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INHIBITION OF SUPEROXIDE-GENERATING NADPH OXIDASE OF HUMAN NEUTROPHILS BY LAZAROIDS (21-AMINOSTEROIDS AND 2-METHYLAMINOCHROMANS)

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Abstract—Lazaroids (21-aminosteroids and 2-methylaminochromans) are a new series of drugs designed and demonstrated to protect against tissue damage after trauma and/or ischemia. It has been suggested that the protective effects of lazaroids are derived from their potent actions to inhibit iron-dependent lipid peroxidation, but whether this is sufficient to explain their therapeutic effects is unknown. In an effort to better understand their mechanism of action, these drugs were tested for other modes of antioxidant activity such as scavenging superoxide and hydroxyl radicals and inhibition of production of oxygen free radicals by human neutrophils stimulated with phorbol myristate acetate. Using an ESR spin-trapping technique, with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap for superoxide and hydroxyl radicals, we found that the lazaroids U74500A and U78518F are, at best, weak scavengers of superoxide radicals whereas U78518F is a strong scavenger of hydroxyl radicals. In addition, lazaroids were found to be strong inhibitors (60–80% inhibition at 50 μM) of the superoxide-generating NADPH oxidase of human neutrophils. Inhibition of NADPH oxidase by lazaroids in cell-free systems suggested the action to be on the activated enzyme rather than on the process of activation. This may represent an important mode of action of lazaroids and suggests their potential use in ischemic/inflammatory conditions involving oxygen free radical production by activated phagocytic cells.

Lazaroids‡ (21-aminosteroids and 2-methylaminochromans) are a class of compounds recently developed for use in the treatment of central nervous system trauma and ischemia [1–3]. Experiments with animal models have demonstrated that these compounds can prevent or limit the extent of tissue damage after brain or spinal cord injury [4, 5], as well as from ischemia–reperfusion injury in a variety of organs [6–10]. Their potent lipid antioxidant activity has been suggested as a possible mechanism of action [1, 2, 4]. However, lipid peroxidation is only one of several consequences of oxygen free reactions in cells [11–13]. Furthermore, the antioxidant effects of an agent may derive from action at one or more levels of oxygen free radical production and/or action.

Our interest in examining the mechanisms of action of lazaroids emerged from the following observations. Recent studies from one of our laboratories (A.R.) indicated that the cyto-destructive effects of cytokines on rat pancreatic islet β-cells, a possible mechanism for the autoimmune destruction of islet β-cells in insulin-dependent diabetes, can be inhibited significantly by a mixture of citiolone (an inducer of superoxide dismutase) and dimethylthiourea (a scavenger of hydroxyl radicals) [14], and also by the lazaroid compound U78518F [15]. The protective effect of these antioxidants may arise from a direct scavenging of oxygen free radicals (superoxide and/or hydroxyl) or alternatively by the inhibition of lipid peroxidation resulting from the action of oxygen free radicals. Lipid peroxidation products as well as oxygen free radicals (independent of lipid peroxidation) have been shown to damage cells, and some models of oxygen free radical injury to cells consider lipid peroxidation only as a secondary event propagating the initial injury caused by oxygen free radicals [11–13]. Hence, it was of interest to see whether the protective actions of lazaroids included other modes of antioxidant activity (activities other than the inhibition of lipid peroxidation), such as direct scavenging of oxygen free radicals and possibly inhibition of oxygen free radical production.

In the present study we assessed the effects of lazaroids on several aspects of oxygen free radical metabolism: the superoxide-generating xanthine–xanthine oxidase reaction implicated in ischemia–reperfusion injury [16]; the superoxide-generating NADPH oxidase of human neutrophils implicated in the bactericidal activity of phagocytes in general [17]; the H_2O_2-generating NADPH oxidase of rat liver microsomes [18–20]; and the hydroxyl radical-generating Fenton reaction implicated in many pathological processes [11–13]. Two types of lazaroids were used in these studies. Compounds U74500A and U75412E are lazaroids with a 21-aminosteroid moiety (first generation lazaroids) and

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‡ The term "lazaroid" is commonly used to refer to the series of 21-aminosteroids and related non-steroidal antioxidants (2-methylaminochromans) synthesized by the Upjohn Co., U.S.A., recently. The individual compounds used in this study are denoted by their numbers, namely U74500A, U75412E U78518E, and U78518F.

compounds U78518E and U78518F are non-steroidal lazaroids with a 2-methylaminochroman moiety (second generation lazaroids). U78518E and U78518F are identical compounds except for their salt form. The results suggest that a strong inhibition of the superoxide-generating NADPH oxidase of phagocytic cells is likely to be at least one important mode of action of lazaroids.

MATERIALS AND METHODS

Materials. Lazaroids U74500A, U75412E, U78518E, and U78518F were gifts from Drs. J. M. McCall and J. M. Braughler of the Upjohn Co., Kalamazoo, MI, U.S.A. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO*), phorbol myristate acetate (PMA), NADPH, ATP, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), cytochrome *c* (bovine heart, type III), xanthine oxidase (bovine milk), xanthine, adrenalin, and arachidonic acid were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Ficoll–Paque was the product of Pharmacia Fine Chemicals, Sweden. SOD was obtained from the Carlsberg Laboratories, Denmark. All other chemicals used were of analytical grade.

