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Pharmacology of Lazaroids and Brain Energy Metabolism: A Review

ROBERTO FEDERICO VILLA[a] AND ANTONELLA GORINI

Institute of Pharmacology, Faculty of Science, University of Pavia, Pavia, Italy

I. Introduction

A. Background

A considerable body of experimental evidence indicates that lipid peroxidation (Komara et al., 1986; Dexter et al., 1989), the presence of iron (Riederer et al., 1989; Hirsh et al., 1991) and the depletion of natural antioxidants (Sato and Hall, 1992) seem to be a common epiphenomena of some pathologies in the central nervous system (CNS).[b]

The role of iron (either free or complexed) in catalyzing oxygen-derived free radical production and, consequently, its role in the peroxidative process (Halliwell and Gutteridge, 1984; Braughler et al., 1986; Minotti and Aust, 1989) is well known, even though the involvement of this biochemical pathway with the pathogenesis of some neuropathologies remains unclear.

[a] Address correspondence to: Dr. Roberto F. Villa, Institute of Pharmacology, University of Pavia, Piazza Botta, 11–27100 Pavia, Italy.

[b] Abbreviations: CNS, central nervous system; alpha-TC, alpha-tocopherol; LOO·, lipid peroxyl radical; LH, (poly)unsaturated fatty acid; $\cdot O_2$ or O_2^-, superoxide free radical or anion; H_2O_2, hydrogen peroxide; ·OH, hydroxyl free radical; OH^-, hydroxide ion; R·, alkyl radical; L·, allylic (poly)unsaturated fatty acid radical (lipid free radical); LOOH, hydroperoxide lipid radical; SOD, superoxide dismutase; CSF, cerebrospinal fluid; C_{inf}, concentration at the end of the infusion; SAH, subarachnoid hemorrhage; Cl, systemic clearance; L_z, Cl corrected for body weight and terminal elimination rate constant; V_d, area estimated from a single dose; $t_{1/2}$, biological half-life; V_{ss}, terminal elimination rate constant; V_{max}, maximal velocity; K_m, kinetics constant; IC_{50}, concentration that inhibits 50%; ADP, adenosine diphosphate; ATP, adenosine triphosphate; *E*, oxidation potential; BBB, blood-brain barrier; DNA, deoxyribonucleic acid; MP, methylprednisolone sodium succinate; MCA, middle cerebral artery; DHBA, dihydroxybenzoic acid; OH·, hydroxyl radical; BCO, bilateral carotid occlusion; SAL, salicylate; PG, prostaglandin; NMDA, N-methyl-D-aspartate; TBARS, thiobarbituric acid-reactive substances; NADH, reduced nicotinamide adenine dinucleotide.

The radical-initiated peroxidation of neuronal, glial, vascular cell membranes and myelin is catalyzed by free iron released from hemoglobin, transferrin and ferritin by either lowered tissue pH or oxygen radicals. If unchecked, lipid peroxidation is a geometrically progressing process that will spread over the surface of the cell membrane, causing impairment to phospholipid-dependent enzymes, disruption of ionic gradients and, if severe enough, membrane lysis.

Natural or synthetic compounds with scavenger and/or chelating properties have been found in in vitro and in vivo experimental models, aimed to protect the nervous tissue from the lipid peroxidative attack (Jacobsen et al., 1990; Hara et al., 1990a; Hall et al., 1991a; Ciuffi et al., 1992), but only some of them seem to be efficient for the activity in vivo (Hall, 1987; Hall and Braughler, 1989).

Lipid peroxidation normally proceeds as a radical-driven chain reaction involving oxygen, where the lipid peroxyl radical (LOO·), formed through initiation, attacks a second unsaturated fatty acid (LH). An important endogenous inhibitor of lipid peroxidation in membranes is alpha-tocopherol (alpha-TC), that inhibits lipid peroxidation by scavenging (LOO·):

$$\text{LOO}\cdot + \text{alpha-TC} \rightarrow \text{LOOH} + \text{alpha-TC}\cdot \quad [1]$$

thus preventing lipid radical chain reaction from occurring (Braughler and Pregenzer, 1989). The alpha-TC· radical decomposes to tocopherolquinone and effectively terminates the chain reaction (Braughler and Pregenzer, 1989). In addition, the alpha-TC radical is much less reactive in attacking adjacent fatty acid side chains and can be converted back to alpha-TC by vitamin C.

Studies with intact membranes indicate that the 21-aminosteroids are as potent as alpha-TC as inhibitors of iron-dependent lipid peroxidation, and their reactivity is less than that for alpha-TC in scavenging (LOO·).

The chemistry of free radical formation provides several sources that may cause cell generation of superoxide free radical ($\cdot O_2$) and that use (H^+) to undergo spontaneous dismutation reaction (reaction 2). Hydrogen peroxide (H_2O_2), which results from this spontaneous dismutation, in the presence of another superoxide radical (anion), can undergo reduction to form a highly reactive hydroxyl free radical (·OH), molecular oxygen and the hydroxide ion (OH^-) (reaction 3). Hydroxide anion is also released when hydroxyl free radicals are formed from a series of reactions involving ($Fe^{3+/2+}$) metal ions (reactions 4 and 5):

$$\cdot O_2 + \cdot O_2 + 2H^+ \rightarrow O_2 + H_2O_2 \quad \text{(Farber)} \quad [2]$$

$$\cdot O_2 + H_2O_2 \rightarrow O_2 + \cdot OH + OH^- \quad \text{(Beauchamp and Fridovich)} \quad [3]$$

$$Fe^{3+} + \cdot O_2 \rightarrow Fe^{2+} + O_2 \quad \text{(Haber-Weiss)} \quad [4]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad \text{(Fenton)} \quad [5]$$

The reaction (reaction 5) is known also as the Fenton reaction, certainly occurring in vitro.

The free radicals ($\cdot O_2$) and (·OH) and the compound (H_2O_2) are generated by all aerobic cells, and their antioxidant defenses prevent these species from causing cell injury. The clamping effects occur when the rate of formation of these free radical species is increased and/or the antioxidant defenses of cells are weakened.

In these conditions, excessive production and concentrations of radical species (R·) can initiate lipid peroxidation by attacking and removing an allylic hydrogen from a (poly)unsaturated fatty acid (LH) of membrane phospholipids (reaction 6); rearrangements of the double bonds results in the formation of conjugated dienes. The resulting allylic (poly)unsaturated fatty acid radical (lipid free radical) (L·) reacts with O_2 dissolved within the membrane to form a strong oxidizing species, lipid peroxyl radical (LOO·) (reaction 7), which can extract a second allylic hydrogen (reaction 8) ion from another methylene carbon.

This autocatalytic process converts the carbons of fatty acids of membrane phospholipids to unstable and highly reactive hydroperoxide lipids radical (LOOHs), which are further fragmented to a variety of lower molecular weight products, resulting in the destruction of unsaturated fatty acids of membrane phospholipids, including malondialdehyde, ethane and pentane.

The $Fe^{3+/2+}$ can react directly with the hydroperoxide radical of lipids (reactions 9 and 10):

$$\text{R}\cdot + \text{LH} \rightarrow \text{RH} + \text{L}\cdot \quad \text{R}\cdot = \cdot\text{OH}; \cdot\text{O}_2 \quad [6]$$

$$\text{L}\cdot + \text{O}_2 \rightarrow \text{LOO}\cdot \quad \text{R}\cdot = \text{LOO}\cdot; \text{L}\cdot \quad [7]$$

$$\text{LOO}\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}\cdot \quad [8]$$

$$\text{LOOH} + (Fe^{3+}\text{-complex}) \rightarrow \text{LO}\cdot + (Fe^{IV}=O^-\text{-complex}) + H^+ \quad [9]$$

$$\rightarrow \text{LOO}\cdot + (Fe^{2+}\text{-complex}) + H^+$$

$$\text{LOOH} + (Fe^{2+}\text{-complex}) \rightarrow \text{LO}\cdot + (Fe^{3+}\text{-complex}) + OH^- \quad [10]$$

It was suggested that the absolute ratio of Fe^{3+} to Fe^{2+} was the primary determining factor for the initiation of lipid peroxidation reactions on the order of 1:1 to 7:1 (Braughler et al., 1986).

It should be noted that the reaction of peroxysulphenyl radical will lead to formation of the superoxide anion, thus providing a new source of H_2O_2 by dismutation reaction of the superoxide (reactions 11 and 12):

$$2\text{R-SOO}\cdot + 2\text{R-SH} \rightarrow 2\text{R-S-S-R} + 2\cdot O_2 + 2H^+ \quad [11]$$

$$\cdot O_2 + \cdot O_2 + 2H^+ \rightarrow H_2O_2 + O_2 \quad [12]$$

In our opinion, at first it is very important to stress that (R-SH) groups are present in many proteins with catalytic properties, i.e., enzymes; thus, their function may be altered directly. Secondly, malondialdehyde reacts with the ϵ-amino groups of lysine, causing the cross-

linking of proteins and with other primary amino groups on phospholipids and nucleic acid.

As indicated, the chemistry of radicals is very complex, and it should be stressed that many of the indicated reactions have been studied in vitro. An important first concern is that the reaction (reaction 2) really happens as:

$$2O_2 + 2e^- \rightarrow 2{\cdot}O_2 + 2H^+ \rightarrow 2HO_2 \quad [13]$$

$$2HO_2 \rightarrow O_2 + H_2O_2 \quad [14]$$

thus stressing the fact that, for each H_2O_2 formed, two electrons (e^-) are necessary (reaction 13). To make the reaction (reaction 3) possible, an additional (e^-) is required to form the third superoxide radical (that properly is an anion); therefore $3e^-$ are required for coupling (reaction 2) *plus* (reaction 3) reactions; reactions 4 and 5 require ($3e^-$), too.

Regarding the role of lipid peroxidation in the injury of the CNS, the mosaic lipid peroxidation is mediated by the free radical in the neurons, which forms lipid peroxides within cell membranes and organelles. These oxidized lipids alter the structure and function of membranes by tissue injury. During periods of ischemic metabolism, superoxide anion is produced by mitochondrial dysfunction, as a by-product of various enzyme-substrate reactions. Electron transport chain in mitochondria and endoplasmic reticulum are major sources of superoxide. When mitochondrial function breaks down, some of the electrons "leak" from the usual electron carriers onto oxygen, forming superoxide anion (reaction 13). This is paradoxically augmented by postischemic reperfusion, especially under hyperoxic conditions (Hall et al., 1994).

Superoxide anion is not itself particularly reactive, and it does not cross cell membranes very well. However, it can become more dangerous by either accepting a proton or by dismutating to hydrogen peroxide (reactions 2, 13 and 14). During ischemia, lactic acidosis can lead to protonation of some of the superoxide anion, and protonated superoxide anion can better penetrate the membrane, where it can initiate lipid peroxidation.

The CNS is particularly susceptible to lipid peroxidation (LeBel and Bondy, 1991) for several reasons. First, the membrane lipids of the brain are rich in polyunsaturated fatty acids, which have particularly reactive hydrogens that can participate willingly in either the initiation or the propagation phases of lipid peroxidation. Second, the brain has only modest antioxidant capacity relative to other organs; it is poor in catalase and weak in superoxide dismutase (SOD) and glutathione peroxidase (Cohen, 1988). Third, several areas of the brain are rich in intracellular iron that is released during the injury process (Youdim and Ben-Schachar, 1988). Fourth, cerebrospinal fluid (CSF) contains much less transferritin than plasma and thus does not bind excess-released iron; the transferrin that is present is essentially saturated (Halliwell and Gutteridge, 1992). Finally, the CNS is rich in monoamine neurotransmitters (dopamine, epinephrine and norepinephrine): these produce H_2O_2 when they are oxidized by monoamine oxidase.

Recently, a family of steroid compounds, 21-aminosteroids, was developed; although this family derived from glucocorticoids, it lacks glucocorticoid and mineralocorticoid activities (Jacobsen et al., 1990). The compounds in this family were shown to scavenge lipid peroxyl radicals and to inhibit iron-dependent lipid peroxidation (Braughler et al., 1988a; Braughler and Pregenzer, 1989). Moreover, they were observed to improve survival, to preserve neurons and to reduce cerebral edema in animal models of focal cerebral ischemia (Hall et al., 1988a; Young et al., 1988a).

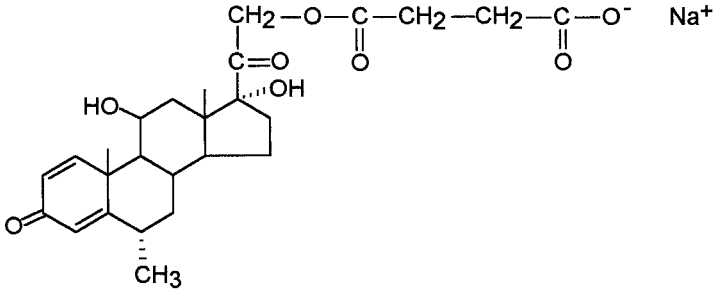
Therefore, the 21-aminosteroids or "lazaroids," a novel series of lipid peroxidation inhibitors, were designed to be devoid of glucocorticoid receptor interactions, while simultaneously retaining a propensity for cell membrane localization and having improvements in lipid peroxidation inhibitory efficacy in comparison with methylprednisolone. In particular, one of these compounds, U-74006F (United States and United Kingdom generic name is tirilazad mesylate) was selected for clinical development, as a parenterally administered acute neuroprotective agent. The chemical structural formula of these compounds (series: F-A-E) is indicated in fig. 1. Among these compounds, U-74500A was reported to inhibit the cytotoxicity and lipid peroxidation of iron-loaded cultured endothelial cells that had been submitted to an exogenous or endogenous oxidant attack (Balla et al., 1990). Moreover, this compound reduces H_2O_2 generation by stimulated human polymorphonuclear leukocytes and decreases both chemiluminescence and H_2O_2 produced by monocytes, which are harvested from the blood of patients affected by multiple sclerosis (Fischer et al., 1990, 1991).

However, apart from the pharmacological characteristics of specific compounds, it is clear that these "lazaroids" represent very interesting molecules with important pharmacological features not yet explored. In the following chapters, these pharmacological actions will be revised with particular relevance to the brain tissue and related pathologies.

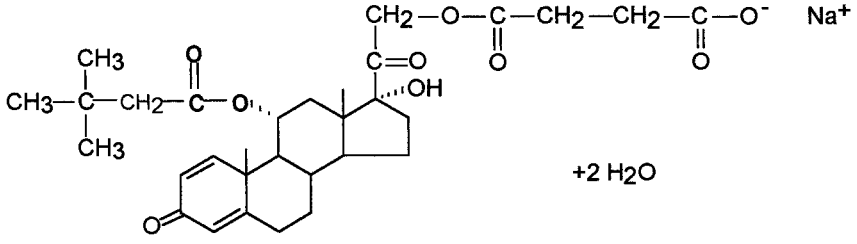
B. Bioavailability and Metabolism

As far as the chemicophysical characteristics and bioavailability of lazaroids, these were studied using the in vivo model (Ciuffi et al., 1994) of Wistar rat treated i.p. or s.c.: (*a*) 12 mg/kg every 24 h, i.p.; (*b*) 48 mg/kg every 48 h, i.p.; (*c*) 48 mg/kg every 48 h, s.c.; and (*d*) s.c., one-fifth of the total dose dissolved in citrate vehicle. Half an hour later, the animals received the remaining part of the drug, now dissolved in PEG 4000 (1.4% aqueous solution, retard preparation).

METHYLPREDNISOLONE SODIUM SUCCINATE



U-72099E NON-GLUCOCORTICOID STEROID



21-AMINOSTEROIDS (LAZAROIDS)

U-74006F (TIRILAZAD MESYLATE)

U-74500A

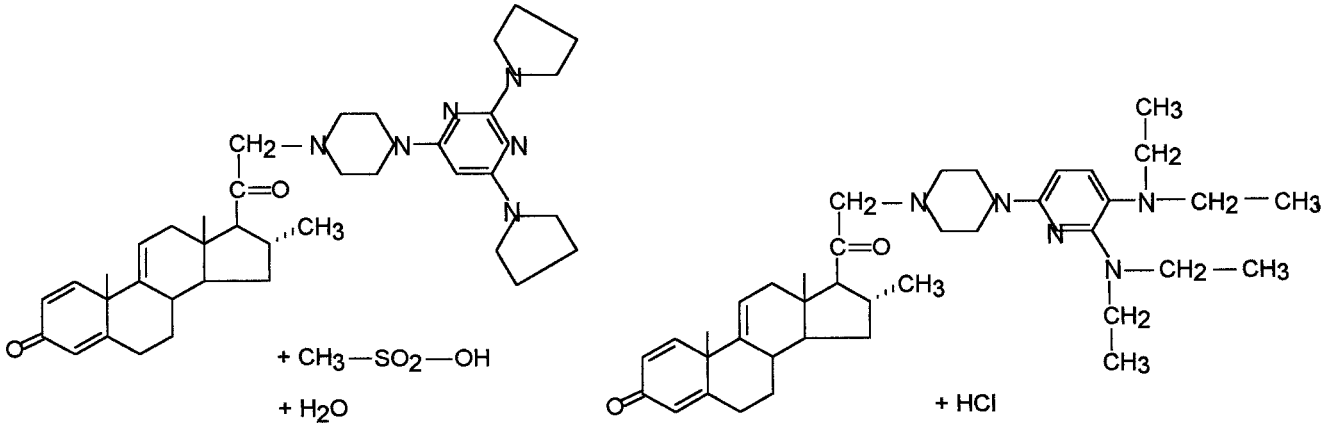


FIG. 1. Chemical structures of the glucocorticoid methylprednisolone, the nonglucocorticoid steroid U-72099E, the 21-aminosteroid U-74006F (tirilazad mesylate) and the 21-aminosteroid U-74500A (lazaroids).

