrations of both NADPH and GSSG (17). A high concentration of NADPH inhibits and a low amount of GSSG activates GR.

Increased production of antioxidants and elevated activity of antioxidative enzymes, like GR, seem to be a general strategy to improve tolerance against toxic oxidation. This hypothesis has been supported by work of other research groups. Lee *et al.* (15) have shown higher amounts of vitamin C in O_3-tolerant plants after fumigation with the oxidant O_3. Smith *et al.* (22, 23) reported that a barley mutant, highly sensitive to light, could not exceed a certain level of glutathione seemingly necessary for detoxification of toxic O_2 species liberated during photorespiration in barley. In algae, the herbicide paraquat has been found to increase the enzyme superoxide dismutase that detoxifies reactive O_2 species produced by the herbicide (20). Further, Burke *et al.* (1) found enhancement of GR activity by dryness in field-grown cotton that protected against paraquat toxicity. However, more research is necessary in the future to support the hypothesis that tolerance of plants against peroxidation mainly depends on the increase of both activity of antioxidative enzymes and production of antioxidants.

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LITERATURE CITED

1. Burke JJ, PE Gamble, J Hatfield, JE Quisenberry 1985 Plant morphological and biochemical responses to field water deficits. Plant Physiol 79: 415–419
2. Burton GW, A Joyce, KU Ingold 1983 Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? Arch Biochem Biophys 221: 281–290
3. Finckh BF, KJ Kunert 1985 Vitamin C and E: an antioxidative system against herbicide induced lipid peroxidation in higher plants. J Agric Food Chem 33: 574–577
4. Foster JG, JL Hess 1982 Oxygen effects on maize leaf superoxide dismutase and glutathione reductase. Phytochemistry 21: 1527–1532
5. Foyer CH, B Halliwell 1976 The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25
6. Frear DS, HR Swanson, ER Mansager 1983 Acifluorfen metabolism in soybean: diphenylether bond cleavage and the formation of homoglutathione, cysteine, and glucose conjugates. Pestic Biochem Physiol 20: 299–310
7. Gillham DJ, AD Dodge 1986 Hydrogen-peroxide-scavenging systems within pea chloroplasts. Planta 167: 246–251
8. Grill D, H Esterbauer, U Klösch 1979 Effect of sulphur dioxide on glutathione in leaves of plants. Environ Pollut 19: 187–194
9. Halliwell B, CH Foyer 1978 Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139: 9–17
10. Kenyon WH, SO Duke 1985 Effects of acifluorfen on endogenous antioxidants and protective enzymes in cucumber (*Cucumis sativus* L.). Plant Physiol 79: 862–866
11. Kunert KJ, P Böger 1984 The diphenyl ether oxyfluorfen: action of antioxidants. J Agric Food Chem 32: 725–728
12. Kunert KJ, C Homrighausen, H Böhme, P Böger 1985 Oxyfluorfen and lipid peroxidation: protein damage as a phytotoxic consequence. Weed Sci 33: 766–770
13. Kunert KJ, G Sandmann, P Böger 1986 Modes of action of diphenyl ethers. Rev Weed Sci. In press
14. Law MY, SA Charles, B Halliwell 1983 Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. Biochem J 210: 899–903
15. Lee EH, JA Jersey, C Gifford, J Bennett 1984 Differential ozone tolerance in soybean and snapbeans: analysis of ascorbic acid in O_3-susceptible and O_3-resistant cultivars by high-performance liquid chromatography. Environ Exp Bot 24: 331–341
16. Loewus FA 1980 L-Ascorbic acid: metabolism, biosynthesis, function. *In* J Preiss, ed, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 77–99
17. Mata AM, MC Pinto, J Lopez-Barea 1985 Redox interconversion of glutathione reductase from *Escherichia coli*. A study with pure enzyme and cell-free extracts. Mol Cell Biochem 67: 65–76
18. McCay PB 1985 Vitamin E: interactions with free radicals and ascorbate. Ann Rev Nutr 5: 323–340
19. Meister A 1985 The fall and rise of cellular glutathione levels: enzyme based approaches. Curr Top Cell Regul 18: 21–57
20. Rabinowitch HD, DA Clare, JD Crapo, I Fidovich 1983 Positive correlation between superoxide dismutase and resistance to paraquat toxicity in the green alga *Chlorella sorokiniana*. Arch Biochem Biophys 225: 640–648
21. Schmidt A, KJ Kunert 1986 Antioxidative systems: defense against oxidative damage in plants. *In* C Arntzen, C Ryan, eds, Molecular Strategies for Crop Protection. UCLA Symposia on Molecular and Cellular Biology, New Series, Vol 48. Alan R. Liss, New York, In press
22. Smith IK, AC Kendall, AJ Keys, JC Turner, PJ Lea 1984 Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare* L.). Plant Sci Lett 37: 29–33
23. Smith IK 1985 Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol 79: 1044–1047
24. Summerfield FW, AL Tappel 1984 Vitamin E protects against methyl ethyl ketone peroxide-induced peroxidative damage to rat brain DNA. Mutat Res 126: 113–120
25. Tel-Or E, M Huflejt, L Packer 1985 The role of glutathione and ascorbate in hydroperoxide removal in cyanobacteria. Biochem Biophys Res Commun 132: 533–539
26. Tappel AL 1980 Measurement of and protection from in vivo lipid peroxidation. *In* WA Pryor, ed, Free Radicals in Biology, Vol IV. Academic Press, New York, pp 1–44