Preparation of neutrophils and membrane fractions. Neutrophils were isolated from human blood freshly drawn from healthy volunteers by standard procedures. Briefly, the buffy coat and part of the erythrocytes below it, obtained by low speed centrifugation of whole blood, were subjected to Ficoll–Paque separation. The bottom layer containing neutrophils and erythrocytes was subjected to hypotonic lysis and several washings to obtain neutrophils as a pellet. To obtain subcellular fractions from stimulated cells, a combination of different procedures was used as follows [21–23]. The neutrophils were suspended in 10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 5 mM KCl and 1 mg/mL glucose at a concentration of $\sim 10^8$ cells/mL and treated with 1 μg/mL PMA at 37° for 20 min. After the incubation, the cell suspension was cooled on ice, spun (to pellet the cells), washed with 0.34 M sucrose once and suspended in 0.34 M sucrose. The suspension kept on ice, was sonicated with a microtip probe (Branson sonifier) at 30 W for 50 sec, EDTA was added to a concentration of 0.5 mM, and the preparation spun at 800 *g* for 5 min to remove nuclei and unbroken cells. The supernatant was spun at 20,000 *g* for 20 min to obtain a pellet designated as Fraction I. The supernatant was spun again at 48,000 *g* for 1 hr to give another pellet designated as Fraction II. The pellets were suspended in 0.25 M sucrose/0.5 mM EDTA and immediately assayed for NADPH oxidase activity. Fractions I and II are likely to be enriched in granules and plasma membrane, respectively.

Rat liver microsomal fraction was prepared from normally fed Sprague–Dawley rats weighing 200–250 g according to the method described earlier [24] and stored at −20° until used.

* Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylene triaminepentaacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PMA, phorbol myristate acetate; and PBS, phosphate-buffered saline.

Spin-trapping assay. Superoxide and hydroxyl radicals were measured by spin-trapping with DMPO in a quartz flat cell at room temperature (23°). The resulting ESR spectra were recorded on a Brucker ESP300 spectrometer operating at 9.78 GHz with a modulation frequency of 100 kHz, modulation amplitude of 1 G, microwave power of 10 mW, and receiver gain of 5×10^4. The central field was set at 3477 G and the spectra were obtained by adding three scans made at a sweep rate of 0.596 G/sec over a sweep width of 100 G. All the ESR spectra were recorded at the above settings.

Spectrophotometric assays. The effects of lazaroids on the xanthine–xanthine oxidase reaction were assessed spectrophotometrically by measuring the reduction of ferricytochrome *c* [25]. The reaction was carried out in 50 mM potassium phosphate (pH 7.8) containing 100 μM EDTA and 20 μM cytochrome *c*. The absorbance at 550 nm was monitored for 3 min and the rate of change (ΔA_{550}/min) was calculated from a linear fit of the data.

The biochemical assay of NADPH oxidase activity of subcellular fractions from neutrophils was carried out by measuring the disappearance of NADPH (decrease in absorbance at 340 nm) as described earlier with some minor modifications [21, 22]. An NADPH concentration of 0.2 mM was used and the protein (fraction) concentration was adjusted to give an initial absorbance reading of 2 or less. The reaction was followed for 3 min and the rate of the reaction was obtained from the slope of the linear fitting of the data.

NADPH oxidase of rat liver microsomes was measured by the formation of H_2O_2 [18, 19] and also by the oxidation of adrenalin to adrenochrome [20] To measure H_2O_2, rat liver microsomes (0.759 mg protein) were incubated in 50 mM HEPES buffer, pH 7.4, containing 5 mM sodium azide, 10 mM $MgCl_2$ and 0.5 mM NADPH in a total volume of 0.5 mL at room temperature (23°) for 4 min. The reaction was terminated by adding 0.1 mL of 1.8 M trichloroacetic acid, cooled in ice for 10–15 min, and spun in a microfuge for 10 min. The supernatant (0.4 mL) was measured for H_2O_2 by the thiocyanate method [18, 19]. The oxidation of adrenalin to adrenochrome was measured at room temperature by monitoring the absorbance at 480 nm of the assay mixture containing rat liver microsomes (0.759 mg protein), 50 mM HEPES (pH 7.4), 0.2 mM sodium azide, 5 mM $MgCl_2$, 0.4 mM adrenalin, and 0.2 mM NADPH in a total volume of 1 mL. The rate of adrenochrome formation was calculated from the slope of a linear fit of the data points between 1 and 5 min of incubation.

Protein was measured according to the method of Lowry *et al.* [26], using bovine serum albumin as standard.

Lazaroids were added from a stock solution of 5 mM prepared in methanol or 0.05 N HCl and used within 1–2 days. An equivalent volume of methanol or HCl was added to the control experiments. The addition of HCl did not change the pH of the buffer. Methanolic solution was used for most of the studies because this was the diluent for the lazaroids used for the studies with pancreatic islet cells [15]. Solution