U-74500A, following administration in the retard form, showed an inhibitory effect on lipid peroxidation in the iron-saccharate-injected brain hemicortices. On the contrary, the aminosteroid dissolved in the buffer (0.02 M citric acid monohydrate, 0.0032 M sodium citrate dihydrate, 0.077 M NaCl, pH 3) appears to be ineffective; this is probably ascribable to the short half-life of this drug (less than 10 min) once it reaches the blood.

It seems that an adequate concentration in the brain tissue that is able to inhibit the continuous iron-induced lipid peroxidation can only be achieved with a retard preparation, and it may be regarded as a possible ther-

apeutical tool in neuropathologies that are characterized by a peroxidative attack. However, it should be noted that, in this form, U-74500A inhibits lipid peroxidation at a step before diene conjugation, and diene formation is considered to be evidence of an early or moderate alteration of the structure of polyunsaturated lipids, as a result of free radical attack (Klein, 1970); in fact, the diene measurement showed a clear decrease in iron-injected drug-treated animals (Ciuffi et al., 1994). Nevertheless, the total iron content of the brains submitted to intracortical injection was not significantly modified by the 21-aminosteroid administration. It has been reported that this lipophylic drug with chelating activity displays spectral changes in the ultraviolet (UV) range in the presence of Fe^{2+} (Braughler et al., 1988a) and inhibits in vitro iron-dependent lipid peroxidation of intact phospholipid membranes (Braughler et al., 1987a). These observations would appear to contrast with the above quoted results; however, it is to be considered that, in this model, 7 days after operation, the iron injected into the brain was almost completely readsorbed.

Tirilazad mesylate has been studied in animal models for the prevention of neuronal damage due to head trauma (Hall et al., 1988b), subarachnoid hemorrhage (SAH) (Kanamaru et al., 1990), spinal cord injury (Anderson et al., 1988) and stroke (Hall et al., 1988a). In these systems, tirilazad mesylate, administered intravenously, appears to reduce the moiety rate and promote neurological recovery after acute insult. Doses ranging from 0.03 to 30 mg appear to have beneficial effects in animal model free radical-induced injury after head trauma (Hall et al., 1988b).

The purpose of the trial of Fleishaker et al. (1993a), was to evaluate in humans the tolerability, pharmacological effects and pharmacokinetics of tirilazad mesylate. Doses of 0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg were administered as intravenous infusions over 10 min or 30 min. The final concentration of tirilazad mesylate administered was 0.375 mg/mL, except in those subjects receiving 10 min infusions, who received the drug at a 1.5 mg/mL concentration. No significant effects of tirilazad mesylate on blood pressure, pulse or temperature were observed. No statistically, clinically significant effects of tirilazad mesylate on cardiac rhythm, as assessed by Holter recording, were observed. No effect of tirilazad mesylate on plasma cortisol or adrenocorticotrophic hormone was observed. As regards the total lymphocyte content, a statistically significant treatment-time interaction was observed: this was due to increases in lymphocyte count seen at 24 h and 48 h in the 2.0-mg/kg dose group. No significant differences among treatments in monocyte or eosinophil counts were observed. No significant treatment effects on general serum or urine chemistry or hematology assays were identified. In general, detectable tirilazad mesylate plasma concentrations were observed up to 2 h, 4 h, 8 h and 12 h for the 0.25-mg/kg, 0.5-mg/kg, 1.0-mg/kg and 2.0-mg/kg doses, respectively.

These results (Fleishaker et al., 1993a) show that tirilazad mesylate was well tolerated at the doses administered. No clinically significant effects of tirilazad mesylate on cardiovascular function or on clinical laboratory determinations were apparent. Thus, single doses of tirilazad mesylate appear to be devoid of glucocorticoid and mineralocorticoid activity in healthy male volunteers, and no safety concerns for single-dose tirilazad mesylate were identified from this study.

The pharmacokinetics of tirilazad mesylate are dose-independent for corrected values (C_{inf}) under single-dose conditions at doses up to 2.0 mg/kg. Tirilazad mesylate and related compounds appear to have high affinity for peripheral tissues (Cox et al., 1989). These data suggest that several tissues in humans may also have high affinity for tirilazad that was rapidly cleared from the plasma in humans. The systemic clearance of tirilazad mesylate in human approximates hepatic plasma flow and will likely be affected by those factors that affect liver blood flow. The terminal half-life observed for the two higher doses in this study was 3.7 h. In the rat, the value was of 50 h (Cox et al., 1989). A prolonged elimination phase may impact on tirilazad mesylate accumulation on multiple dosing (Fleishaker et al., 1993a). All kinetics parameters are summarized in table 1.

Multiple dose administration, however, has been used in animal models of head injury, SAH and cerebral ischemia (Anderson et al., 1988; Silvia et al., 1987; Kanamaru et al., 1990), and multiple-dose administration is anticipated also for therapeutic intervention in humans.

The purpose of the trial of Fleishaker et al. (1993b) was to evaluate, in humans, the tolerability, pharmacological effects and pharmacokinetics of tirilazad mesylate after multiple-dose administration. The dosage of 0.5 mg/kg/day, 1 mg/kg/day, 2 mg/kg/day, 4 mg/kg/day and 6 mg/kg/day were administered in equally divided doses every 6 h as intravenous infusions over 10 min or over 30 min. The final concentration of tirilazad mesylate administered was 0.375 mg/mL. A total of 21 doses were administered, and the subjects remained in the clinic through 48 h after the last dose. As reported after single-dose administration (Fleishaker et al., 1993a), the most commonly reported medical event was local pain at the injection site. The frequency of this event increased with the increase of the dose of drug infused. The lack of differences between vehicle control and tirilazad groups suggests that the local side effect are due to the drug vehicle, rather than to the drug itself (Fleishaker et al., 1993b). The approximately even distribution of systemic medical events between active and placebo groups indicated that no systemic medical events could be attributed to tirilazad mesylate treatment. No clinically significant changes in blood pressure, pulse or cardiac rhythm were observed.

TABLE 1
Pharmacokinetic parameters of U-74006F in humans and rats

Author	Dose in mg/kg	Administration Route	Plasma Concentration	Half-life in Hours	Side Effects	Steady State After Days	V_d Area in L/kg	Excretion
Fleishaker et al. (1993a)	0.25–0.50	intravenous infusions over 10 or	20 ng/mL[a] >99%	human 3.7	local pain at injected site due to		3.33	majority in feces less
	1.00–2.00	30 min at final concentration of 1.5 mg/mL or 0.375 mg/mL, respectively	bound to proteins in humans	rat 50[b]	vehicle and increased lymphocyte counts at 24–40 min in 2.0 mg/kg group			than 12% in urine[c]
Fleishaker et al. (1993b)	0.50–1.00	intravenous infusions every 6 h	20 ng/mL[a]	35	as above and increase in liver	5	from 17	as above
	2.00–4.00	over 10 or 30 min (multiple			enzyme activities (alanine		to 31	
	6.00–(day)	dosing)			transaminase) in 6 mg/kg group			
Laizure et al. (1993)	10	intravenous injections after 0–10–20–40 min and 1.5–2.0–3.0–4.0–6.0–8.0 h from injection		rat 2.4 brain 1.9 heart 1.6 liver 1.5			4.8	clearance 6.1 mL/min
Hulst et al. (1994)	1.5	single infusions over 10 min	10 ng/mL[a]	human 8.1	note[d]			clearance is 25% lower
	3.0			14.7				in the elderly

[a] Assay limit.
[b] Cox et al., 1989.
[c] Stryd et al., 1992.
[d] Male volunteers: mean age 33 years (from 23 to 42)—mean body weight. 67.4 kg; female volunteers: mean age 70 years (from 65 to 85)—mean body weight 68.2 kg. A gender-related effect for tirilazad pharmacokinetics was noted: clearance was higher in women than in men, probably because of 5-alpha-reductase activity (liver) 5-fold higher in female rats than in male rats (Wienkers et al., 1995).

Thus, tirilazad mesylate was clinically well tolerated in these volunteers. Several subjects experienced transient increases in liver enzymes. The proportion of subjects with these transient increases was greatest in the 6 mg/kg/day-dose group (50%). Therefore, the cause of these liver function abnormalities remains unknown, but these observations suggest the need for surveillance of alanine transaminase levels in future clinical trials of tirilazad (Fleishaker et al., 1993b). Similar to results obtained previously, tirilazad mesylate exhibited no glucocorticoid, mineralocorticoid nor gonadotropic effects.

Although the analysis of concentrations of plasma tirilazad mesylate show that steady state is achieved after 5 days of dosing, longer durations of administration should not result in substantially greater accumulation of tirilazad mesylate. The pharmacokinetic parameters of tirilazad mesylate were proportional to dose, under both single-dose and multiple-dose conditions. The previous single-dose pharmacokinetic study showed linear behavior over doses of 0.25 to 2.0 mg/kg, based on evaluation of dose-corrected concentration at the end of the infusion (C_{inf}), systemic clearance (Cl), Cl corrected for body weight and terminal elimination rate constant (L_z) among dose groups (Fleishaker et al., 1993a). In this study (Fleishaker et al., 1993b), only data up to 6 h after the first dose were available. Thus, calculated values of L_z, plasma concentration-time curve (area under the curve), clearance and volume distribution must be viewed as suboptimal. In this case, linearity after the first dose can only really be assessed using dose-corrected C_{inf}. Analysis of this parameter suggested linear behavior. Because steady state was achieved by the fifth day of dosing, a more rigorous pharmacokinetics analysis could be performed using data collected after the last dose (Fleishaker et al., 1993b). The linear pharmacokinetics of tirilazad mesylate in humans contrasts with the reduced clearance exhibited at high doses in dogs.

On multiple dosing at 2 mg/kg/day or above, a terminal half-life of approximately 35 h is observed; the terminal half-life observed after a single 2-mg/kg dose is approximately 3.7 h (Fleishaker et al., 1993a). The reason for this discrepancy is that plasma tirilazad mesylate concentrations in this terminal phase fall below detectable levels after a single dose. In other words, the terminal phase is there, but it is only after accumulation of tirilazad mesylate on multiple dosing that this terminal phase may be obtained (Fleishaker et al., 1993b). Clearance of tirilazad mesylate approached liver plasma flow (Fleishaker et al., 1993a).

Tirilazad mesylate is extensively distributed in body tissues: area estimated from a single dose (V_d) was 3.33 L/kg (Fleishaker et al., 1993a). The estimate obtained using multiple-dose data ranges from 17 to 31 L/kg. The majority of tirilazad mesylate is recovered in the feces as various metabolites, and less than 12% of the dose is recovered in the urine (Stryd et al., 1992).

However, because ischemic stroke occurs primarily in an elderly (age > 65 years) population, the safety of tirilazad mesylate should be shown in this setting. Physiological changes, such as decreased cardiac output, blunted homeostatic mechanisms and diminished hepatic and renal function, occur with increasing age (Dawling and Crome, 1989). Liver size and liver blood flow also decrease with age in humans (Woodhouse and Wynne, 1988). These physiological changes in the elderly result in altered pharmacokinetic properties of several drugs. It is likely that, based on its pharmacokinetic properties in young volunteers, tirilazad mesylate will exhibit altered pharmacokinetics in the elderly (Hulst et al., 1994).

Thus, the objectives of the study of Hulst et al. (1994) were to compare the pharmacokinetics of two doses of tirilazad mesylate (1.5 and 3.0 mg/kg, as single infusions administered over 10 min) in healthy young and elderly volunteers and to assess gender-related effects on the pharmacokinetics of this drug. A secondary objective of the study was to assess the tolerability of tirilazad mesylate administration to these volunteers. Twelve healthy young volunteers and 13 healthy elderly volunteers (six men and six women in each age group) were enrolled for this study. The age range of the young volunteers was from 23 to 42 years (mean age, 33 years), and their weights ranged from 52.7 to 89.4 kg (mean weight, 67.4 kg). The age range of the elderly volunteers was from 65 to 85 years (mean age, 70 years), and their weights ranged from 49.1 to 87.5 kg (mean weight, 68.2 kg). The results of this study support the dose proportionality of tirilazad pharmacokinetics through a dose of 3.0 mg/kg.

However, as previously observed, there is a dose dependency of the observed tirilazad biological half-life ($t_{1/2}$) after single-dose administration. This has been attributed to limited assay sensitivity, which precludes the detection of the prolonged elimination phase in the concentration-time profile for tirilazad mesylate, which can be seen after higher single dose or after multiple dosing (Stryd et al., 1992). The results of this study, taken with the results of the previous single-dose study, further support this hypothesis. After a single 2.0 mg/kg dose administered to healthy young men, the mean terminal $t_{1/2}$ obtained was 3.7 h with use of an assay with a limit of quantity of 20 ng/mL (Fleishaker et al., 1993a).

However, in the study of Hulst et al. (1994), an assay with a limitation of 10 ng/mL was used, and the mean $t_{1/2}$ values of tirilazad mesylate in young male subjects were 8.1 and 14.7 h for the 1.5 mg/kg and 3.0 mg/kg doses, respectively. The longer $t_{1/2}$ values measured were attributable to the improvement in assay sensitivity, which allowed a better characterization of the terminal phase of the log concentration-time profile. The pharmacokinetics of tirilazad mesylate appear to be linear through single doses of 3.0 mg/kg, and the apparent dose dependencies of the terminal elimination rate constant (V_{ss}) and $t_{1/2}$ are an artifact of limited assay sensitivity (Hulst et al., 1994).

In any case, the results were consistent with decreased clearance of tirilazad in the elderly. Clearance was approximately 25% lower in the elderly volunteers than in young volunteers. Tirilazad mesylate concentrations at the end of the infusion were also higher in elderly volunteers. No significant differences were observed in $t_{1/2}$, but differences in $t_{1/2}$ may be obscured by the lack of sufficient assay sensitivity to fully characterize the terminal phase.

A study by Laizure et al. (1993) was conducted in Sprague-Dawley rats to determine the basic pharmacokinetics and distribution of tirilazad into the brain, heart, and liver. Rats were killed in groups of five at 0, 10, 20 and 40 min, and at 1.5, 2, 3, 4, 6 and 8 h after intravenous administration of 10 mg/kg of tirilazad mesylate. Tirilazad was assayed in plasma, heart, liver and brain tissue by high performance liquid chromatography. Tirilazad was rapidly eliminated from the plasma with a half-life of 2.4 h and clearance of 6.1 mL/min. The volume of distribution at steady state was large: 4.8 L/kg. The concentrations of tirilazad were highest in the liver and heart and lowest in the plasma and brain. Elimination from tissues paralleled elimination from plasma with half-lives of 1.9, 1.6 and 1.5 h in the brain, heart and liver, respectively. Tirilazad appears to be a highly extracted, hepatically eliminated drug, suggesting its clearance is dependent on liver blood flow, and alterations in plasma protein binding are unlikely to affect its clearance but may affect the fraction unbound (Laizure et al., 1993).

The decreased clearance in the elderly was primarily attributable to lower clearance in elderly women compared with young women, because clearance did not differ significantly between young and elderly men. Tirilazad has been characterized as a high extraction ratio compound, because clearance is dependent primarily on hepatic blood flow (Fleishaker et al., 1993a; Cox et al., 1989). Both liver weight and liver blood flow decrease with age in humans (Woodhouse and Wynne, 1988) and the apparent decrease in tirilazad clearance in elderly women may be a consequence of decreased liver blood flow in older women.

In addition to the effect of age on tirilazad pharmacokinetics, a gender-related effect was also observed (Hulst et al., 1994). Clearance of tirilazad was higher in women than in men, whereas plasma concentration-time curve and C_{inf} were lower in women. The gender-related effect was much more dramatic in the younger volunteers than in older volunteers. The mechanism for this gender-related effect is not known but, based on the pharmacokinetic properties of tirilazad, it may involve gender-related differences in hepatic blood flow. However, gender-related effects on blood flow have not been reported (Yonkers et al., 1992).

In fact, to elucidate these open questions, very recently, the biotransformation of tirilazad has been investigated in liver microsomal preparations from adult male and female Sprague-Dawley rats (Wienkers et al., 1995). Tirilazad metabolism in male rat liver microsomes resulted in the formation of two primary metabolites. Structural characterization by mass spectrometry demonstrated that one metabolite was formed by reduction of the delta-4-double bond in the steroid A-ring, whereas the other arose from the oxidative desaturation of one pyrrolidine ring.

Comparison of calculated intrinsic formation clearances (maximal velocity (V_{max})/kinetics constant (K_m)) for both metabolites indicates that the female rat possessed a greater in vitro metabolic capacity for tirilazad biotransformation than did the male rat. Therefore, the clearance of tirilazad mesylate in the rats is mediated primarily by rat liver 5-alpha-reductase, and the capacity in the female rat is five-fold the capacity in the male. These observations correlate with documented differences in 5-alpha-reductase activity and predict a gender difference in tirilazad hepatic clearance in vivo (Wienkers et al., 1995).