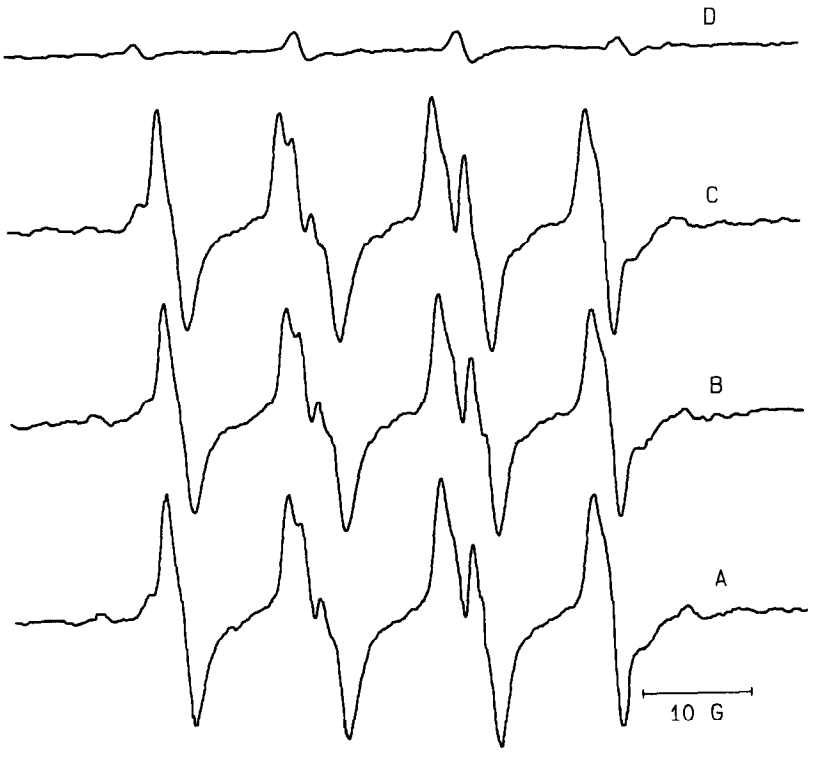

Fig. 1. Representative ESR spectra showing the effects of lazaroids on the xanthine–xanthine oxidase reaction. The reaction mixture consisted of 100 mM DMPO, 0.1 mM DTPA, 0.4 mM xanthine, and 140 mU/mL of xanthine oxidase in 50 mM sodium phosphate buffer, pH 7.8. Lazaroids were added as a methanolic solution. Spectra in the presence of 1% methanol (control) (A), 50 μM U74500A (B), 50 μM 75412E (C), and 2 μM superoxide dismutase (D) are shown. Lazaroids had no effect on DMPO-OOH peaks (A_N = 14.2 G, A_H = 11.6 G, $A_H{}^{\gamma}$ = 1.25 G).

in HCl was used whenever there was interference from methanol in the assay.

RESULTS

The effects of lazaroids on the xanthine–xanthine oxidase reaction are given in Fig. 1. Under these conditions, superoxide generated in the reaction is expected to be trapped quantitatively by the excess (100 mM) of DMPO added [27, 28]. The control spectrum shows the characteristic peaks of the superoxide radical [27, 28]. Identity of these peaks as superoxide radical was further supported by the ability of superoxide dismutase to obliterate the peaks. The lazaroids U74500A and U75412E, at a concentration of 50 μM, had no influence on the superoxide peaks. There was less than a 3% difference between control and lazaroid peak intensities as measured by double integrals of the peaks. The lazaroid U78518E also gave the same result (data not shown). This suggests that lazaroids do not inhibit the production of superoxide by the xanthine–xanthine oxidase reaction. However, it is not possible to absolutely rule out a superoxide scavenging role for lazaroids because of the 2000-fold difference in concentration between DMPO and lazaroids in the assay mixture. To resolve this question, another set of spectra was taken with a lower concentration of DMPO (10 mM) and a lower concentration of reactants, and the results are shown in Fig. 2. Here, some changes (in relative peak heights) were observed in the spectra on adding lazaroids to the xanthine–xanthine oxidase system. Thus, integration of peaks (second set from left) revealed a 30–40% decrease in intensity (compared to the spectrum in the presence of methanol) when either U74500A or U78518F was added. Although the results were obscured partially by the low signal to noise ratio, these findings suggest the possibility of, at best, a weak superoxide scavenging activity by the lazaroids. This possibility was tested further by monitoring superoxide levels by the cytochrome *c* (reduction) assay. The results shown in Table 1 show no significant scavenging activity, although U78518F dissolved in HCl resulted in a 10% decrease in superoxide levels ($P < 0.004$). Therefore, taken together, the results shown in Fig. 2 and Table 1 indicate that lazaroids can be considered as only very weak scavengers of superoxide, at best.

Figure 3 shows the effects of lazaroids on superoxide production by PMA-stimulated human neutrophils. Both U74500A and U78518E inhibited superoxide production in a concentration-dependent manner (only one spectrum for U78518E is given). This is similar to the reported effect of U74500A on hydrogen peroxide production by human neutrophils