Although metabolic pathways for tirilazad mesylate have not yet been completely elucidated in humans, a possible pathway may be metabolism at the steroid portion of the molecule. Lew et al. (1993) reported that a 46% higher ideal body weight normalized clearance of methylprednisolone in women compared with men. Thus, metabolic differences in metabolism at the steroid portion of tirilazad mesylate molecule may contribute to the gender-related effect on pharmacokinetics observed by Hulst et al. (1994). Further work is necessary to test this hypothesis.

Clearly, evaluation of the protein binding of tirilazad mesylate would provide an estimate of the effect of age and sex on unbound tirilazad mesylate concentrations, which would be more therapeutically relevant. Based on an estimate obtained in delipidized serum, tirilazad mesylate appears to be $>$ 99% bound to plasma proteins in humans. Because of the lipophil properties of tirilazad and its adsorbability to surfaces, routine determinations of tirilazad protein binding in native serum from different patient populations are not currently possible. Therefore, effects of changes in tirilazad mesylate protein binding on clearance and on V_{ss} that were attributable to age and gender could not be assessed as part of this trial (Hulst et al., 1994).

C. Mechanism of Action of 21-Aminosteroids

The 21-aminosteroid mechanism of action has been studied using both in vitro and in vivo experimental models. In cell-free systems, the 21-aminosteroids are potent inhibitors of lipid peroxidation, having an IC_{50} of 2 to 60 μM in rat brain homogenate (Braughler et al., 1987a). Lazaroids seem to inhibit lipid peroxidation by a mechanism similar to vitamin E. In addition, as a group, these drugs containing an NC=CN fragment, such as U-74500A, also possess the ability to interact with ferrous ions (Braughler and Pregenzer, 1989).

As previously reported, tirilazad mesylate is a nonglucocorticoid 21-aminosteroid that is a potent inhibitor of oxygen radical-induced, iron-catalyzed lipid peroxidation. It is a very lipophil compound that distributes preferentially to the lipid bilayer of cell membranes. It appears that the compound exerts its antilipid peroxidation action through cooperative mechanisms: (*a*) a radical scavenging action and (*b*) a physicochemical interaction with the cell membrane that serves to decrease membrane fluidity.

As regards the antioxidant effects in membrane systems, in vitro, the 21-aminosteroids are potent inhibitors of lipid peroxidation of rat brain homogenate, crude rat brain synaptosomes as the lipid source (Braughler et al., 1988a) and also rat brain synaptic plasma membranes (Braughler et al., 1987a). However, when such compounds are added in organic solution to physiological buffer, they microprecipitate. Emulsion delivery is probably a delivery technique for compounds of this class. A preliminary report suggests that U-74500A differs from U-74006F in that the former appears to interact with iron in some manner (Braughler et al., 1987a). Indeed, the concentration that inhibits 50% (IC_{50}) of U-74500A to inhibit iron-dependent lipid peroxidation in rat brain homogenates is lower in the presence of 10 μM Fe^{2+} than in presence of 200 μM Fe^{2+} (Braughler et al., 1988a). Furthermore, U-74500A has been demonstrated to display spectral changes in the ultraviolet range that are dependent upon the concentration of Fe^{2+}. No iron-dependent spectral changes have been observed for U-74006F in aqueous solution. This does not rule out the possibility that U-74006F might bind iron in some manner within the membrane environment (Braughler and Pregenzer, 1989). U-74500A and tirilazad act to slow the oxidation of vitamin E during linoleic acid peroxidation and potentiate vitamin E's antioxidant efficacy (Braughler and Pregenzer, 1989). U-74500A is actually a better antioxidant than is tirilazad, especially in iron-driven peroxidation systems, possessing a lower oxidation potential than tirilazad, and it has the ability to interact with ferrous iron and to lessen its oxidation, in contrast with tirilazad, which does not (Ryan and Petry, 1993).

The effects of two 21-aminosteroids (U-74500A and U-74006F) on the oxidation and reduction of iron were investigated (Ryan and Petry, 1993). U-74500A completely prevented adenosine diphosphate (ADP):Fe(II) autoxidation, whereas U-74006F had only a slight inhibitory effect. In particular, the inhibition of Fe(II) oxidation by U-74500A was concentration-dependent, with 100% inhibition occurring at concentrations equal to or greater than 25 μM in systems containing 50 μM Fe(II). When the Fe(II)-specific chelator Ferrozine (Sigma Chemical Co. St. Louis, MO), was added to incubations containing U-74500A and ADP:Fe(II), formation of the

Ferrozine:Fe(II) chromophore was slow, suggesting that U-74500A chelates Fe(II) with substantial affinity. Twenty minutes were required for complete formation of the Ferrozine:Fe(II) chromophore in the presence of U-74500A, whereas complexation in its absence was instantaneous. This phenomenon was not observed with U-74006F or ascorbate. In a system containing 25 μM ADP:Fe(II), both U-74500A (25 μM) and U-74006F (25 μM) reduce iron at rates approximately 2 and 0.1 μM/min, respectively (Ryan and Petry, 1993). U-74500A fluorescence was quenched in a concentration-dependent manner upon the addition of Fe(III), further demonstrating interactions between this compound and iron.

The substructures of U-74500A consist of a steroid (U-76911) and a complex amine (U-82902E). When these compounds were assayed individually, it was found that U-82902E exhibited activities similar to those of U-74500A, whereas the free steroid had no effect (Ryan and Petry, 1993). Studies using cyclic voltametry revealed that U-74500A had relatively low oxidation potential (E = 228 mV), whereas U-74006F was much less susceptible to oxidation (E = 810 mV) (Ryan and Petry, 1993). Taken together, these data suggest that subtle effects on iron redox chemistry, which would in turn inhibit or eliminate the initiation of undesired oxidative reactions, may contribute to the potent antioxidant activities of U-74500A and U-74006F.

Tirilazad also can interact with hydroxyl radicals generated during in vitro Fenton reaction (5) (Althaus et al., 1991). In vivo studies, using the salicylate (SAL) trapping method for measurement of hydroxyl radical, have demonstrated that tirilazad administration decreases brain hydroxyl radical levels in a model of concussive head injury in mice (Hall et al., 1992, 1993a) and global cerebral ischemia/reperfusion injury in gerbils (Andrus et al., 1991). Tirilazad has also been reported to lessen the increase in hydroxyl radical concentration in rat brain produced by infusion of glutamate (Boisvert and Schreiber, 1992).

As an antioxidant in whole cells, tirilazad is effective in an in vitro model for predicting a compound's ability to prevent cell damage during periods of energy failure. Iodoacetic acid was administered (50 mM) to the cultured human astroglial cells for 4 h. This agent shuts down glycolysis and leads to subsequent irreversible breakdown of cellular membranes and, ultimately, to cell death. During the first hours, iodoacetic acid rapidly depleted cellular level of adenosine triphosphate (ATP) and decreased active uptake of tritiated aminoisobutyric acid. Subsequent irreversible cellular injuries were characterized by the release of large amounts of free arachidonic acid into extracellular medium, massive calcium influx and leakage of cytoplasmic contents. The appearance of 15-hydroxy eicosatetraenoic acid in membrane phospholipids and loss of cellular thiol groups indicated the cell constituents were being assaulted by oxidative species. These manifestations of iodoacetic acid-induced cell damage were inhibited by tirilazad, which also decreased the release of arachidonic acid (Hall et al., 1994).

The 21-aminosteroids tirilazad and U-74500A have potent stabilizing effects on cell membranes. The compounds have high affinity for the lipid bilayer because of their lipophilia and are incorporated into the lipid bilayer, where they occupy strictly defined positions and orientations (Hinzmann et al., 1992). Tirilazad is a very lipophilic compound that localizes in and protects cell membranes from peroxidative damage. Not surprisingly, this compound has been shown also to exert physicochemical effects on endothelial cell membranes. It has high affinity for vascular endothelium and protects the blood-brain barrier (BBB) against either a trauma-induced or SAH-induced permeability increase. Tirilazad appears to poorly penetrate the BBB in rats after intracarotid injection, because the penetration of tirilazad into brain parenchyma is enhanced after injury, apparently by virtue of the trauma-induced disruption of the BBB (Hall et al., 1992). The endothelial localization and protection probably is not confined to the CNS but also occurs at the hepatic level. Tirilazad does not exert any glucocorticoid receptor-mediated actions and, actually, the only demonstrated cerebroprotective mechanism of the 21-aminosteroids concerns their ability to block oxygen radical-induced lipid-peroxidation.

U-74500A is actually a more potent inhibitor of iron-catalyzed lipid peroxidation than is U-74006F, but it has not been chosen for development due to pharmaceutical instability and rapid elimination in vivo (Hall, 1992a). In addition, brief mention is made of more recently discovered antioxidants, the 2-metylaminochromans, in which the steroid moiety of U-74006F has been replaced by the more potent antioxidant chromanol structure of vitamin E (i.e., alpha-TC): U-78517F (Hall, 1992a).

In regards to effects on cerebral metabolism, tirilazad (1 mg/kg i.v. at 30 min postinjury *plus* 0.5 mg/kg 2 h later, in cats severely head-injured) improved the metabolic profile within the injured hemisphere measured at 4 h (Dimlich et al., 1990), particularly reducing post-traumatic lactic acid accumulation in both the cerebral cortex and the subcortical white matter.

To conclude, Hall and Braughler (1993) reviewed the current state of knowledge regarding the occurrence and possible role of oxygen radical generation and lipid peroxidation in experimental models of acute CNS injury. Although much work remains, four criteria that are logically required to establish the pathophysiological importance of oxygen radical reactions have been met, at least in part. First, oxygen radical generation and lipid peroxidation appear to be early biochemical events subsequent to CNS trauma. Second, a growing body of direct and circumstantial evidence suggests that oxygen radical formation and lipid peroxidation are linked to pathophysiological processes such as hypoperfusion, edema, axonal conduction failure, failure of energy me-

tabolism and anterograde (Wallerian) degeneration. Third, there is a striking similarity between the pathology of blunt mechanical injury to CNS tissue and that produced by chemical induction of peroxidative injury. Fourth, compounds that inhibit lipid peroxidation or scavenge oxygen radicals can block posttraumatic pathophysiology and promote functional recovery and survival in experimental studies (Hall and Braughler, 1993).

Nevertheless, the significance of oxygen radicals and lipid peroxidation ultimately depends on whether it can be demonstrated that early application of effective antifree radical or antiperoxidative agents can promote survival and neurological recovery after CNS injury and stroke in humans. The results of the NASCIS II clinical trial, which have shown that an antioxidant dosing regimen with methylprednisolone begun within 8 h after spinal cord injury can significantly enhance chronic neurological recovery, strongly support the significance of lipid peroxidation as a posttraumatic degenerative mechanism. However, phase III trials with the more selective and effective antioxidant U-74006F (tirilazad mesylate) will give a more clear-cut answer as to the therapeutic importance of inhibition of posttraumatic free radical reactions in the injured CNS (Hall and Braughler, 1993).

D. Toxicity

Very little is known about other biological effects of lazaroids, except that they improve endothelial cell viability at 4°C, with U-74500 being the most effective (Killinger et al., 1992). Furthermore, they inhibit growth of cultured Balb/c 3T3 clone A31 fibroblast (Singh and Bonin, 1991). U-75412E caused inhibition of cellular growth of human epithelial cell line (Wish), that was both concentration-dependent and time-dependent (Mattana et al., 1994). In particular, drug-treated cells showed a remarkable number of vacuoles and mitochondria, which were smaller, rounded and showed a widening of the intercrystal spaces in treated cells.

The flow cytometry analysis confirmed the antiproliferative effect, a large number of cells were blocked in the G2/M phase, without apparently degenerative phenomena (Mattana et al., 1994). Different phases of nuclear fragmentation (apoptosis) were also evident when the cells were incubated with 6 μM U-75412E for 48 h. Reduced deoxyribonucleic acid (DNA) stainability observed in apoptotic cells was a consequence of a partial loss of DNA due to activation of endogenous endonuclease (Darzynkiewicz et al., 1992; Hotz et al., 1992). Cell growth inhibition by lazaroids was probably due to a cytotoxic action of the compounds in these experimental conditions. The release of the intracellular enzyme lactate dehydrogenase, used as an indicator of cytotoxicity, confirmed these data (Mattana et al., 1994).

Furthermore, scanning electron microscopy experiments showed that treated cells exhibited damage to the cell surface that could be ascribed to the high lipophilia of the molecule. In addition, U-75412E caused ultrastructural damage, as shown by transmission electron microscopy, indicating that tubulin could be quite a specific target for the lazaroid toxicity. In conclusion, these data suggest that lazaroid U-75412E has a cytotoxic effect at concentrations above 1 μM in Wish cells (Mattana et al., 1994).

II. Central Nervous System Trauma

A. Background

Lipid hydrolysis with subsequent eicosanoid production is an early pathochemical event in the injured spinal cord (Anderson et al., 1985; Demediuk et al., 1985; Hsu et al., 1985; Jonsson and Daniell, 1976). However, lipid hydrolysis with the release of arachidonate may be closely tied to peroxidation-induced changes in membrane calcium permeability. In addition, a synergistic interaction between calcium and lipid peroxidation during cell damage has been demonstrated (Braughler et al., 1985; Malis and Bonventre, 1986).

Many factors are involved in the pathogenesis of traumatic brain edema. The initial mechanical disruption of capillary endothelial cells (Long, 1982) allows excess movement of fluid into the brain, but vascular thrombosis quickly prevents further edema formation from this source (Tornheim, 1985). A more important cause of traumatic brain edema appears to be the release or activation of chemical mediators, such as bradykinin, serotonin, histamine, arachidonic acid, leukotrienes, excitatory amino acids and free radicals, and failure of the BBB (Black and Hoff, 1985; Chan et al., 1984; Wahl et al., 1988). Although it is accepted that chemical mediators play a role in brain edema development, the importance of each mediator has yet to be determined. In CNS trauma, tissue hemorrhage initiates free radical formation, and iron compounds catalyze the generation of the highly reactive hydroxyl radical and stimulate membrane lipid peroxidation (Halliwell and Gutteridge, 1985).

Lipid peroxides and oxygen reactive species are thought to be involved in major physiological or pathological events, such as inflammation, radiation damage, mutagenesis, cellular aging and reperfusion damage (Halliwell, 1987). Evidence of the potential role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be used in the therapy or prevention of these diseases.

The 21-aminosteroids, specifically designed to localize within cell membranes and to inhibit lipid peroxidation reactions (Braughler et al., 1987a,b, 1988a,b; Braughler and Pregenzer, 1989), have shown activity in in vivo models of experimental CNS trauma (Anderson et al., 1988; Hall et al., 1988b; Braughler et al., 1989). In contrast to metylprednisolone, U-74006F has no steroidal side effects or peripheral vasodilator activity, is more

potent (Hall et al., 1988a) and has no deleterious effect on blood pressure. These characteristics make this compound a candidate for possible treatment of CNS injury (Sanada et al., 1993).

Regarding the effects on BBB permeability, free radicals are known to increase BBB permeability. It is possible that the effect of tirilazad to protect the BBB is due to either a reduced formation of hydroxyl radicals or perhaps a protection of the microvascular endothelium from hydroxyl radical-induced lipid peroxidation.

Preventing and reducing secondary brain injury have been foci of recent research on CNS trauma (Sanada et al., 1993). Although the precise mechanism of delayed injury after mechanical trauma is unclear, several metabolic derangements have been implicated. These include influx of calcium ions (Hubschmann and Nathanson, 1985; Siesjo and Wieloch, 1985), tissue lactic acidosis, free radical generation and tissue peroxidation (Kontos and Wei, 1986), production of prostaglandins and leukotrienes (Kiwak et al., 1985) and membrane depolarization by release of excitatory amino acid neurotransmitters (Choi and Tecoma, 1988). A variety of antioxidants, or free radical scavengers, have been proposed to treat CNS injury (Faden, 1985). Among the agents tested are vitamins C and E, selenium, coenzyme Q_{10}, megadose corticosteroids and high-dose opiate antagonists. None of these agents, however, has led to major improvement in neurological function after CNS trauma (Sanada et al., 1993).

Mechanical trauma of the spinal cord causes destruction of gray and white matter with consequent loss of function (Anderson et al., 1988). Nerve cells and axons can be damaged directly by the physical deformation of the spinal cord and/or by a cascade of pathochemical events that are initiated by the original mechanical trauma. It is this biochemical injury that would be susceptible to pharmacological treatment if the mechanisms were understood (Anderson et al., 1988). Lipid peroxidation (Anderson et al., 1985; Demediuk et al., 1985; Hall and Braughler, 1982; Malis and Bonventre, 1986), phospholipid hydrolysis with production of eicosanoids (Anderson et al., 1985; Demediuk et al., 1985; Hsu et al., 1985) and depletion of energy stores with increased lactic acid formation (Anderson et al., 1976, 1985; Braughler and Hall, 1983) are the earliest biochemical events detected thus far in injured spinal cord tissue. Disruption of cell membranes by peroxidative and hydrolytic process may be intimately involved in the initiation and/or propagation of the posttraumatic autodestruction of spinal cord tissue.