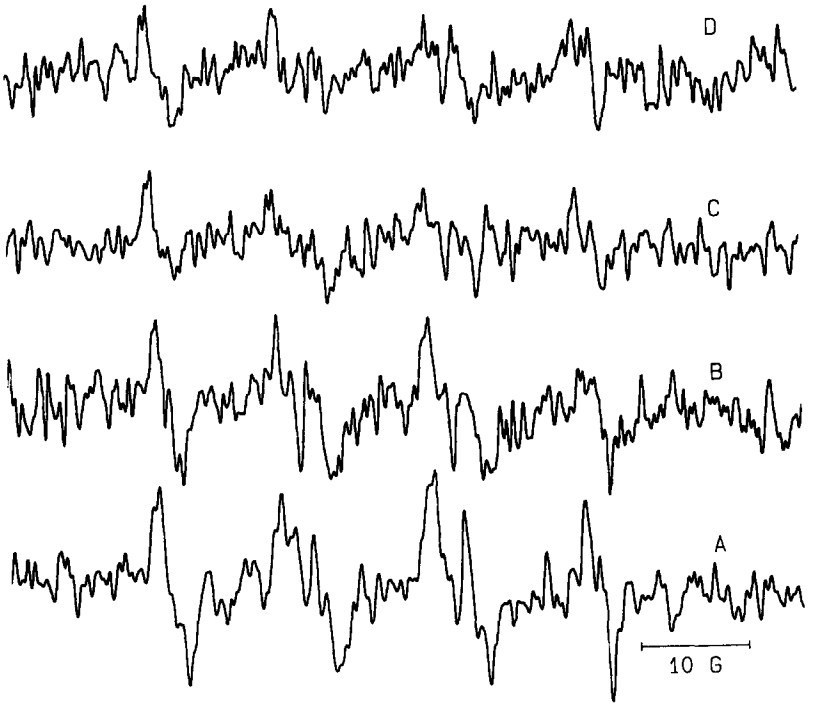

Fig. 2. ESR spectra measuring the superoxide scavenging capacity of lazaroids. The reaction mixture consisted of 10 mM DMPO, 0.1 mM DTPA, 0.05 mM xanthine, and 17.5 mU/mL of xanthine oxidase in phosphate-buffered saline (PBS). Spectra without any additions (A), and in the presence of 1% methanol (control) (B), 50 μM U74500A (C), and 50 μM U78518F (D) are shown.

Table 1. Cytochrome *c* assay for the scavenging of superoxide radical by lazaroids

Addition	Rate of appearance of superoxide (nmol/min)	
	Expt. I	Expt. II
None	1.22 ± 0.02	3.00 ± 0.03 (100)
Methanol (1%)	1.09 ± 0.04 (100)	
U74500A (50 μM)	1.21 ± 0.01 (111)	3.01 ± 0.03 (100)
U78518F (50 μM)	1.13 ± 0.01 (104)	2.70 ± 0.05 (90)
Superoxide dismutase (2 μM)	0.02 ± 0.01 (2)	0.07 ± 0.01 (2)

The assay mixture contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 50 μM xanthine, 3.5 mU/mL xanthine oxidase, and 10 μM cytochrome *c* (Expt. I) or 20 μM cytochrome *c* (Expt. II). Lazaroids were dissolved in methanol (Expt. I) or 0.05 N HCl (Expt. II). Values are means ± SEM from three measurements. The values in parentheses give the rates as percent of control.

[29]. The effect was detectable at a lazaroid concentration of 10 μM. The peak intensities (double integrals) showed 35–45 and 65–75% decreases with 10 and 50 μM concentrations of lazaroids, respectively. This effect was not due to any defects of lazaroids on cell viability because it was found to be the same (99%) in the absence and presence of 50 μM lazaroids, as measured by trypan blue exclusion. Since the lazaroids lacked a significant superoxide scavenging activity (Fig. 2 and Table 1), the results in Fig. 3 point to a potent inhibitory effect of lazaroids on NADPH oxidase, the enzyme system in the neutrophil plasma membrane responsible for the production of superoxide. To confirm this possibility, NADPH oxidase was assayed by measuring the utilization of NADPH in membrane fractions isolated from stimulated neutrophils. The rate of cyanide-insensitive consumption of NADPH is expected to give a better measure of the rate of production of superoxide because the consumption of NADPH will not be affected by the possible weak superoxide scavenging activity of lazaroids, whereas the spin-trapping assay (used above) and the conventional ferricytochrome *c* reduction assay will be affected. The results in Table 2 reveal that lazaroids were strong inhibitors of NADPH oxidase activity in human neutrophils. This inhibitory action of lazaroids on NADPH oxidase, therefore, would explain the action of these compounds to inhibit superoxide production (Fig. 3), independent of weak superoxide scavenging effects of the lazaroids (Fig. 2) that might have accounted for a minor decrease in the amplitude of the superoxide peaks in Fig. 3. Lazaroids are potent inhibitors of NADPH oxidase activity also when stimulated with arachidonic acid in a cell-free system as seen from the ESR spectra given in Fig. 4 and the data given in Table 2. To test whether the inhibition by lazaroids is reversible,