Thus, agents that protect cell membranes by quenching these peroxidative reactions and/or by limiting lipolysis should be effective in improving neurological recovery (Anderson et al., 1988). However, it appears that a major portion of posttraumatic neuronal necrosis after spinal cord (or brain) injury does not result from differences in primary injury, but rather occurs as a secondary pathophysiological process. The injury is due to a series of molecular events that lead to gradual derangements, e.g., vascular and neuronal degeneration, thus destroying the anatomic substrate necessary for the neurological recovery. Thus, the functional recovery can be facilitated by appropriate therapies targeted to interrupt the molecular processes involved in the secondary degeneration phenomena.

High doses of methylprednisolone sodium succinate (MP) promote functional recovery in animals with spinal cord injury (Braughler et al., 1987b). A primary action of MP in the injured or ischemic CNS is believed to be its ability to inhibit lipid peroxidation and to preserve the structural and functional integrity of biological membranes (Anderson and Means, 1985; Anderson et al., 1985; Braughler, 1985). Treatment of human CNS injuries with MP has been complicated by its biphasic dose-response characteristics (Braughler and Hall, 1982, 1983; Hall, 1985; Hall et al., 1984) and its glucocorticoid receptor-mediated activity (Braughler and Hall, 1985; Hall and Braughler, 1982). The realization that the membrane-protective capabilities of MP were separate from its hormonal activity stimulated an intensive drug-development effort to identify and prepare unique compounds specifically targeted for the treatment of human CNS trauma and ischemia. U-74006F has proved to be a substantially more potent and effective treatment than MP in several different models of acute CNS trauma and ischemia (Hall, 1988; Hall et al., 1988a,b). The slow CNS tissue uptake of vitamin E requires chronic dosing, making it an impractical agent for treatment of acute neuronal injury.

B. Selected Experimental Data

There are many data about the effects of lazaroids in experimental spinal cord and head injury, evaluating a variety of functional or biochemical parameters. Spinal cord white matter blood flow was measured by hydrogen clearance in the injured segment before and at various times up to 4 h after injury. After 4 h postinjury, spinal cord white matter blood flow was decreased by 63.5%, whereas the spinal cord white matter blood flow measured 4 h postinjury in cats treated with a single 10-mg/kg dose of U-74006F was of about normal value. The mechanism of action of U-74006F in antagonizing posttraumatic development is believed to involve the ability of the compound to inhibit iron-dependent lipid peroxidation in CNS (Hall, 1988).

Initial studies of the efficacy of U-74006F in experimental acute head injury have been carried out to determine the ability of the compound to improve early neurological recovery and survival of head-injured mice (Hall et al., 1988b). Unanesthetized male CF-1 mice were subjected to a 900-gcm head injury produced by a 50-g weight that was dropped 18 cm (Hall, 1985). Administration of a single dose of i.v. U-74006F significantly improved the 1 h postinjury neurological status

(grip test score) over a broad range of dosages (0.003 to 30 mg/kg). A 1 mg/kg i.v. dose given within 5 min and again at 1.5 h after a severe injury, in addition to improving early recovery, increased the 1-week survival to 78.6% compared with 27.3% in vehicle-treated mice. The compound was also effective in enhancing early recovery after a more moderate injury.

The study of Anderson et al. (1988) demonstrates the remarkable effectiveness of a nonglucocorticoid 21-aminosteroid, U-74006F, administered through the venous cannula, in enhancing neurological recovery in female adult mongrel cats traumatized by compression of the spinal cord with a 180-gm weight for 5 min. Beginning at 30 min after injury, cats were given an intravenous bolus of either vehicle or U-74006F. Two hours later, the treated cats received a second intravenous bolus of one-half the original loading dose; at 6 h postinjury, the cats received a third intravenous bolus, again one-half of the original loading dose. Immediately after this last injection, a continuous intravenous infusion was started and continued for 42 h. Thus, the animals were treated for the first 48 h postinjury. The cats were divided into nine groups: one vehicle-treated group and eight U-74006F-treated groups. The dose levels tested (the initial loading dose and total dose) were: 0.01 mg/kg (0.048 mg/kg/48 h); 0.03 mg/kg (0.16 mg/kg/48 h); 0.1 mg/kg (0.48 mg/kg/48 h); 1.0 mg/kg (4.8 mg/kg/48 h); 3.0 mg/kg (16.0 mg/kg/48 h); 10 mg/kg (48 mg/kg/48 h) and 30 mg/kg (160 mg/kg/48 h). All cats were allowed to recover for 4 weeks, and their functional recovery was evaluated on a weekly basis. The neurological evaluation procedure used is based on observing and rating the mobility of a freely moving animal in various controlled situations. Over the initial 2 weeks following injury, there was no statistically detectable recovery in any of the U-74006F-treated groups as compared with vehicle-treated controls. However, at 2 weeks, the mean recovery scores for all drug-treated groups, with the exception of the lowest dose, tended to be higher than the vehicle-treated groups, with the exception of the lowest dose. By 3 weeks postinjury, all treatment groups receiving total U-74006F doses of 1.6 m/kg/48 h and higher (with exception of the group receiving 16.0 mg/kg/48 h) showed statistically better recovery than the vehicle-treated cats. This pattern of recovery was sustained through the fourth and final week of evaluation.

The molecular mechanism(s) by which U-74006F promoted neurological recovery in this model of spinal cord injury is not known (Anderson et al., 1988). U-74006F completely lacks any glucocorticoid, mineralocorticoid or other hormonal activity (Braughler et al., 1988b; Hayes and Murad, 1980). Hence, it is unlikely that any CNS protective functions of U-74006F are mediated through glucocorticoid receptors. Physiologically, U-74006F has been shown to prevent the development of white matter ischemia following a severe contusion of the spinal cord (Hall, 1988). Moreover, U-74006F has the ability to partially restore posttraumatic spinal cord blood flow, even after it has declined significantly (Hall, 1988).

Some data are consistent with the dose-response findings for U-74006F in less complex models of CNS trauma (Hall et al., 1984; McCall et al., 1987). The broad range of effective doses for U-74006F, its remarkable potency, its lack of glucocorticoid receptor-mediated activity and the lack of any adverse side effects should make the clinical utility for this 21-aminosteroid significantly greater than that of MP for the treatment of human CNS trauma. The beneficial effect of antioxidant doses of methylprednisolone, administered within 8 h after spinal cord injury, can improve 3-month, 6-month and 12-month neurological recovery in humans (Bracken et al., 1990, 1992). This observation supports the view that posttraumatic lipid peroxidation is a critical degenerative mechanism that can be effectively interrupted with an antioxidant agent.

The study of Dimlich et al. (1990) was designed to evaluate further the effect of U-74006F on the acute pathophysiology of experimental head injury, involving severe unilateral cerebral contusion in cats. The parameters tested included magnitude and territory of vasogenic edema, brain swelling and cerebral metabolic function. Conditioned mongrel female cats were anesthetized with ketamine hydrochloride and, at 30 min after head or sham injury, each cat was intravenously injected with either 1 mg/kg of U-74006F or a comparable volume of its vehicle (0.02 M citric acid; 0.003 M sodium citrate; 0.08 M sodium chloride). A second treatment (0.5 mg/kg) was administered 2.5 h after injury. Four hours after injury, a styrofoam cup was fixed to the calvaria, and liquid nitrogen was poured over the skull for 20 min for in situ fixation of brain tissue. The frozen heads were coronally sliced at 5-mm intervals in a −20°C cold room. Brain samples (5 to 10 mg) were weighed and extracted (Wagner et al., 1985). Lactate, ATP and phosphocreatine were assayed in perchloric acid extracts using enzymatic fluorometric techniques (Lowry and Passonneau, 1972). Glucose and glycogen were determined in non-perchlorate-treated samples of homogenate, using the fluorometric procedure of Passonneau and Lauderdale (1974). Metabolites and edema (specific gravity) were measured bilaterally in the cerebral cortex and white matter. The magnitude of edema and metabolites in tissue with vasogenic edema was similar in vehicle-treated and drug-treated cats. By contrast, the cortex and nonedematous white matter neighboring contusion in drug-treated cats had lactate, glucose and glycogen levels that suggested an improved metabolic state over vehicle treatment. Most metabolites were not affected by trauma or treatment in the uncontused hemisphere. These results suggest that postinjury treatment with the nonglucocorticoid steroid U-74006F may benefit the metabolism of nonedematous tissue adjacent to contusion (Dimlich et al., 1990).

Regarding the effect on cerebral metabolism, tirilazad (1 mg/kg i.v. at 30 min postinjury, plus 0.5 mg/kg 2 h later, in cats severely head-injured) improved metabolic profile within the injured hemisphere measured at 4 h (Dimlich et al., 1990). In particular, the drug reduced posttraumatic lactic acid accumulation in both the cerebral cortex and the subcortical white matter.

The study of McIntosh et al. (1992), evaluated the effect of the nonglucocorticoid 21-aminosteroid U-74006F, an inhibitor of iron-dependent lipid peroxidation, on the development of regional cerebral edema after lateral fluid-percussion brain injury. Male Sprague-Dawley rats were anesthetized and subjected to fluid-percussion brain injury of moderate severity centered over the left parietal cortex (2.5 to 2.6 atms). Fifteen minutes after brain injury, animals randomly received an i.v. bolus of either U-74006F (3 mg/kg) followed by a second bolus (3 mg/kg) at 3 h or buffered sodium citrate vehicle. An additional group of 12 surgically prepared but uninjured animals served as preinjury controls. At 48 h after injury, animals were sacrificed, and brain tissue was assayed for water content and regional cation concentrations. With the use of specific gravimetric techniques, no significant differences were observed in posttraumatic cerebral edema between drug-treated and control-treated animals. However, using wet weight/dry weight methodology, McIntosh et al. (1992) found that administration of U-74006F significantly reduced water content in the right *hippocampus* (controlateral to the site of injury) compared with saline-treated animals. U-74006F also significantly prevented the postinjury increase in sodium concentrations in the ipsilateral *hippocampus* and *thalamus*. Regional concentrations of potassium were unaltered after drug treatment. Administration of U-74006F significantly reduced postinjury mortality, from 28% in control animals to zero in treated animals. These results suggest that lipid peroxidation may be involved in the pathophysiological sequelae of brain injury and that 21-aminosteroids may be beneficial in the treatment of brain injury (McIntosh et al., 1992).

The purpose of the study of Sanada et al. (1993) was to further evaluate the effect of U-74006F on neurological outcome and cerebral edema after head injury in rats. The rats were anesthetized with chloral hydrate (0.35 g/kg, i.p.). Through a 4.0-mm diameter craniectomy in the right temporal region just above the zygoma, a flanged polyethylene tube filled with isotonic saline was placed over the dura, securely fixed to the skull and connected to the fluid percussion device (Sullivan et al., 1976). The rats were subjected to an impact pressure of 4.5 atm for 15 msec and immediately transferred into a chamber supplied continuously with 7% oxygen. The PaO_2 was maintained at a hypoxic level for 45 min and then normoxia was restored. The animals were treated with U-74006F at 1, 3, 10 or 30 mg/kg intravenously at 3 min and at 3 h after impact injury. Neurological function was evaluated 24 h after injury, and three categories were scored: motor function, rotaroad walking and activity. The measurement of water content was determined by microgravimetry in the coronal slices obtained from the impact site, from the frontal, temporal and parietal cortex, and from caudate *putamen* and *thalamus* from both ipsilateral and controlateral hemispheres. In this study, only the 10-mg/kg dose (20 mg/kg total) of U-74006F significantly improved motor function 24 h after fluid percussion-hypoxic brain injury, but no improvement was evident when the dose was increased to 30 mg/kg (60 mg/kg total), which may indicate a relatively narrow effective dose range for rats.

Whether U-74006F has any adverse effects at doses higher than 30 mg/kg in rats has yet to be demonstrated. Steroids show biphasic actions on cell membranes, stabilizing them at relatively low concentrations and lysing them at higher concentrations (Lewis et al., 1970).

U-74006F had a differential effect on the three categories of the neurological evaluation. A statistically significant improvement was detected in motor score, but not in rotaroad walking nor activity, and the brain water content was not reduced by U-74006F at any dose (Sanada et al., 1993). This compound has reduced arachidonic acid-induced vasogenic edema and ischemic edema in rats subjected to middle cerebral artery (MCA) occlusions (Hall et al., 1988a; Wahl et al., 1988). Despite its ability to scavenge or inhibit the formation of free radicals, U-74006F did not reduce brain water content in this study.

Lipid peroxidation, however, is only one of the events in the complex process that results in traumatic cerebral edema. Oxygen free radicals have been implicated as a causal factor in neuronal cell loss following both cerebral ischemia and head injury. In this research, Althaus et al. (1993) studied simultaneously the effect of lazaroid U-74006F both in incomplete ischemia and in head injury. The conversion of SAL to dihydroxybenzoic acid (DHBA) in vivo was used to study the formation of hydroxyl radical (OH·) following CNS injury. Bilateral carotid occlusion (BCO) in gerbils and concussive head trauma in mice were selected as models of brain injury. The lipid peroxidation inhibitor, tirilazad mesylate (U-74006F), was tested for its ability to attenuate (OH·) formation in these models. In addition, U-74006F was studied as a scavenger of (OH·) in an in vitro assay based on the Fenton reaction. For in vivo experimentation, (OH·) formation was expressed as the ratio of DHBA to SAL (DHBA/SAL) measured in brain. In the BCO model, (OH·) formation increased in whole brain with 10 min of occlusion followed by 1 min of reperfusion. DHBA/SAL was also found to increase in the mouse head injury model at 1 h postinjury. In both models, U-74006F (1 or 10 mg/kg) blocked the increase in DHBA/SAL following injury (Althaus et al., 1993). In vitro, reaction of U-74006F with (OH·) gave a product with a molecular weight that was 16-fold greater than U-74006F, indica-

tive of (OH·) scavenging. The authors speculate that U-74006F may function by blocking oxyradical-dependent cell damage, thereby maintaining free iron (which catalyzes hydroxyl radical formation) concentrations at normal levels (Althaus et al., 1993). It is believed that U-74006F acts at the cell membrane level during reperfusion by inhibiting lipid peroxidation and significantly reduces the incidence of postischemic spinal cord injury following temporary aortic occlusion (Fowl et al., 1990), as well as locomotor function in cats (Anderson et al., 1991).

The aim of a recent study (Schneider et al., 1994) was to determine whether brain edema induced by a cryogenic injury can be influenced by the 21-aminosteroid U-74389F. A cortical freezing lesion was applied to the right parietal region of Sprague-Dawley rats under ketamine-xylazine anesthesia. Systemic blood pressure was monitored in the peritraumatic period. Four different doses (A to D) of U-74389F were studied for their effect on posttraumatic brain swelling and edema. Respective control groups received only the solvent, citric acid buffer. The doses were as follows: (A) 3 mg/kg b.w., i.p. (total dose) 30 min before, 1 and 12 h posttrauma; (B) 9 mg/kg b.w., i.v. 30 min before, 1 and 12 h posttrauma; (C) 25 mg/kg b.w., i.v. 30 min before, 1, 6 and 12 h posttrauma; and (D) 50 mg/kg b.w., i.v. 15 min before, 15 and 30 min as well as 1, 2, 6 and 12 h posttrauma. Twenty-four h after trauma, brains were removed, and hemispheric swelling and water content were determined from the difference between wet weight and dry weight. Application of the 21-aminosteroid U-74389F (Schneider et al., 1994) moderately reduced posttraumatic brain swelling in all treatment groups: (A) 5%, (B) 9%, (C) 12% and (D) 14%. In parallel with this, the increase in water content of traumatized hemisphere was marginally lowered by U-74389F in all groups: in (C) e.g., from 1.9 ± 0.1% to 1.7 ± 0.1%, $P = 0.07$. These two findings taken together indicate that the 21-aminosteroid U-74389F moderately reduces posttraumatic swelling and edema (Schneider et al., 1994).

The neurochemical sequelae of traumatic brain injury and their therapeutic implications have been reviewed recently and extensively by McIntosh (1994). A general comment at the end of this paragraph is that in head injury, major foci regarding the supported therapeutic efficiency of lazaroids have been the membrane damage resulting from the free radical cascade and the disruption in cellular ionic homeostasis, with less attention to other factors related to the pathological mechanisms of this disease: for example, the excitotoxic effects of pathological release of amino acid neurotransmitters.

At present, the final analyses of phase III trials of the antagonism of the initiation and propagation of the free radical cascade by tirilazad and polyethylene glycol-bound superoxide dismutase are nearing completion, as reported by Marshall and Marshall (1995).

III. Subarachnoid Hemorrhage

A. Background

Sustained cerebral arterial narrowing, occurring days after SAH, is commonly referred to as cerebral vasospasm (Findlay et al., 1991) and is defined as a reduction in vessel caliber of 10% or greater as compared with the baseline value (Kanamaru et al., 1991). This effect is widely accepted as an important complication of SAH (Allcock and Drake, 1965). The demonstration of a delayed onset (Weir et al., 1978) provided a potential therapeutic "window of opportunity " that is lacking in other ischemic strokes. The introduction of computed tomography in the 1970s, and its use in SAH, established the clear relationship between thick blood clots in the basal subarachnoid cisterns and subsequent vasospasm (Fischer et al., 1980; Kistler et al., 1983). The clinical observations that vasospasm (*a*) has a delayed onset, (*b*) is a major cause of morbidity and mortality after SAH, and (*c*) is predictable according to the amount of subarachnoid blood detected on initial computed tomography have made its prevention and treatment a tempting goal for neurosurgeons over the years (Findlay et al., 1991).