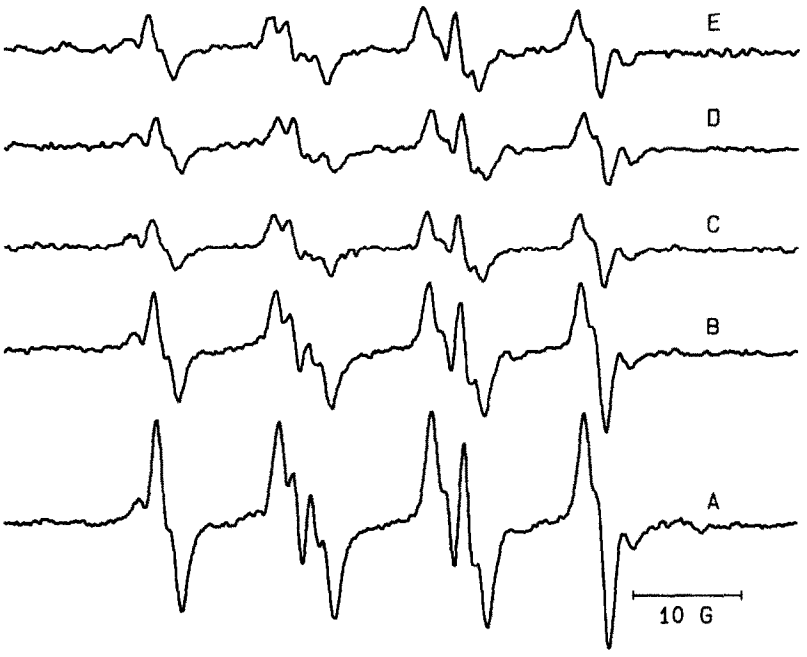

Fig. 3. Effects of lazaroids on the production of superoxide by PMA-stimulated (intact) human neutrophils. The assay system in PBS contained 100 mM DMPO, 10^6 neutrophils/mL, and 100 ng/mL PMA. ESR spectra for control (A), and in the presence of 10 μM U74500A (B), 30 μM U74500A (C), 50 μM U74500A (D), and 50 μM U78518E (E) are shown.

Table 2. Effects of lazaroids on NADPH oxidase activity of post-nuclear supernatant from human neutrophils and membrane fractions from PMA-stimulated neutrophils, as measured by NADPH utilization

Fraction	Addition	NADPH oxidase activity (nmol/min/mg protein)
Fraction I	None	137 ± 5
	Methanol (1%)	134 ± 3 (100)
	U74500A (50 μM)	102 ± 3 (76)
	U78518F (50 μM)	56 ± 1 (42)
Fraction II	None	167 ± 4
	Methanol (1%)	204 ± 24 (100)
	U74500A (50 μM)	77 ± 7 (38)
	U78518F (50 μM)	31 ± 3 (15)
P.N. Supt.	None	2.75, 3.26
	Methanol (1%)	3.35, 3.07 (100)
	U74500A (50 μM)	1.53 ± 0.43 (48)
	U78518F (50 μM)	0.87 ± 0.34 (27)

The assay mixture contained 65 mM sodium potassium phosphate (pH 5.5), 125 mM sucrose, 0.5 mM $MnCl_2$, 2 mM KCN, 0.2 mM NADPH, and the subcellular fraction. Fraction I (20,000 *g* pellet) and Fraction II (48,000 *g* pellet) are likely to be enriched in granules and plasma membrane, respectively. In the case of post-nuclear supernatant (P.N. Supt.), the assay mixture also contained 1 mM ATP, 1 mM $MgCl_2$ and 0.1 mM arachidonic acid. Lazaroids were added as a methanolic solution. Individual values are given for duplicate measurements; other values are means ± SEM from three to four measurements. Values in parentheses give the activities as percent of control.

one of the membrane fractions was preincubated with lazaroids for 10 min at room temperature, lazaroids were washed off, and the membranes were assayed for NADPH oxidase activity. The results given in Table 3 show that U78518F, which is the better inhibitor, was a reversible inhibitor whereas U74500A was an irreversible inhibitor. The apparent irreversibility of inhibition by U74500A may be a

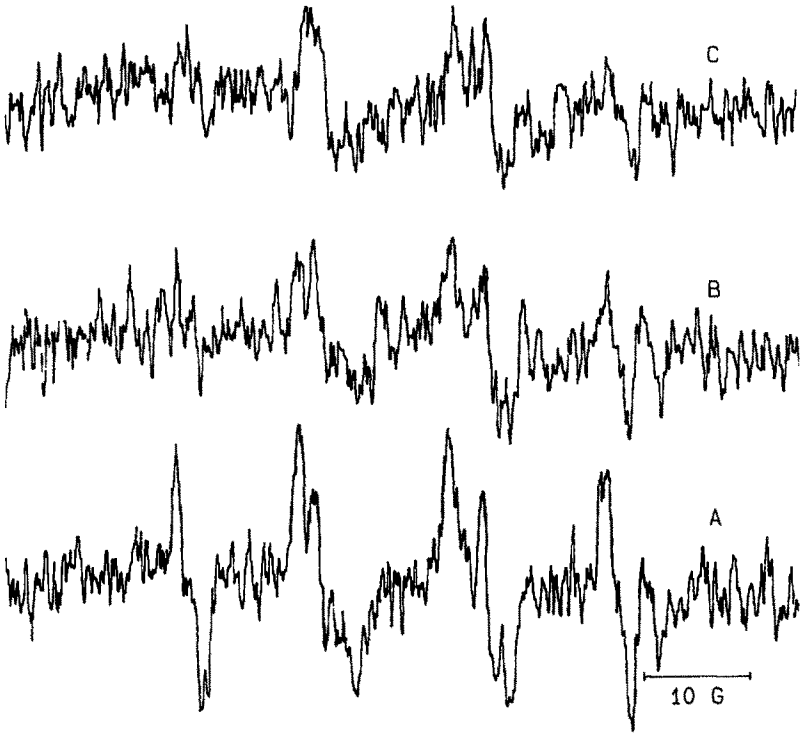

Fig. 4. Effects of lazaroids on superoxide production by arachidonic acid-stimulated human neutrophil homogenate (post-nuclear supernatant). The assay system contained 800 *g* supernatant from neutrophil homogenate (0.178 mg protein), 136 mM sucrose, 0.2 mM EDTA, PBS, 60 mM DMPO, 1 mM ATP, 1 mM $MgCl_2$, 0.2 mM NADPH, and 0.2 mM arachidonic acid. Lazaroids were added as a methanolic solution. ESR spectra in the presence of 1% methanol (control) (A), 50 μM U74500A (B), and 50 μM U78518F (C) are given.