There is little question that the etiology of vasospasm involves subarachnoid blood clots (Asano et al., 1990; Findlay et al., 1989; Seifert et al., 1989). The mechanism by which lumen narrowing develops following SAH is controversial and revolves around two hypotheses. One is that vasospasm may be prolonged vasoconstriction (Findlay et al., 1989). With time, vasospastic arteries develop pharmacological (Bevan et al., 1987; Kim et al., 1989; Vorkapic et al., 1990) and ultrastructural (Duff et al., 1986; Findlay et al., 1989; Mayberg et al., 1990) abnormalities and are resistant to vasodilator drugs (Wilkins, 1986). The structural theory of vasospasm, however, hypothesizes that these changes in some way cause vasospasm (Kassel et al., 1985). Initially, the structural change believed to underlie vasospasm was intimal proliferation (Clower et al., 1981). Vasospasm usually resolves by 14 days after SAH and shows intimal proliferation $<$ 3 weeks after SAH (Clower et al., 1981; Findlay et al., 1989). The structural theory now revolves around whether other factors, such as inflammation and arterial wall fibrosis, contribute to prolongation of vasospasm (Bevan et al., 1987; Findlay et al., 1989; Smith et al., 1985).

Findlay et al. (1991), believe that vasospasm is best characterized as prolonged intense vasoconstriction, which leads secondarily to smooth muscle cell damage. This induces a change in smooth muscle phenotype, with subsequent migration of these cells into *tunica intima*, followed by their proliferation. There may be some collagen deposition in *tunica media* in association with smooth muscle phenotype change. These changes would be expected to correlate with the severity of angiographic vasospasm and with factors responsible for va-

sospasm. Studies of vasospastic smooth muscle provide evidence for a mechanism of contraction that is prolonged and temporally irreversible (Findlay et al., 1991). Contraction by intracellular calcium release probably involves activation of protein kinase C by diacylglycerol (Asano et al., 1990; Takayasu et al., 1986). These investigators speculated that vasospasm might result from continuous activation of protein kinase C by high levels of diacylglycerol produced continuously by lipid peroxidation within the smooth muscle cell membrane.

Spectrophotometric examination of CSF and pathological study of the subarachnoid space following SAH reveal that the most prominent process occurring in CSF during vasospasm is the erythrocyte hemolysis, with attendant release of oxyhemoglobin, which potently inhibits endothelium-dependent relaxation and its breakdown products, bilirubin, methemoglobin (Duff et al., 1988) and other vasoactive agents. Sano (1988) detected significantly higher levels of lipid peroxides in CSF of patients after SAH. Elevated levels of vasoconstrictor prostaglandins (PG) PG_s, PGE_2, $PGF_{2\alpha}$, and PGD_2, occur in lumbar CSF following SAH (Chehrazi et al., 1989; Cook and Schulz, 1990; Weir et al., 1978).

Much evidence has been accumulated concerning the possible role of free radical reactions in the pathogenesis of prolonged cerebral vasospasm following SAH from ruptured intracranial aneurysms (Sakaki et al., 1986; Asano et al., 1984). Active species of oxygen produced during autooxidation (Sutton et al., 1976; Winterbourn et al., 1976), or produced on the surface of leukocytes during their phagocytotic activities (Allen et al., 1974), readily oxidize polyunsaturated fatty acids derived from the lysis of erythrocyte membranes to form lipid peroxides.

However, many other experiments have shown that enzymes such as SOD, catalase or glutathione peroxidase protect the cellular structures against lipid peroxidation initiated by active species of oxygen and that antioxidants are also responsible for the inactivation of lipid peroxides (Mills, 1960; Zimmerman et al., 1973; Winterbourn et al., 1976; Vladimirov et al., 1980).

In a previous study (Sakaki et al., 1986), the relationship between free radical reactions and the biological defense mechanisms in the CSF in patients with ruptured intracranial aneurysms were studied. There was a close correlation between the increase in the amount of lipid peroxides and the decrease in the activity of SOD in the CSF. It is likely that the increased level of lipid peroxides in the CSF may cause lipid peroxidation in the arterial wall, resulting in prolonged vasospasm. The study of Sakaki et al. (1988) on adult mongrel dogs reveals that lipid peroxidation in the cerebral arterial wall contributes to the genesis of prolonged cerebral vasospasm following SAH. In particular, the results of the angiographic study provided data on the most appropriate times to kill the animals following SAH for the biochemical analysis of the CSF, the arterial wall and the brain parenchyma. The maximum vasoconstriction was elicited in the basilar artery on the 5th day, the vasoconstriction subsided slightly on the 8th day, and the relaxation of vasoconstriction was observed on the 14th day (Sakaki et al., 1988).

Among several mechanisms that may be involved in the pathogenesis of prolonged vasospasm, the authors assume from these results and from the study by others (Sasaki et al., 1981), in which they demonstrated experimental vasospasm similar to that in humans by intracisternal injection of lipid hydroperoxide, that lipid peroxidation in the arterial walls after SAH plays an important role in the genesis of vasospasm. The primary free radical reaction must occur because of an active species of oxygen produced during autooxidation of the oxyhemoglobin in the subarachnoid space in the presence of polyunsaturated fatty acid and catalytic metals, producing various lipid peroxides.

In addition to these nonenzymatic reactions, enzymatic reactions, such as lipoxygenase of platelets or leukocytes and cyclooxygenase, may involve the polyunsaturated fatty acids in the lipid peroxidation chain (Siegel et al., 1979; Sasaki et al., 1981). Lipid peroxides produced in the subarachnoid space can initiate or propagate successive free radical reactions in the arterial wall. The existence of micropores in the arterial *adventitia* may facilitate the progression of the reaction from the subarachnoid space to the arterial wall (Zervas et al., 1982).

The inhibition of the principal biological mechanisms regulating lipid peroxidation in the cells has been demonstrated in the arterial wall. It is known that the activity of glutathione peroxidase can be impaired by the inactivation of its conjugate enzymes glucose-6-phosphatase and glucose-6-phosphate dehydrogenase in the presence of lipid peroxides (Tsai et al., 1976; Benedetti et al., 1979). Under this impairment of the controlling mechanisms against free radical reactions, lipid peroxidation can progress further, involving the principal elements of the biological membranes in the destruction of the cells. The involvement of free radicals in the brain may be the cause of brain edema, resulting in the prolonged impairment of neuronal dysfunction after SAH (Aritake et al., 1983).

B. Selected Experimental Data

The ability of the nonglucocorticoid 21-aminosteroid U-74006F to antagonize acute progressive cerebral hypoperfusion following experimental SAH was examined in chloralose-anesthetized cats. The SAH was produced by injection of 0.5 mL/kg of unheparinized autologous blood into the *cisterna magna* after prior withdrawal of an equivalent volume of CSF (Hall and Travis, 1988). In untreated animals, the SAH caused a progressive decline in caudate nuclear blood flow (−51.4% by 3 h) and an increase in intracranial pressure (+18.5% mm Hg by 3 h). In comparison, in cats that received a 1 mg/kg i.v.

dose of U-74006F at 30 min after SAH, there was a complete prevention of the fall in caudate nuclear blood flow and a significant attenuation of the rise in intracranial pressure. Furthermore, the drug reduced a concomitant fall in the mean arterial blood pressure and cerebral perfusion pressure. Although not as effective as the 1 mg/kg dose, a 0.1 mg/kg dose also significantly attenuated the post-SAH fall in caudate nuclear blood flow. These results support a role of lipid peroxidation in the acute pathophysiology of SAH and suggest that U-74006F may be useful in the early treatment of this disorder (Hall and Travis, 1988).

The effects of subarachnoid injection of blood on BBB permeability to albumin was assessed in a rat model (Zuccarello et al., 1989). Subarachnoid injection of blood caused a significant, six-fold increase in Evans blue extravasation, whereas sham operation or NaCl injection had no effect. In addition, subarachnoid injections of arachidonic acid or $FeCl_2$ increased BBB permeability to Evans blue 16-fold and 10-fold, respectively. The capillary permeability after subarachnoid injection of blood was normalized by pretreatment with a novel 21-aminosteroid, U-74006F, which has antioxidant and antilipolytic activity. Pretreatment with U-74006F also reduced the vascular leakage induced by subarachnoid injection of arachidonic acid or $FeCl_2$ by 50% and 45%, respectively. The authors (Zuccarello et al., 1989) conclude that damage to membrane lipids by peroxidative and/or lipolytic processes is involved in the SAH-induced BBB opening and that U-74006F protects the BBB against the effects of SAH by preventing or limiting these pathological membrane lipid changes.

The efficacy of the 21-aminosteroid U-74006F was investigated using different dosages in a restricted, randomized, placebo-controlled trial (Kanamaru et al., 1990). Forty *Cynomolgus* monkeys were divided into five groups of eight. There were two groups given treatment with placebos: one group was given saline, and other group was given the vehicle in which U-74006F was delivered. There were three U-74006F treatment dosage groups: 0.3, 1.0 and 3.0 mg/kg. Each monkey underwent baseline cerebral angiography followed by right-side craniectomy and subarachnoid placement of a clot around the MCA. Treatment was administered intravenously every 8 h for 6 days. Seven days after experimental SAH, angiography was repeated, and the animals were sacrificed. In both saline placebo treatment groups, significant vasospasm occurred on the clot side in the extradural internal carotid artery (C3), the intradural internal carotid artery, the precommunicating segment of the anterior cerebral artery (A1) and the MCA. After U-74006F treatment, significantly less vasospasm developed in A1 on the clot side (0.3 mg/kg U-74006F treatment group) and the MCA (all U-74006F treatment groups). When the percentages of changes from the baseline for the vessel diameters on the clot side were compared, vasospasm was attenuated in A1 and MCA of all U-74006F treatment groups, as compared with placebo groups; only 0.3 mg/kg of U-74006F significantly prevented vasospasm in C3. Although the 0.3 mg/kg dosage appeared to have the most favorable effect, no significant differences were observed among the three dosage groups. Electron microscopy of the MCA on the clot side in the animals treated with U-74006F still showed luminal convolutions and morphological changes in the endothelial cells. These changes appeared less prominent in those MCAs with milder vasospasm. If these results in primates are applicable to humans, U-74006F would be useful in reducing vasospasm after aneurysmal SAH (Kanamaru et al., 1990).

The study of Matsui et al. (1994) examined the effect of tirilazad mesylate (U-74006F) on the intraluminal narrowing of basilar artery subjected to SAH in beagle dogs. An intravenous bolus injection of either vehicle or U-74006F (0.5, 1.5 and 3.0 mg/kg) was repeated every 8 h after an induction of the first SAH until the animals were sacrificed. A dose of 0.5 mg/kg U-74006F provided the greatest beneficial effect. An intravenous infusion of 100 mL of saline containing either vehicle or U-74006F (0.3 and 1.0 mg/kg) was given at the same time (every 8 h after first SAH). Post-SAH treatment of U-74006F, at a dosage of approximately 0.5 mg/kg, showed a beneficial effect by infusion as well as by bolus administration. This study demonstrates that U-74006F has an ability to prevent chronic vasospasm in the canine SAH model (Matsui et al., 1994).

It is believed that vasospasm is the leading treatable cause and cerebral ischemia is the final common pathway in most of the death and disability, resulting from aneurysmal SAH. Tirilazad mesylate is a potent scavenger of oxygen free radicals and an inhibitor of lipid peroxidation processes, which may play a central role in the development of the arterial narrowing of vasospasm, as well as in the final cascade of ischemic cell death (Braughler et al., 1989). In experimental models, tirilazad has been shown to ameliorate vasospasm associated with SAH (Steinke et al., 1989) and to reduce infarct size in models of focal cerebral ischemia (Wilson et al., 1992).

In human volunteers, the compound is well-tolerated and lacks the usual glucocorticoid side effects. Because it does not affect blood pressure or heart rate and would, therefore, be complementary to other forms of therapy for vasospasm, the study of Kassel et al. (1993) was undertaken to test the safety of a range of doses of tirilazad mesylate in patients with aneurysmal SAH and to develop pilot information for designing definitive trials of efficacy. This small dose-escalation safety study suggests that tirilazad mesylate, at doses up to 6.0 mg/kg/day for 10 days, is safe in a contemporaneously managed cohort of patients with aneurysmal SAH. A relatively high incidence of infusion site complications were observed in both the tirilazad and vehicle-treated groups, likely owing to the acidity (pH = 3) of the citrate

vehicle needed to solubilize the drug. Although there were no statistically significant differences in outcome between any of the treatment groups and controls, trends suggested a benefit of tirilazad at 2.0 mg/kg/day in reducing symptomatic vasospasm and improving favorable outcome at 3 months following SAH. Caution must be used in interpreting these data, however, because randomization among treatment groups (i.e., tirilazad doses) was not concurrent, and the vehicle groups were combined to increase statistical power.

Recently, MacDonald and Weir (1994) reviewed the relationship between the cerebral vasospasm and the role of free radicals. Although this condition has features of a prototypical free radical-mediated disease and a plausible theory can be outlined, data to support the theory are limited. An association of lipid peroxidation with vasospasm has been observed, but more sophisticated techniques for detection of free radical damage to arterial wall proteins and nucleic acids have not been used. Therefore, these are conflicting reports about efficacy of various antioxidant treatments for vasospasm. In these studies, concomitant experiments usually have not confirmed that the treatments have decreased free radicals or lipid peroxides in CSF.

Because smooth muscle contraction is involved in vasospasm, it would be interesting to investigate the actions of free radical on smooth muscle cells using, for example, isometric tension recordings and patch clamp techniques (MacDonald and Weir, 1994). Studies of cardiac myocytes indicate that free radicals alter conductances through potassium and calcium channel and through the sodium-calcium exchanger and may result in elevations in intracellular calcium. Few studies have been performed on cerebral smooth muscle cells. In one study, exposure of cerebrovascular smooth muscle cells to free radicals resulted in increased outward currents, decreased membrane resistance, cell contraction, appearance of membrane blebs and cell death.

Finally, in a recent study (Smith et al., 1996), the authors compared the effects of tirilazad on acute BBB damage in rats subjected to SAH via injection of 300 μL of autologous nonheparinized blood under the dura of the left cortex. The rats were treated by intravenous administration of either 0.3 or 1.0 mg/kg of tirilazad or U-89678, the main metabolite formed when the 4–5 double bond in the A-ring is reduced, 10 min before and 2 h after SAH. BBB damage was quantified according to the extravasation of the protein-bound Evans' blue dye into the injured cortex 3 h after SAH. The results revealed that 0.3 and 1.0 mg/kg tirilazad significantly reduced SAH-induced BBB damage 35.2% ($P < 0.05$) and 60.6% ($P < 0.001$), respectively, in comparison with treatment with the vehicle. On the basis of these findings, the authors conclude that the tirilazad metabolite, U-89678, possesses vasoprotective and neuroprotective properties that are essentially equivalent to the parent 21-aminosteroid. Therefore, U-89678 probably contributes to the protective effects of tirilazad in SAH and other insults to the CNS.

In summary, more investigations using more sophisticated experimental techniques are required before free radicals and reactions induced by them can be said with certainty to be the primary cause of vasospasm, even if they participate to pathogenetic molecular mechanisms.

IV. Hypoxia

A. Background

Release of excitatory amino acids and oxygen free radical-mediated membrane lipid peroxidation have been postulated to play a role both in neurodegenerative diseases and in cerebral ischemia (Choi and Rothman, 1990). Various reports have shown a protective influence of the nonglucocorticoid 21-aminosteroids on different models of cerebral ischemia (Perkins et al., 1991). The effects of the excitatory amino acid antagonists, especially of the N-methyl-D-aspartate (NMDA) receptor subtypes, are equally well established. In particular, competitive and noncompetitive NMDA receptor antagonists are reported to ameliorate the hypoxia-induced functional failure in rat hippocampal slices (Grigg and Anderson, 1990).

B. Selected Experimental Data

In preliminary studies, U-74006F blocked the release of arachidonic acid from cultured pituitary cells in response to hypoxia or lipid peroxidation (Braughler et al., 1988b).

In the study of Domenici et al. (1993), the authors analyze the effects of the noncompetitive NMDA receptor antagonist, dizocilpine (MK-801), and the 21-aminosteroids U-74500A and U-78517F on the hypoxia-induced electrophysiological changes in rat hippocampal slices. Hippocampal slices of male Wistar rats were constantly perfused with an artificial CSF saturated with 95% O_2 and 5% CO_2 at 33°C. Hippocampal slices were subjected to hypoxia by varying superfusion, from O_2-CO_2 to N_2-CO_2, for 45 min. The slices were then subjected to a recovery period of reoxygenation by varying superfusion from N_2-CO_2 to O_2-CO_2 for 45 min. The lipid peroxidation inhibitors, U-74500A (100 to 200 μM) and U-78517F (50 to 100 μM), or the NMDA receptor antagonist, MK-801 (50 μM), were superfused 15 min before, during the hypoxic and recovery or reoxygenation periods. This study showed a different influence of two 21-aminosteroids, U-74500A and U-78517F, on hypoxia-induced functional electrical failure in rat hippocampal slices.

U-78517F, but not U-74500A, was able to significantly ameliorate the recovery of the electrical responses during reoxygenation. This different influence probably depends on the different potency of the drugs in inhibiting membrane-lipid peroxidation (Braughler et al., 1987a; Hall et al., 1991b). This indicates that the oxygen free-

membrane-lipid peroxidation contributes to the development of the neuronal injury in rat hippocampal slices subjected to a hypoxic insult (Domenici et al., 1993). Under the same experimental conditions, the NMDA receptor antagonist, MK-801, was also able to improve the electrical recovery from hypoxia. The effects of low concentrations of MK-801 improved the protective activity of ineffective low concentrations of U-78517F (Domenici et al., 1993).