Table 3. Reversibility of NADPH oxidase inhibition by lazaroids

Pretreatment	NADPH oxidase activity (nmol/min/mg protein)
Methanol (1%)	134 ± 5
U74500A (50 μM)	58 ± 4
U78518F (50 μM)	117 ± 1*

Fraction I from PMA-stimulated neutrophils (1 mg protein/mL) was incubated with 1% methanol, 50 μM U74500A or 50 μM U78518F (both lazaroids dissolved in methanol) for 10 min at room temperature in 65 mM sodium potassium phosphate buffer (pH 5.5) containing 125 mM sucrose. Membranes were sedimented by centrifuging at 48,000 *g* for 10 min and the supernatant was removed carefully. The pellet was rinsed a few times with 0.25 M sucrose/0.5 mM EDTA and finally suspended in it. NADPH oxidase activity was assayed by measuring NADPH consumption as in Table 2. Values are means ± SEM from three measurements.

* Significantly different from methanol-pretreated membranes at the level of $P < 0.011$.

reflection of the inability to remove the drug from the membranes by washing (due to the hydrophobicity of the drug) rather than a direct irreversible interaction of the drug with the enzyme.

Rat liver microsomes possess an NADPH oxidase activity producing H_2O_2 [18–20]. Since H_2O_2 by itself is a reactive oxygen metabolite and is the substrate for the Fenton reaction generating the more damaging hydroxyl radical, it was of interest to see the effects of lazaroids on this system as well. The results obtained by two different methods are shown in Table 4. Contrary to the NADPH oxidase of human neutrophils, the NADPH oxidase of rat liver microsomes was not inhibited at all by lazaroids; surprisingly, U78518F *stimulated* (+71%) H_2O_2 production. The absence of an effect when adrenochrome formation was monitored could be due to the difference in the nature of the reaction when adrenalin is added to the system [20].

Lazaroids were tested for their ability to inhibit the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$$

producing hydroxyl radicals ($HO^{\cdot}$) and their ability to scavenge hydroxyl radicals. The iron chelators EDTA and diethylene triaminepentaacetic acid (DTPA) are known to stimulate the Fenton reaction [30]. One of the mechanisms previously suggested for the lipid antioxidant activity of lazaroids is a possible iron binding capacity [1]. The above

Table 4. Effects of lazaroids on NADPH oxidase activity of rat liver microsomes

Addition	NADPH oxidase activity	
	H_2O_2 (nmol/min/mg protein)	Adrenochrome (nmol/min/mg protein)
None (control)	22.82 ± 0.51 (100)	9.39 ± 0.39 (100)
U74500A (50 μM)	24.52 ± 0.61 (107)	9.87 ± 0.23 (105)
U78518F (50 μM)	38.94 ± 0.37 (171)	9.34 ± 0.15 (99)
Superoxide dismutase (2 μM)		1.17 ± 0.60 (12)

The activities generating H_2O_2 (in the absence of adrenalin) and adrenochrome from adrenalin were measured as described in Materials and Methods. Lazaroids were added from a stock solution in 0.05 N HCl (lazaroids were not tested in methanolic solution because methanol was found to interfere with the assay). Values are means ± SEM from five measurements for H_2O_2 and three measurements for adrenochrome. The values in parentheses give activities as percent of control.

considerations suggest the importance of looking at the influence of lazaroids on the Fenton reaction. Hence, the effects of lazaroids on the Fenton reaction were tested along with some iron chelators (as positive controls) and the results are given in Fig. 5. Since a high concentration (20 mM) of DMPO was used, the trapping of hydroxyl radical would be expected to be quantitative and the peak intensities should reflect the rate of formation of hydroxyl radical and/or scavenging by the agent added. EDTA (50 μM) stimulated the reaction as reported earlier [30]. Also, addition of 50 μM DTPA resulted in an 8-fold increase in peak intensities (spectrum not shown). On the other hand, desferrioxamine, a much stronger iron chelator, resulted in the reduction of peak intensities by 75–80%. Lazaroids U74500A

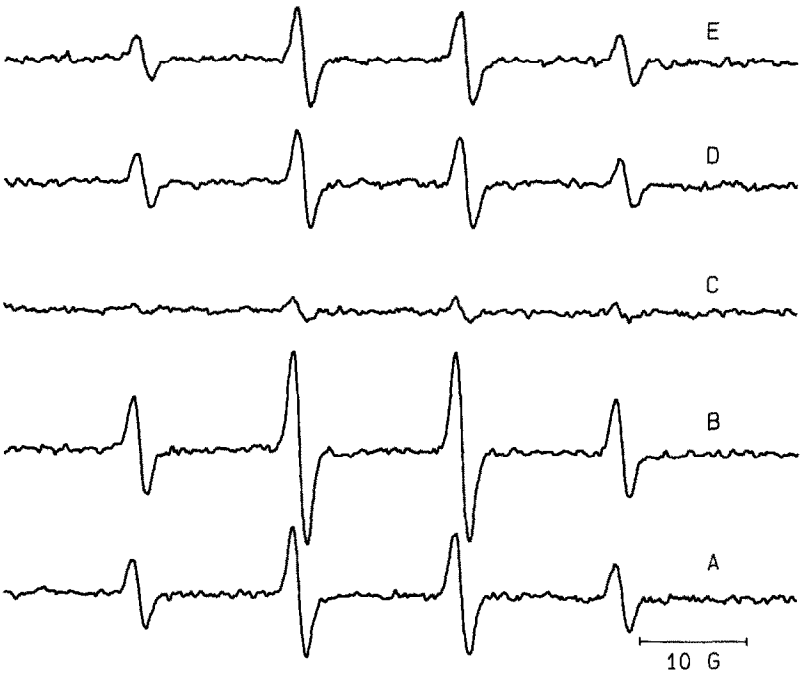

Fig. 5. Typical ESR spectra showing the effects of lazaroids on hydroxyl radical production by Fenton reaction. The reaction mixture contained 20 mM potassium phosphate (pH 7.4), 150 mM KCl, 20 mM DMPO, 0.1 mM H_2O_2, and 20 μM Fe^{2+} (as ferrous ammonium sulfate). Lazaroids were added to the mixture as a solution in 0.05 N HCl and the same amount of HCl was added to all the other systems. (Lazaroids were not tested in methanolic solution because methanol was found to scavenge hydroxyl radical.) Spectra for control (A), 50 μM EDTA (B), 50 μM desferrioxamine (C), 50 μM U74500A (D), and 50 μM U78518F (E) are shown. Lazaroids had no effect on DMPO-OH peaks ($A_N = A_H = 14.9$ G).

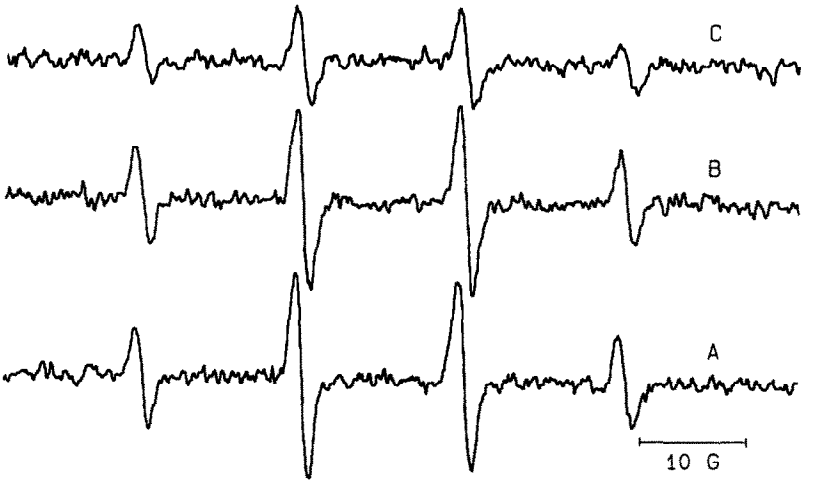

Fig. 6. Scavenging of hydroxyl radical by lazaroids. The reaction was the same as in Fig. 5 except that 0.5 mM DMPO was used. Typical ESR spectra for control (A), 50 μM U74500A (B), and 50 μM U78518F (C) are shown.

and U78518F were found to have no significant effect on peak intensities, thereby ruling out the possibility that lazaroids could slow down the rate of formation of hydroxyl radical in the Fenton reaction. However, since there was a large (400-fold) difference in the concentration of DMPO and lazaroids, the results do not exclude completely the possibility of hydroxyl radical scavenging by lazaroids. Therefore, we tested lazaroids at a much lower DMPO concentration (0.5 mM) and the results are given in Fig. 6. Again, U74500A had a negligible effect, if any. However, U78518F resulted in a 60–70% decrease in peak intensities, thereby suggesting that whereas the production of hydroxyl radical by the Fenton reaction was not inhibited there was good scavenging of the hydroxyl radical. Testing U74500A at higher concentrations for possible hydroxyl radical scavenging activity was precluded by the poor solubility of the drug.

DISCUSSION

The recent emphasis on the role of oxygen free radicals and lipid peroxidation in pathological processes such as diabetes, atherosclerosis, cancer, stroke and aging gives special significance to the concept of oxidation therapy and hence the development and study of novel antioxidant drugs like lazaroids [11–15, 31–33]. Since some of the lazaroids are currently being evaluated in clinical trials, the present studies are intended to provide mechanistic information that could be helpful for clinical application. The only mechanism known so far to explain the action of lazaroids is their strong lipid antioxidant activity attributed to iron chelation, chain breaking (antioxidant activity) like that of α-tocopherol, and possibly the alteration of membrane fluidity [1, 34, 35]. We also have observed the lipid antioxidant activity when arachidonic acid micelles were peroxidized with $FeSO_4$ (data not given). The present results, however, show that in addition to the lipid antioxidant activity, other antioxidant activities, such as alteration of the rate of production of oxygen free radicals, and possibly the scavenging of hydroxyl radicals, are also to be considered.