However, it is clear that these are in vitro experimental models and that further experiments are required to assess the efficacy of these 21-aminosteroids.

V. Ischemia

A. Background

The implication of lipid peroxidation in cerebral ischemia is supported by considerable evidence. Biochemical assays for the consumption of intrinsic antioxidants such as ascorbate (Flamm et al., 1978), alpha-TC (Hall et al., 1991b, 1993b; Yoshida et al., 1982) or glutathione (Cooper et al., 1980; Rehnrona et al., 1980) and indices of lipid peroxidation such as conjugated dienes (Watson et al., 1984) have been used as indirect evidence in models of cerebral ischemia.

As reported by Sutherland et al. (1991), forebrain ischemia of short duration damages neurons in discrete regions of the CNS (Pulsinelli et al., 1982; Smith et al., 1984a). Neuronal injury progressively worsens during the reperfusion period following a transient ischemic insult, a process termed "ischemic maturation" (Pulsinelli et al., 1982; Smith et al., 1984a,b). Several processes occurring both during and following an ischemic insult determine the extent of neuronal injury, and calcium fluxes possibly play a central role (Siesjo, 1982). In addition, postischemic increases in tissue lactate (Peeling et al., 1989) indicate abnormal mitochondrial function that could result in generation of toxic free radicals due to univalent reduction of oxygen (Halliwell and Gutteridge, 1985). Postischemic lipid peroxidation has been observed in rats subjected to 30 min of global ischemia (Watson et al., 1984; Yoshida et al., 1980) and in rats given an embolic stroke to one hemisphere (Kogue et al., 1982). Other investigators did not find significant postischemic changes in those brain phospholipids or fatty acids that would reflect free radical chain reactions (Renchrona et al., 1982). The latter experiments were conducted on large multiregional or whole brain samples; therefore, significant regional changes could have been masked, yielding false negative results. Alternatively, in those studies, either free radical generation did not occur or the brain was capable of mobilizing intrinsic cellular defense mechanisms that protect against free radical-related membrane injury (Sutherland et al., 1996).

In view of this controversial evidence for impaired oxidative metabolism during and following ischemia (Pulsinelli and Duffy, 1983; Siesjo, 1982), Sutherland et al. (1991a) have measured regional lipid peroxidation and SOD activity evaluated in tissue supernatant following transient forebrain ischemia in rat. After transient (10 min) forebrain ischemia (BCO *plus* controlled hypotension) the *hippocampus*, frontal lobes, parietal or occipital lobes and *cerebellum* were rapidly dissected and stored in liquid nitrogen. The evaluations were performed after 1 h, 24 h and 7 days postischemic time intervals. This model, together with its anesthetic management (pentobarbital 20 mg/kg, cloral hydrate 150 mg/kg and atropine 0.5 mg/kg), produces neuronal damage confined to selectively vulnerable brain regions (Sutherland et al., 1988). All regions showed a progressive increase in SOD activity with increasing postischemic time intervals. In regard to regional lipid peroxidation, the *hippocampus* had the highest inherent (nonischemic) value of thiobarbituric acid-reactive substances (TBARS) (Sutherland et al., 1991a).

Apart from the alteration in neuronal cells, adaptation is seen in glial cells also following ischemia. Compared with cerebral neurons, glial cells have greater SOD activity/mg protein (Geremia et al., 1990; Savolainen, 1978). Glia are not injured following 10 min of ischemia and, in fact, proliferate after such an insult.

Global changes in brain blood flow and metabolism take place following ischemic insults of short duration (Slater, 1984), and such changes contribute to the establishment of a hypometabolic state (Pulsinelli and Duffy, 1983). The controversial phenomenon is operative here, i.e., global recruitment of brain glial SOD activity. The mechanism of this metabolic mobilization may be related to potassium and pH changes (Chesler and Kraig, 1989; Hertz, 1965). Ischemia causes an abrupt rise in extracellular potassium. Passive and active uptake of potassium into adjacent glia are the principle means by which extracellular potassium is lowered (Chesler and Kraig, 1989; Hertz, 1965). This uptake would be accompanied by an increase in glial pH (Chesler and Kraig, 1989). Global changes in brain potassium, pH and possibly lactate levels that accompany ischemia would be expected to induce generalized activation of glia. It is interesting to speculate that in situations in which glial pH becomes acidotic, postischemic SOD probably would not increase. Glial SOD, despite a several-fold increase, does not protect neurons in the selectively vulnerable brain regions, where mechanisms other than those related to free radicals might be responsible for tissue damage (Sutherland et al., 1991a).

Despite a significant increase in neocortical TBARS, the lack of similar changes in the *hippocampus* further emphasizes the role of other mechanisms of tissue injury in that region (Sutherland et al., 1991a). Measurement of TBARS, when used alone as an absolute index of lipid peroxidation, has been suggested to be nonspecific (Slater, 1984). Under controlled conditions, however, this method can reflect adequately the relative changes

in lipid peroxidation (Bose et al., 1989). In this study, the authors (Sutherland et al., 1991a) ascertained a sustained global elevation in SOD activity with only mild regional increases in lipid peroxidation in the postischemic rat brain. It is of interest to note that even though they did not attain statistical significance, the levels of lipid peroxidation where highest in the *hippocampus*, consistent with hippocampal susceptibility to ischemic injury. Because the degree of lipid peroxidation was not markedly increased, however, its significance in the overall genesis of ischemic neuronal injury is difficult to determine. The dramatic increase in SOD activity possibly reflects global activation of glia, resulting from changes in potassium and pH during and following ischemia (Sutherland et al., 1991a).

B. Selected Experimental Data

A more convincing method for evaluating the role of free radical and lipid peroxidation in cerebral ischemia, with and without reperfusion, is to determine the effect of antioxidants upon the severity of neuronal injury. Indeed, to date, several such studies have demonstrated an ameliorating action of free radical scavengers or antioxidants on postischemic neurological, biochemical and pathological recovery (Abe et al., 1988; Beck and Bielenberg, 1991; Imaizumi et al., 1990; Liu et al., 1989). Several previous investigations have examined the effect of U-74006F in cerebral ischemia in various animal models (Hall et al., 1988a; Young et al., 1988a; Beck and Bielenberg, 1990; Lesiuk et al., 1991; Buchan et al., 1992). Although many of these reports have shown a protective effect with preischemia administration of U-74006F, the results are not uniformly conclusive.

A previous study using a rat forebrain ischemia model showed neocortical protection with U-74006F, whereas injury within either the *striatum* or the *hippocampus* was not significantly changed (Lesiuk et al., 1991). Such an observation implies that the free radical mechanisms of tissue injury may play a more significant role in the neocortex than within other selectively vulnerable brain regions (Sutherland et al., 1993).

While increased oxygen radical formation probably is initiated during an ischemic episode, it is greatly amplified following the reoxygenation of the tissue following reperfusion. Protective effects of the 21-aminosteroid, tirilazad, have been observed in several experimental models of focal cerebral ischemia with reperfusion. These models mimic the clinical situation of thromboembolic stroke. In a model of temporary hemispheric cerebral ischemia produced in the Mongolian gerbil by unilateral occlusion of a carotid artery, tirilazad (i.p., 10 min before and again immediately after a 3-h temporary occlusion of the right carotid artery), improved both 24-h and 48-h survival compared with vehicle-treated animals. Histological examination of vehicle-treated animals revealed a marked neuronal cell loss in the *hippocampus* and lateral cerebral cortex. In contrast, tirilazad-treated animals showed a statistically significant preservation of neurons in both brain regions.

Other results (Hall et al., 1988a) provide evidence that the ability of the 21-aminosteroid antioxidant tirilazad to reduce postischemic neuronal degeneration is due to an inhibition of postischemic lipid peroxidation, as judged from the significant drug-induced maintenance of endogenous vitamin E content and postischemic preservation of ascorbic acid levels (Sato and Hall, 1992). The most likely explanation is that after the ischemic insult, tissue vitamin E and ascorbate are being used to quench postreperfusion membrane lipid peroxidative reactions, whereas tirilazad spares tissue antioxidant content by assuming this pharmacological role. Moreover, the drug acts to preserve cellular processes responsible for the reversal of the ischemia-triggered intracellular calcium accumulation (gerbil, 3-h unilateral carotid occlusion model). Tirilazad has been shown to attenuate neurophilic influx into the reperfused hemisphere (Oostveen and Williams, 1992).

U-74006F has attracted attention also for its protective properties against the circulatory, biochemical and behavioral consequences of cerebral ischemia (Hall and Yonkers, 1988; Hall et al., 1988a,b; Silvia et al., 1987). Although beneficial effects on the Na^+/K^+ ion shifts following MCA occlusion and reduction of infarct size after a reversible 1 h occlusion of the MCA were demonstrated (Silvia et al., 1987; Young et al., 1988a), conflicting results exist if U-74006F is in fact reducing the number of necrotic neurons after global ischemia.

Young et al. (1988a) investigated the effects of U-74006F on the early ionic edema produced by MCA occlusion in rats. Intravenous doses of 3 mg/kg U-74006F were given 10 min and 3 h after occlusion. Tissue concentrations of Na^+, K^+ and water at and around the infarct site were measured by atomic absorption spectroscopy and by wet-dry weight measurements 24 h after occlusion. Compared with vehicle treatment, U-74006F treatment reduced brain water entry, Na^+ accumulation, K^+ loss and net ion shift by 25 to 50% in most brain areas sampled in the frontal and parietal cortex. However, reductions of ionic edema were most prominent and reached significance mostly in the frontoparietal and parietal cortex areas adjacent to the infarct site. These findings suggest that a steroid drug without glucocorticoid or mineralocorticoid activity can reduce edema in cerebral ischemia but that the effects are largely limited to tissues in which collateral blood flow may be present (Young et al., 1988a).

The possible efficacy of U-74006F in attenuating postischemic mortality and neuronal necrosis was examined in gerbils following 3 h of unilateral carotid artery occlusion (Hall et al., 1988a). Male mongolian gerbils received two intraperitoneal injections of either vehicle or U-74006F (3 or 10 mg/kg), the first injection 10 min before and the second injection at the end of the 3-h ischemic episode. In an initial series of experiments,

vehicle-treated gerbils displayed 60.9% survival 24 h after ischemia, which decreased to 34.8% at 48 h. In contrast, the 10 mg/kg U-74006F-treated group showed 86.7% survival at 24 h and 80.0% survival at 48 h. In a second series, neurons in the hippocampal CA_1 subfield and in the medial and lateral cerebral cortex were counted in gerbils surviving 24 h after unilateral carotid artery occlusion. Comparison of neuronal densities in the ischemic hemisphere with those in the controlateral nonischemic hemisphere revealed significant neuronal preservation in all three brain regions of gerbils treated twice with 10 mg/kg i.p. U-74006F. These results show that U-74006F can improve survival and attenuate neuronal necrosis in a severe brain ischemia model (Hall et al., 1988a). Because U-74006F is a potent inhibitor of lipid peroxidation, its anti-ischemic efficacy supports an important role of oxygen radical-induced lipid peroxidation in the pathophysiology of brain ischemia with reperfusion. However, other therapeutic mechanisms also may be involved (Hall et al., 1988a).

Although, in a gerbil model of unilateral carotid occlusion, U-74006F decreased significantly the number of necrotic neurons in *hippocampus* and cortex, after a 24-h short-term recovery from ischemia (Hall et al., 1988a), Polek et al. (1989) did not find any improvement of neurological outcome 5 days after a global ischemia in the rat. However, the duration of ischemia in that last study usually was long (30 min), and the preischemic dose was roughly only one-third of that used in the gerbil, factors that might have masked a putative protective action of U-74006F.

In the MCA occlusion model, application of U-74006F 10 min and 3 h postischemia significantly attenuated Na^+ accumulation, K^+ loss and edema formation in the border zone, but not at the infarct site proper (Young et al., 1988a). These effects were attributed to mainly the preservation of the integrity of membranes due to inhibition of lipid peroxidation by U-74006F. However, as previously indicated, a preischemic dose of 3 mg/kg U-74006F and postischemic infusion of 3 mg/kg/h for 3 h failed to improve neurological outcome 5 days after a 30-min ischemia in a rat model of global ischemia (Polek et al., 1989). Low dosage seems hardly responsible for this failure, because a three-fold higher dose of 10 mg/kg did not reduce the number of necrotic pyramidal cells either: measurements were performed 7 days after the insult. Consequently, these data suggest that U-74006F only delays the timepoint when cell death becomes evident on the light microscopic level after global ischemia (Hall et al., 1988a). Whereas U-74006F was reported to attenuate the drop in extracellular Ca^{2+} and reduction of lipid peroxidation measured 2 h postischemia (Hall et al., 1989), the available histological data document a protective effect of U-74006F only up to 24 h after global ischemia (Hall et al., 1988a), thus calling into question whether this compound prevents final hippocampal cell death in the long run, i.e., up to 5 days (Polek et al., 1989) or 7 days postischemia.

These results show that the lipid peroxidation inhibitor U-74006F did not improve the final neurological outcome after transient forebrain ischemia in the rat. Although these results do not rule out the idea that lipid peroxidation contributes to cell damage, they do not support the notion that lipid peroxidation is a major determinant for final cell death in this rat model of cerebral ischemia. Previous reports have provided ambiguous information on the protective properties of U-74006F against cerebral ischemia. Low doses of 1 mg/kg prevented postischemic hypoperfusion in cat cortex (Hall and Yonkers, 1988), and 10 mg/kg injected 10 min before and again immediately after a 3-h ischemia in the gerbil significantly reduced pyramidal cell loss in the CA_1 sector 24 h later.

In the study of Beck and Bielenberg (1990), the transient forebrain ischemia was induced as described by Smith et al. (1984a,b) in male Wistar rats. In the artificially ventilated animals (30% N_2O, 70% O_2), the carotid arteries were clamped, and blood pressure was lowered to 40 mm Hg by withdrawal of blood. After 10 min, blood flow to the brain was reconstituted by releasing the clamps and reinfusion of the shed blood. Rats received 1 mmol/kg $NaHCO_3$ to counteract systemic acidosis. Ten mg/kg U-74006F was given intraperitoneally 10 min before, 3 h after and 24 h after global ischemia. Seven days after ischemia, rats were perfusion-fixed, brains were paraffin-imbedded and coronal sections were taken of the hippocampal region. Necrotic cells were counted within the entire CA_1, CA_3 and CA_4 subfield of the *hippocampus* as well as in the cortex. Severe neuronal damage was measured in the vulnerable CA_1 sector, while CA_3 and CA_4 sectors showed only a slight loss of neurons. Only minor ischemic damage occurred in cortical areas that was not altered by treatment with U-74006F. Application of 20 mg/kg U-74006F resulted in an increase in death rates during the postischemic recovery period and thus was abandoned.

Tirilazad has been documented to reduce infarct size and/or edema in Sprague-Dawley rats subjected to permanent MCA occlusion (Young et al., 1988a; Lythgoe et al., 1990; Beck and Bielenberg, 1991). Additionally, tirilazad has been reported to reduce brain infarct size in a neonatal rat model of permanent carotid occlusion *plus* 2 h of moderately severe hypoxia (Bagenholm et al., 1991). This implies that the relevance of free radical mechanisms, and thus antioxidant protection, is not confined to ischemic situations in which reperfusion takes place. However, it should be noted that the compound has not shown efficacy in two models of permanent MCA occlusion in spontaneously hypertensive rats (Xue et al., 1992) and in hyperglycemic cats (Myers et al., 1990). Because both of these studies only investigated single-dose levels of tirilazad, a dose-response study will be required to completely rule out protective efficacy.

There is some evidence that U-74006F has also mechanistic effects against ischemic brain damage that are independent of a significant penetration of the compound across the BBB into the brain and based upon the fact that it acts to protect the microvasculature. The first vascular mechanism of U-74006F may be a protective effect on the BBB, which could reduce edema and the penetration of potentially deleterious blood-borne products (Young et al., 1988a,b). Also, U-74006F might improve or maintain blood flow in the partially perfused perifocal area.

U-74006F attenuates spreading of depression-induced hypoperfusion brain of anesthetized Sprague-Dawley rats (Hall and Smith, 1991). Spreading depression has been postulated to play a role in exacerbating ischemic damage. In permanent focal cerebral ischemia, although ischemia is dense in the "focus," there is a perifocal "penumbra" zone with a more moderate degree of ischemia (Beck and Bielenberg, 1991). The "penumbra" could be salvaged by blocking the spreading depression-induced hypoperfusion. It has been reported (Wilson et al., 1992) that U-74006F improved cerebral blood flow in a rabbit model of embolus-induced cerebral ischemia. In the study of Park and Hall (1994), the most consistent and numerically greatest reductions were observed in caudal stereotaxic coronal planes and on the dorsomedial portion of the frontoparietal lobes. The difficulty has been to delineate physically a perifocal penumbral zone, defined as an area with reduced cerebral blood flow but with viable cells with maintained ion homeostasis.

In models of global cerebral ischemia with reperfusion, which mimic the clinical situation of cardiac arrest/resuscitation, tirilazad has produced mixed, but generally positive, results. A reduction in delayed (7-day) postischemic hippocampal CA_1 damage has been observed in the widely used gerbil brief (10 min) BCO model, but only with maintained dosing; acute perischemic dosing is ineffective (Hall et al., 1994). Using the same model, pretreatment with tirilazad has been shown to reduce the early postreperfusion increase in brain levels of hydroxyl radical measured with the SAL trapping method (Andrus et al., 1991).

Selectively vulnerable neurons in either the *hippocampus* or *striatum* were not protected (Lesiuk et al., 1991). This implies that free radical-related mechanisms of neuronal damage are more pronounced in the neocortex or, alternatively, that the free radical burden within the *hippocampus* or *striatum* is so overwhelming that U-74006F would not be effective at any dose. In this model of forebrain ischemia, cerebral blood flow decreases to below 0.03 mL/g/min in the fronto-parietal neocortex and 0.04 mL/g/min in the *hippocampus* and *striatum* (Kozuka et al., 1989). Such values have been shown to be accompanied by energy failure, dissipative ion fluxes and many metabolic perturbations (Siesjo, 1984). In the report of Sutherland et al. (1991a), early postischemic elevations in lipid peroxidation were confined to the neocortex and evidence for an oxidative stress was reflected by global elevations in SOD activity.

Intrinsic scavenging mechanisms seem to be sufficient in the *hippocampus* and *striatum* to prevent significant increases in lipid peroxidation in these regions. The detrimental effects of other ischemia-induced mechanisms of tissue injury seem therefore to be more significant in the *hippocampus* and *striatum,* which are markedly damaged in this model compared with the relatively mild neocortical injury. Among other factors, these may include impaired postischemic protein synthesis (Nowak et al., 1985), protein kinase C activation (Hara et al., 1990b), neural tubule/filament degradation (Jenkins et al., 1979) and alteration in excitatory receptor/channels (Choi, 1988). It is difficult to account for the lack of protection with the 10 mg/kg dose of U-74006F. This may be related in part to the increased volume of the acidic carrier necessary to deliver the 10 mg/kg dose.

The lack of correlation between the various studies also may be explained by different models (focal versus global), animal species, strains and methodology. In contrast, the highly sensitive hippocampal CA_1 region has not been effectively protected by tirilazad in the same model (Hoffman et al., 1991; Beck and Bielenberg, 1990; Lesiuk et al., 1991; Pahlmark et al., 1991) or in the rat 15-min, four-vessel occlusion model (Buchan et al., 1992). This discrepancy between antioxidant protection of cortex and the *hippocampus* has led to the suggestion that the mechanisms of postischemic neuronal damage may differ in those two regions, with lipid peroxidation being more relevant in the cortex.

The mixed results with tirilazad regarding its ability to successfully impact the cortical damage associated with brief global ischemia (Lesiuk et al., 1991), but not the more severe hippocampal damage (particularly the highly vulnerable CA_1 region) (Hoffman et al., 1991; Lesiuk et al., 1991; Pahlmark et al., 1991; Buchan et al., 1992), may have an explanation in the several observations of accentuated postischemic free radical production and lipid peroxidation in the *hippocampus* in comparison with the cortex (Hall et al., 1993b). Thus, the dose level found to salvage the gerbil *hippocampus* is noteworthy (Hall et al., 1994). The exploration of tirilazad doses as high as those used in the gerbil, or the administration of the compound preferably by the i.v. route at the time of reperfusion, may show better protection in the *hippocampus* than is thus far reported.

In a dog model of 10-min normothermic cardiac arrest, tirilazad has been documented to improve 24-h neurological recovery and survival (Natale et al, 1988). Nevertheless, tirilazad failed to improve early neurophysiological (i.e., somatosensory evoked potentials) and metabolic (i.e., magnetic resonance phosphorus spectroscopic measurement of ATP, phosphocreatine and pH) recovery in a nearly identical model of 10 min of complete global ischemia (Helfaer et al., 1992).

Interestingly, the latter group of investigators observed a striking improvement in early neurophysiological and metabolic recovery in a more severe dog model of 30 min of incomplete global ischemia in hyperglycemic animals produced via raised intracranial pressure (Maruki et al., 1993). A similar improvement in recovery of brain energy metabolism and acid-base balance has been reported in the model of rat two-vessel occlusion *plus* hypotension forebrain ischemia (Haraldseth et al., 1991; Vande Linde et al., 1993).

The neuroprotective effects of tirilazad in global cerebral ischemia, in the absence of effects on systemic blood pressure, are not associated with direct actions on cerebral blood flow (Natale et al., 1988). This is similar to the lack of correlation of blood flow effects with the neuroprotection observed in models of focal cerebral ischemia (Xue et al., 1992; Wilson et al., 1992).

Haraldseth et al. (1991) used phosphorous-31 nuclear magnetic resonance spectroscopy in a rat model of 10 min of severe incomplete forebrain ischemia (two-vessel occlusion with hypotension) to study the effects of preischemic and postischemic treatment with 3 mg/kg i.v. U-74006F on the recovery of high-energy phosphates and intracellular pH during early reperfusion. The mean ± standard deviation time to 85% recovery of phosphocreatine was 14.1 ± 8.4 min in the control group compared with 6.6 ± 3.5 min in the preischemic and 4.2 ± 1.0 min in the postischemic treatment groups. The mean ± standard deviation time to 80% recovery of adenosine triphosphate (ATP) was 15.4 ± 8.5 min in the control group compared with 6.3 ± 1.8 and 5.4 ± 2.8 min in the preischemic and postischemic treatment groups, respectively. There were no differences in intracellular pH between the control and either of the treatment groups.

As reported by Haraldseth et al. (1991), recovery of high-energy phosphates during reperfusion is not a biochemically reliable predictor of the energy metabolic state of neurons that may influence the final neuronal survival or functional outcome. It is probable that ATP can recover initially in cells with irreversible damage and that events at later stages during reperfusion have an impact on the final outcome. However, the quicker early recovery with treatment with U-74006F suggests an immediate beneficial effect of the drug on brain tissue during the first 30 min of reperfusion. Almost all ATP in the brain is generated in the mitochondria (Erecinska and Silver, 1989). It is, however, not possible to conclude that the improved recovery of ATP with U-74006F in the study of Haraldseth et al. (1991) was due to a direct effect on the mitochondria. The mechanism of action could have been improved microcirculation, which has been proposed by some authors to be the main target of free radical damage during reperfusion after cerebral ischemia (Demopoulos et al., 1980; Siesjo et al., 1990). The quicker recovery of high-energy phosphates was also found when U-74006F was injected after the ischemic insult. This is in accordance with the concept of free radical damage as a reperfusion injury, when oxygen returns to brain tissue after ischemia (Siesjo et al., 1989). These results give the impression that postischemic treatment is even better than preischemic treatment. However, the study did not allow for any comparison between the two treatment groups (Haraldseth et al., 1991).

The study of Lesiuk et al. (1991) describes the effect of the 21-aminosteroid U-74006F on transient forebrain ischemia in rats. Acute-treatment rats received either 3 mg/kg U-74006F or carrier vehicle intravenously 30 min before ischemia. Sustained-treatment rats received the same treatment before ischemia, followed by 3 mg/kg U-74006F or carrier vehicle intraperitoneally every 6 h for 48 h, and control rats received no injection. Coronal magnetic resonance images were obtained daily for 3 days, followed by the histological examination of the perfusion-fixed brains. Control rats demonstrated magnetic resonance image changes in the *hippocampus* and neocortex at 48 h. No significant effect of U-74006F treatment on striatal or hippocampal injury was demonstrated. However, both the acute and sustained U-74006F treatments produced a significant reduction in the severity of neuronal damage in the neocortex. These results (Lesiuk et al., 1991) suggest that U-74006F is of benefit in ameliorating ischemic neuronal injury, particularly in the neocortex, and raises the possibility of regional variability in lipid peroxidation following an ischemic insult (Lesiuk et al., 1991).

In rat model of brief (10–12 min) BCO *plus* hypotension, a reduction in cortical neuronal loss has been reported (Lesiuk et al., 1991; Sutherland et al., 1993). In this study (Sutherland et al., 1993), male Sprague-Dawley rats received U-74006F at a dosage of 0.3, 1.0, 3.0, 7.0, and 10 mg/kg i.p. 30 min before ischemia. Seven days following ischemia, the rats were perfusion-fixed with 1 L of 10% buffered formaldehyde (pH 7.25). The brains were removed and placed in the same fixative for 2 weeks before sectioning into 1.5-mm-thick slices. In the *hippocampus* and neocortex, the frequency of ischemic neurons was calculated by dividing the number of acidotic and/or pyknotic neurons by the total number of neurons. Striatal damage was graded using established methods, with (*a*) <10% necrotic neurons given a grade of 1; (*b*) 10 to 50% necrotic neurons at grade 2; (*c*) 50 to 100% necrotic neurons at grade 3 (Sutherland et al., 1991b). For this region, a nonparametric analysis was performed. Protection against injury was limited to the neocortex, reaching significance in the 7 mg/kg-treated group compared with control or 1 and 3 mg of U-74006F. Striatal damage was not significantly different between groups.

While in experimental models of temporary focal cerebral ischemia, U-74006F appeared to reduce ischemic brain damage and promote survival (Hall et al., 1988a; Hall, 1990; Silvia et al., 1987; Xue et al., 1990, 1992), in

models of permanent focal cerebral ischemia, conflicting results exist (Braughler and Hall, 1989; Myers et al., 1990; Xue et al., 1992). Postischemic evoked potential recovery correlates with acidosis during ischemia and early reperfusion. Acidosis promotes lipid peroxidation in vitro. In this study, the authors (Maruki et al., 1993) tested the hypothesis that the 21-aminosteroid tirilazad mesylate (U-74006F), an inhibitor of lipid peroxidation in vitro, ameliorates somatosensory evoked potential recovery and acidosis during reperfusion after severe incomplete cerebral ischemia. Cerebral perfusion pressure was reduced to 11 ± 1 mm Hg for 30 min by cerebral ventricular fluid infusion in anesthetized dogs.

Cerebral intracellular pH and high-energy phosphates were measured by magnetic resonance spectroscopy. Dogs were randomized to receive vehicle (citrate buffer) or tirilazad (1 mg/kg) before ischemia in a blinded study. Cerebral blood flow was reduced to 6 ± 1 mL/min per 100 g during ischemia, resulting in nearly complete loss of high-energy phosphates and an intracellular pH of 6.0 to 6.1 in both groups. Initial postischemic hyperemia was similar between groups but lasted longer in the vehicle group. Tirilazad accelerated mean recovery time of intracellular pH from 31 ± 5 to 15 ± 3 min and inorganic phosphate from 13 ± 2 to 6 ± 1 min. Recovery of somatosensory evoked potential amplitude was greater with tirilazad (49 ± 3%) than with vehicle (33 ± 6%), and fractional cortical water content was less with tirilazad (0.819 ± 0.003) than with vehicle (0.831 ± 0.002). Thus, tirilazad attenuates cerebral edema and improves somatosensory evoked potential recovery after incomplete ischemia associated with severe acidosis. Accelerated pH and inorganic phosphate recovery indicates that this antioxidant acts during the early minutes of reperfusion (Maruki et al., 1993).

In the study of Park and Hall (1994) using male Sprague-Dawley rats, focal cerebral infarction was achieved using a modification of the permanent MCA occlusion model (Tamura et al., 1981). Four groups of rats were studied: vehicle-administered controls ($n = 7$), and U-74006F-treated animals at doses of 0.3 mg/kg ($n = 7$), 1.0 mg/kg ($n = 7$) and 3.0 mg/kg ($n = 7$) (i.v. 15 min, 2 h and 6 h after occlusion, and 3.3 times higher than the first three doses, i.p. 12 h after occlusion). Twenty-four hours after surgery, the animals were subjected to neurological examination using a grading scale of 0 to 3 and were sacrificed to assess ischemic damage by means of tetrazolium chloride staining. Immediately after sacrifice, the brain was removed and frozen at −10°C for 10 min, and the forebrain was cut into coronal slices. The present study indicates that, in this model, U-74006F provides significant protection against ischemic brain damage in a dose-dependent manner and ameliorates postischemic neurological deficits, although the reduction of infarct volume in the cerebral hemisphere amounted to only 25 to 36% at the two highest doses.

On the other hand, Karlsson et al. (1994) studied the effect of tirilazad, an aminosteroid with radical scavenging effect, or its vehicle on cerebral blood flow and neuronal death when given before 15 min of severe global ischemia achieved by hypotensive bilateral carotid clamping in rats. Ischemic blood flow less than 1% of the nonischemic values were seen in the forebrain regions. Hypoperfusion occurred in all regions 60 min after the insult, with low values by 21 to 58% of those in the nonischemic group. Tirilazad had no effect on cerebral blood flow in the nonischemic rats, nor in those decapitated during or after the insult. Five days postischemia, neuronal damage had developed in all regions examined, but no significant differences were seen between the tirilazad-treated and the vehicle-treated rats (Karlsson et al., 1994).

Thus, the protective effects of the 21-aminosteroid tirilazad mesylate (U-74006F), one of the most efficacious inhibitors of free radical-initiated lipid peroxidation, against brain damage, particularly in permanent focal cerebral ischemia, remain controversial.

The effect of the antioxidant drug tirilazad mesylate (U-74006F) on histopathological and neurological outcome 3 days after permanent MCA occlusion was evaluated in rats. Previous studies (Hall et al., 1988a; Young et al., 1988a; Lesiuk et al. 1991) have demonstrated the efficacy of tirilazad in reducing infarct size when administered before and during MCA occlusion, whereas posttreatment administration may be less effective in permanent focal ischemia. Thus, the authors sought to determine whether a protective effect of tirilazad could be demonstrated when administered only after the insult. Either U-74006F (3 mg/kg, i.v.) or sterile vehicle was randomly given to rats 10 min and 3 h after permanent MCA occlusion produced by intracranial proximal electrocauterization (Hellstrom et al., 1994). There was no significant difference in infarct volume, volume of noninfarcted tissue, nor neurological score between the tirilazad-treated and placebo-treated rats. The results support the concept that posttreatment with tirilazad mesylate is not efficacious in reducing infarct size in permanent focal ischemia, whereas pretreatment, as reported by other groups, appears to be effective in both permanent and temporary focal ischemia models. In temporary focal ischemia, the limited data available also suggest that posttreatment with tirilazad may prove to be neuroprotective.

However, transient global ischemia may lead to persistent production of reactive oxygen species in selected brain regions, thereby contributing to selective vulnerability to ischemia. Using cerebral microdialysis, Zhang and Piantadosi (1994) assessed the production of the highly reactive hydroxyl radical (OH·) in rat *hippocampus* during global ischemia and reperfusion. During ischemia and reperfusion perfusate containing salicylic acid was collected and analyzed for nonenzymatic hydroxylation of SAL to 23-DHBA. Because 21-aminos-

teroids can attenuate excitatory amino acid-mediated OH· production in the brain, the authors repeated the experiments after administration of the 21-aminosteroid, U-74389G. The data indicate that the 23-DHBA level increased progressively between 15 and 60 min after reperfusion, reaching values nearly the baseline value at 60 min. U-74389G, given 30 min before ischemia, greatly attenuated the increase in 23-DHBA during reperfusion. This is the first evidence for prolonged OH· production in the *hippocampus* after reperfusion in vivo that can be prevented by 21-aminosteroids (Zhang and Piantadosi, 1994).

Takeshima et al. (1994) tested the hypothesis that administration of the antioxidant tirilazad mesylate improves electrophysiological recovery and decreases infarct volume after transient focal cerebral ischemia in cats. Halothane-anesthetized cats underwent 90 min of left MCA and bilateral common carotid artery occlusion followed by 180 min of reperfusion. Cats were assigned to receive tirilazad (1.5 mg/kg *plus* 0.2 mg/kg per hour, i.v. infusions) either at the beginning ($n = 9$) or at the conclusion ($n = 9$) of ischemia. Control cats received an equal volume of diluent (citrate buffer, $n = 7$) at the beginning and at the conclusion of ischemia in a blinded fashion. Infarct volume was measured by 2,3,5-triphenyltetrazolium chloride staining. Results show that blood flow of the left temporoparietal cortex decreased to less than 10 mL/min per 10 g with ischemia (but was minimally affected on the right side) and that blood flow distribution during ischemia or reperfusion was not different in the tirilazad-treated groups. No group demonstrated postischemic hyperemia or delayed hypoperfusion. Somatosensory evoked potential recorded over the left cortex was ablated during ischemia and recovered to less than 15% of baseline amplitude at 180 min of reperfusion in all groups. There were no differences among groups in infarct volume of the left hemisphere in this experimental model of focal ischemia involving severe reductions of blood flow followed by reperfusion in cats; administration of tirilazad at the onset of either ischemia or reperfusion does not ameliorate infarct volume assessed during early reperfusion. However, this study (Takeshima et al., 1994) does not address the potential efficacy of tirilazad in the setting of a different dosing strategy or duration of reperfusion.