The superoxide scavenging by lazaroids was assessed by spin-trapping and the conventional spectrophotometric method (Fig. 2 and Table 1). DMPO, cytochrome *c*, and superoxide dismutase scavenge superoxide with (second order) rate constants of 10, 2×10^5, and $2 \times 10^9\ M^{-1}\ sec^{-1}$, respectively [25, 28, 36]. Clearly the scavenging rate for lazaroids should be $< 10^5\ M^{-1}\ sec^{-1}$, the rate for cytochrome *c*. The reduction in peak intensity in Fig. 2 corresponds to a scavenging rate of $\leq 10^3\ M^{-1}\ sec^{-1}$ for lazaroids. The low rate for DMPO coupled with the poor solubility of lazaroids renders more precise measurements very difficult. Though it is a weak scavenger of superoxide, U78518F appeared to be a good scavenger of hydroxyl radical (Fig. 6). Based on the rate constant of $3.4 \times 10^9\ M^{-1}\ sec^{-1}$ for the scavenging of hydroxyl radical by DMPO [28] and the 60–70% decrease in peak intensity seen in Fig. 6, a rate constant of $\sim 6 \times 10^{10}\ M^{-1}\ sec^{-1}$ may be calculated for the scavenging of hydroxyl radical by U78518F. The apparent value compares with, or is even better than, that for the well known hydroxyl scavengers such as ascorbic acid, cysteine, glutathione, guanine, thiourea, albumin and hemoglobin [37]. Despite the high rate of hydroxyl scavenging, the contribution of lazaroids to hydroxyl scavenging in tissues is likely to be insignificant because of the likely low tissue levels of lazaroids (probably in the low micromolar range). The weak superoxide scavenging may also be of no consequence physiologically for the same reason. But these antioxidant activities may become important in vascular endothelium where these drugs are likely to be concentrated.

The most important observation was the ability of

lazaroids to interfere with the production of reactive oxygen metabolites by human neutrophils (Figs. 3 and 4 and Tables 2–4), and this could be physiologically significant because of the low concentrations at which the effects were observed. The superoxide-generating NADPH oxidase of human neutrophils is a multicomponent system and the activation process, though not well understood, may consist of several steps such as the binding of the stimulant to the cell surface receptor, the involvement of G proteins, translocation of protein kinase C, phosphorylation of some cytosolic proteins, rearrangement of the membrane skeletal system and the translocation of cytochrome b_{559} from granules to the plasma membrane [38–44]. Since the inhibitory effect on intact cells is also seen with (cell-free) postnuclear supernatants and isolated membrane fractions, and also with different stimulants (PMA and arachidonic acid), lazaroids may not be affecting any of the above-mentioned steps in the activation of NADPH oxidase. The inhibitory effect most likely results from an interaction of lazaroids with the activated enzyme system. This is in contrast to the inhibitory effect of the antirheumatic drug piroxicam which interferes with the activation process rather than the activity of the activated NADPH oxidase [45]. The interaction between lazaroids and NADPH oxidase can occur as a result of a specific interaction between lazaroids and the protein component(s) of the enzyme system or non-specifically due to a perturbation of the membrane structure around the enzyme system by the lazaroids. Membrane lipid order and lateral mobility may be important in determining the activity of human neutrophil NADPH oxidase [46]. The apparent irreversibility of inhibition by U74500A may be suggestive of such a membrane interaction. However, a direct interaction with the enzyme, including an enhanced proteolysis, cannot be ruled out for U74500A. The possibility of inactivation by enhanced proteolysis can be ruled out at least in the case of U78518F because of the reversibility of its interaction with the membranes.

Whereas the inhibitory effects of lazaroids on oxygen free radical production may contribute to beneficial effects in free radical-mediated tissue injury, undesirable effects may also result. For example, it remains to be seen whether decreased production of superoxide by neutrophils might compromise defense functions of these cells (digesting endocytosed bacteria and other particles). Further, stimulation of H_2O_2 production by the NADPH oxidase of rat liver microsomes by U78518F (Table 4) suggests the possibility of *increased* oxidant stress in certain tissues as a result of lazaroid therapy. Recently, it was reported that the lazaroid U74006F is not capable of improving the neurological outcome after transient forebrain ischemia in the rat, a result contrary to the generally beneficial effect seen in most studies [47]. U74006F was also found to have no protective effect in a canine model of myocardial ischemia–reperfusion injury [48]. Such discrepancies may arise from the differential sensitivities to lazaroids of the various enzyme systems generating reactive oxygen species, ranging from strong inhibition to strong stimulation.

Even though the inhibitory effect of lazaroids on superoxide-generating NADPH oxidase of neutrophils might be harmful from the point of view of the normal phagocytic functioning of these cells (bactericidal activity), it could well be the basis of the protective effects of lazaroids in ischemic and inflammatory injury and in some related pathologies such as atherosclerosis, diabetes, and cancer. Neutrophils infiltrating into a tissue to destroy some initially damaged cells (for different reasons in different cases) will produce oxygen free radicals for this purpose. However, continued production of oxygen free radicals may damage surrounding normal cells and result in the spreading of tissue damage [12–15, 31, 32]. Lazaroids may prevent this process by inhibiting the production of reactive oxygen species by the neutrophils. Also, mononuclear phagocytes (macrophages), fibroblasts, and endothelial cells can produce oxygen free radicals, following stimulation by PMA or immune cytokines [17, 49, 50], and therefore it would be interesting to determine if lazaroids may interfere with free radical production in these cells. Furthermore, it remains to be determined if the protective effect of lazaroids against cytokine-induced damage to pancreatic islet β-cells, associated with inhibition of lipid peroxidation [15], may include inhibition of the generation of reactive oxygen species in the islet cells.

In conclusion, lazaroids are a new class of antioxidants possessing different types of antioxidant activities and pro-oxidant activity in biological systems. In addition to their reported lipid antioxidant activity, other antioxidant activities such as the scavenging of hydroxyl radical and the inhibition of superoxide-generating NADPH oxidase may represent mechanisms of action of these novel drugs.

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