Umemura et al. (1994) evaluated the effect of 21-aminosteroid lipid inhibitor, U-74006F, on ischemic brain tissue damage using the rat MCA occlusion model. Under anesthesia, the left MCA was exposed without cutting the dura mater via a subtemporal craniotomy, under an operating microscope. Photo-illumination (wavelength, 540 nm) was applied to the MCA and then rose bengal (20 mg/kg) was administered intravenously. The MCA was completely occluded by thrombus about 6 min after administration of rose bengal. U-74006F (1.0 mg/kg) was then injected intravenously just after the cessation of illumination. Twenty-four hours after the operation, the extent of ischemic damage was measured by magnetic resonance imaging technique. After measuring the extent of ischemic damage, the brain was removed immediately from animals treated with or without U-74006F for determination of lipid peroxidation and the generation of free arachidonic acid in the brain. U-74006F significantly ($P < 0.01$) reduced the size of ischemic damage. Twenty-four hours after the operation, lipid peroxidation and the concentration of free arachidonic acid in the left hemisphere (infarction side) were significantly ($P < 0.05$) higher than in the right hemisphere. U-74006F significantly ($P < 0.05$) decreased the content of lipid peroxidation products and free arachidonic acid. There was a significant ($P < 0.05$) correlation between the extent of ischemic damage and the concentration of lipid peroxidation products in the left hemisphere 24 h after the operation. In conclusion, U-74006F might reduce the extent of ischemic damage by inhibiting lipid peroxidation in the brain, thus limiting oxidative damage to neural damages (Umemura et al., 1994).

Andrus et al. (1994) measured the production of eicosanoids in the gerbil brain during early reperfusion after either a 3-h unilateral carotid occlusion (a model of focal ischemia) or a 0-min BCO (a model of global ischemia). Arachidonic acid metabolites were examined to determine whether pretreatment with the 21-aminosteroid lipid peroxidation inhibitor U-74006F (tirilazad mesylate) could influence postreperfusion synthesis of brain eicosanoids. In the 3-h unilateral carotid occlusion focal ischemia model, there was an early (5-min) postreperfusion elevation in brain levels of PGF (2 alpha), thromboxane B2 and leukotriene C4 ($P < 0.05$ versus sham for all three eicosanoids). Leukotriene B4 also increased, but not significantly. On the other hand, PGE_2 and 6-keto-PGF (1 alpha) tended to decrease during ischemia and at 5-min postreperfusion ($P < 0.05$ versus sham for PGE_2). Pretreatment with known neuroprotective doses of U-74006F in this model (10 mg/kg, i.p. 10 min before and again immediately upon reperfusion) did not affect the increase in PGF (2 alpha) or thromboxane B2, but significantly blunted the elevations in leukotriene C4 and leukotriene B4. The postreperfusion decrease in PGE_2 was also attenuated. In the 10-min BCO global ischemia model, there was also an increase in each of the measured eicosanoids, except leukotriene B4, at 5 min after reperfusion. Pretreatment with U-74006F (10 mg/kg i.p., 10 min before ischemia) selectively decreased the increase in leukotriene C4, but did not significantly affect the other eicosanoids. The effects of U-74006F on postreperfusion eicosanoid synthesis are consistent with the lipid antioxidant properties of this compound. In particular, the attenuation of leukotriene levels is most likely a reflection of a decrease in postreperfusion lipid peroxidation, because lipid peroxides are potent activators of 5-lipoxygenase (Andrus et al., 1994).

Orozco et al. (1995) evaluated the effects of tissue-type plasminogen activator and 21-aminosteroid (U-74006F) in experimental embolic stroke in rabbits. The MCA of the rabbit was embolized by injecting an arterial ("white") thrombus in the right internal carotid artery. The rabbit treatment was 2 mg/kg of tissue-type plasminogen activator and/or 3 mg/kg of 21-aminosteroid started at 2 h postembolization. The results show that the administration of tissue-type plasminogen activator and/or 21-aminosteroid at 2 h postembolization alone or in simultaneous administration does not significantly reduce the volume of infarction.

Maruki et al. (1995) tested the hypothesis that when acidosis is augmented by hyperglycemia, pretreatment with the 21-aminosteroid tirilazad mesylate (U-74006F) may improve cerebral metabolic recovery. In a randomized, blinded study, anesthetized dogs received either tirilazad mesylate (1 mg/kg *plus* 0.2 mg/kg/h; $n = 8$) or vehicle ($n = 8$). Hyperglycemia (400 to 500 mg/dL) was produced before 30 min of global incomplete cerebral ischemia. Intracellular pH and high energy phosphates were measured by phosphorus magnetic resonance spectroscopy. During ischemia, microsphere-determined cerebral blood flow decreased to 8 ± 4 mL/min/100 g, and intracellular pH decreased to 5.6 ± 0.2 in both groups. During the first 20 min of reperfusion, ATP partially recovered in the vehicle group to 57 ± 21% of baseline, but then decreased progressively in association with elevated intracranial pressure. By 30 min, ATP recovery was greater in the tirilazad group (77 ± 35 versus 36 ± 19%), although postischemic hyperemia was similar. By 45 min, the tirilazad group had a higher intracellular pH (6.5 ± 0.5 versus 5.9 ± 0.6) and a lower intracranial pressure (18 ± 6 versus 52 ± 24 mm Hg). By 180 min, blood flow and ATP were undetectable in seven of eight vehicle-treated dogs, whereas ATP was > 67% and pH was > 6.7% in six of eight tirilazad-treated dogs. The authors concluded that (*a*) tirilazad acts during early reperfusion to prevent secondary metabolic decay associated with severe acidotic ischemia and (*b*) if tirilazad acts by inhibiting lipid peroxidation, then these data are consistent with extreme acidosis limiting recovery by a mechanism involving lipid peroxidation.

In conclusion, generally accepted treatment regimens of hypoxic-ischemic brain damage have not been established so far. Therefore, therapeutic measures should be oriented to pathophysiological mechanisms known at present, including ischemic calcium cascade, excitotoxicity, nitric oxide overformation, and disturbances of recirculation (e.g., no reflow phenomenon) (Prange, 1994).

Bioelectric changes in the brain parenchyma evolving during hypoxia-ischemia become successively apparent as hyperpolarization, failure of synaptic transmission, massive depolarization of cells resembling the spreading depression, neuronal K^+ loss and uptake of large amounts of Na^+, Cl^-, Ca^{2+}, accompanied by H_2O, cause cell swelling. Up to now, the rapid progress of these pathological events has hardly permitted an efficacious treatment (Prange, 1994). In any therapy, the combination of NMDA receptor antagonists, glucocorticosteroids, drugs and heparin could be helpful in preventing the delayed postischemic injury that often occurs after initial apparent recovery, as reported by Prange (1994). However, the therapeutic role of lazaroids, nitric oxide donators and endothelin antagonists still must be defined.

An early assessment of the brain damage subsequent to hypoxia-ischemia is possible by means of somatosensory evoked potentials and serum concentration of neuron-specific enolase, respectively (Prange, 1994). Neuron-specific enolase values exceeding 120 ng/mL during the first 5 days after hypoxia-ischemia point to an unfavorable outcome. In contrast, neuron-specific enolase concentrations below 35 ng/mL mostly indicate a good recovery (Prange, 1994).

In summary, we emphasize the recently recognized pathophysiological mechanisms responsible for brain damage during ischemia and reperfusion and new therapeutic concepts developed on a rational basis. Mediators of secondary damage include excitotoxins such as glutamate, acidosis, free radicals and the disturbance of the microcirculation seen in the early phase of recirculation. Glutamate is an excitatory neurotransmitter, which may turn neurotoxic when the energy supply is limited. Tissue acidosis down to pH 6.0 develops regularly in cerebral ischemia and disturbs a variety of neuronal functions, causing glial swelling and neuronal death. Free radicals attack brain lipids, the cell membrane and myelin in particular, and these molecular chemical species, produced during reperfusion, are only one pathogenetic mechanism of many. Disturbance of the microcirculation aggravates ischemic damage, and suggested therapeutic approaches (see Kempski, 1994, for a review) include glutamate antagonists, normalization of tissue acidosis and use of new diuretics to reduce glial swelling, protection of the brain by free radical scavengers such as 21-aminosteroids, TC, allopurinol or SOD, and hypothermia. Ways of ensuring fast reperfusion, including hypervolemic hemodilution and blood pressure stabilization, are suggested for resuscitation or early stroke. All data available indicate that the combination of several successful therapeutic principles will significantly improve outcome.

VI. Neurodegenerative Disorders

A. Background

Free radicals have been implicated in more than 100 human diseases (Gutteridge, 1993). However, this does not mean the involvement is important (Halliwell and Gutteridge, 1989; Halliwell et al., 1992). In effect, these evanescent chemical species are difficult to assay. Halliwell and Gutteridge (1984) emphasized that oxidative

damage could be just as much a consequence of tissue injury as a cause of it. Indeed, tissue injury (however caused) almost certainly leads to oxidative stress for many reasons. The oxidative stress could then significantly contribute to worsening the tissue injury, or it might be irrelevant. The criteria that are needed to implicate free radicals as important contributors in the cause of disease have been reviewed (Halliwell et al., 1992). At the moment, in neurodegenerative disease, chronic inflammatory disease and cardiovascular disease, evidence is accumulating to show that free radical damage is important. This realization will contribute to the development of new preventive and therapeutic strategies (Halliwell, 1994).

As reported by Jenner (1994), the idea of free radical involvement in Parkinson's disease arose from the concept that chemical oxidation of dopamine produces potentially toxic semiquinones, whereas accelerated metabolism of dopamine by monoamine oxidase B might induce excessive formation of hydrogen peroxide. Necropsy studies provide evidence of increased lipid peroxidation in *substantia nigra* in Parkinson's disease (Dexter et al., 1994a). Concentrations of copper/zinc-dependent and/or manganese-dependent SOD are above normal (Marttila et al., 1988; Saggu et al., 1989), which might be interpreted to be adaptive change to increased free radical load. The nigral content of reduced glutathione is below normal, and this may be important to the production of oxidative damage in Parkinson's disease. The decrease in glutathione may be significant in Parkinson's disease, because it is restricted to *substantia nigra*, does not occur in other basal ganglia degenerative disorders and is not thought to be a consequence of drug treatment (Jenner, 1994).

The cause of the inhibition in mitochondrial complex I activity in *substantia nigra* in Parkinson's disease remains unknown (Shapira, 1994). There is no evidence of 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP)/1-methyl-4-phenylpiridinium (MPP^+)-like toxin in *substantia nigra* in Parkinson's disease (Ikeda et al., 1992), and there is no corroborated evidence for alteration in complex I subunits or of altered mitochondrial DNA encoding for the components of the complex (Shapira, 1994). An alternative explanation might be that oxidative damage inhibits complex I. However, in vitro and in vivo induction of oxidative stress leads to inhibition of complexes I and III and IV.

Spina and Cohen (1989) suggested that increased dopamine elevates brain oxidized glutathione or of the leakage of glutathione from damaged cells. Cleavage of glutathione can itself result in the formation of toxic cysteinyl derivatives, especially in the presence of transition metal ions. There are parallels with the actions of MPP^+ and other mitochondrial poisons, which cause hepatocytes to lose glutathione. Hence, the changes in mitochondrial function and glutathione in Parkinson's disease may be linked (Jenner, 1994).

Some workers suggest that incidental Lewy-body disease represents presymptomatic Parkinson's disease. In these patients, Jenner (1994) found that iron levels were unaltered, suggesting that iron accumulation represents a later and secondary component of cellular destruction (Dexter et al., 1994b). There was a decrease in complex I activity, but this did not reach statistical significance, and glutathione was decreased to the same degree as in advanced Parkinson's disease. Therefore, glutathione is altered early in nigral lesions and to some extent may reflect early changes in mitochondrial function.

In addition, cell death can itself lead to oxidative damage (Halliwell and Gutteridge, 1985), and, because gliosis ensues, the glia might produce free radicals. The relation between the various biochemical changes and the effect of drug treatment on indices of oxidative stress remains to be resolved (Jenner, 1994).

Postmortem studies of the vulnerability of dopamine-containing cells in *substantia nigra* will be particularly relevant, because a specific chemical "fingerprint" of damage to lipids, DNA and proteins can be assayed (Halliwell et al., 1992).

In all these disorders, iron is increased in the lesions (Dexter et al., 1991), which reinforces the belief that iron accumulation is a secondary change associated with neurodegeneration in various diseases and is not specific to Parkinson's disease. Why iron increases remains unknown, but it may be related to gliosis in diseased areas or to changes in the integrity of the BBB caused by altered vascularization of tissue or by inflammatory events (Jenner, 1994).

The altered encoding on chromosome 21q of copper/zinc SOD in amyotrophic lateral sclerosis may be an important indicator of altered free radical activity (Rosen et al., 1993). This defect probably translates into decreased SOD activity, and, in mice transgenic to the mutant for familial lateral sclerosis gene, symptoms similar to those of amyotrophic lateral sclerosis develop (Gurney et al., 1994).

There has been speculation that nitric oxide is implicated in various neurodegenerative disorders (Marx, 1994). In fact, nitric oxide free radical (·ON) can react with superoxide radical to yield highly toxic peroxynitrite (ONOO·), according to reaction 15:

$$\cdot O2 + \cdot ON \rightarrow ONOO\cdot \qquad [15]$$

When peroxynitrite reacts with human body fluids and tissues, nitrotyrosines can be generated (Van der Vliet et al., 1994). In particular, 3-nitrotyrosine detected in human brain (Jenner, 1994) may be increased in neurodegenerative disease as part of the pathological process, especially because glial cells and macrophages might be expected to generate nitric oxide.

Jenner (1994) proposes that they are a common feature of the process of cell death in most, if not all, such illnesses. If so, the therapeutic rewards may be great. It

may become possible to stop or slow the progression of common disorders, such as Alzheimer's or Parkinson's disease, with a single therapeutic approach. In any case, a considerable body of information supports the occurrence and pathophysiological importance of oxygen radical-mediated lipid peroxidation, and such mechanisms have been implicated in chronic neurodegenerative (e.g., Alzheimer's and Parkinson's diseases) and demyelinating (e.g., multiple sclerosis) disorders. Thus, efforts have been directed toward discovery of effective lipid antioxidant compounds that would retard posttraumatic and postischemic neurodegeneration (Hall, 1992b). Consequently, there has been interest in identification of pharmacological agents with potent ability to interrupt oxygen radical formation or cell membrane lipid peroxidative mechanisms (Hall, 1992b).

In Alzheimer's disease, iron and ferritin content increases in cortical regions (Connor et al., 1992), whereas in cortical areas from both senile dementia of the Alzheimer and of the Lewy body types, iron is increased and glutathione is decreased (Jenner, 1994). Complex I activity has not been assessed, although any decrease would reveal close similarity to neurodegeneration in *substantia nigra* in Parkinson's disease. Iron changes also have been detected in multiple sclerosis, spastic paraplegia and amyotrophic lateral sclerosis, once more emphasizing the nonspecific nature of iron's involvement.

In Alzheimer's disease, increased levels of manganese-dependent SOD also have been reported in cortex, although this remains controversial (Marklund et al., 1985). There is accumulating evidence of increased oxidative stress (i.e., increased free radical production) and increased susceptibility to lipid peroxidation in Alzheimer's brains. Recent work has shown that there is a higher baseline content of thiobarbituric acid-reactive lipid-peroxidation products in cerebral cortical tissue from Alzheimer's brain in comparison with age-matched nonAlzheimer's brains (Subbarao et al., 1990). In addition, in vitro induction of lipid peroxidation by iron is more intense in Alzheimer's brains cortical samples, even if such a difference is not observed in *cerebellum*. This observation has been replicated by two other groups (McIntosh et al., 1991; Andorn et al., 1990). In one of these studies (Andorn et al., 1990), increased basal levels of lipid peroxidation-related chemiluminescence (+ 28%) and malonyldialdehyde (+ 42%) in temporal cortical samples from Alzheimer's patients have been reported, and these findings suggest the possible utility of lipid peroxidation inhibitors in treatment of the disease (Hall, 1992b).

B. Selected Experimental Data

The 21-aminosteroid U-74500A has been shown to effectively inhibit iron-induced lipid peroxidation in Alzheimer's brain samples (Subbarao et al., 1990). The IC_{50} in normal brain tissue was 2.5 versus 10.0 μmol/L for Alzheimer's brain samples. Nevertheless, its efficacy against lipid peroxidation in Alzheimer's brain suggests potential utility of lipid antioxidant therapy as a means to slow disease progression (Hall, 1992a).

The effects of the 21-aminosteroids have been examined against the cytotoxic effects of NMDA in cultured mouse cerebral cortical neurons (Monyer et al., 1990). U-74500A, given either before or after NMDA exposure, significantly attenuates neuronal damage by NMDA, while having no effects on NMDA-induced membrane currents. Thus, the mechanism of this protection is indirect. Additionally, both U-74500A and U-74006F reduced neuronal damage produced by peroxidative (i.e., iron-induced), hypoglycemic or hypoxic insult to the cortical cultures. The competitive NMDA antagonist dextromethorphan, although not an antioxidant, also decreased the damaging effects of iron. This finding has led to the suggestion that excitotoxic and lipid peroxidative neuronal injury mechanisms are linked (Hall, 1992a). Thus, excitatory amino acids and oxygen free radicals have been reported to cooperate in the genesis of brain injury in vivo and in vitro. Zuccarello and Anderson (1993) tested the capacity of a noncompetitive NMDA receptor antagonist, MK-801, and a 21-aminosteroid, U-74006F, tirilazad mesylate, to block the opening of the BBB after subarachnoid injection of $FeCl_2$, which is believed to cause a primarily "pure" free radical insult. Subarachnoid injection of $FeCl_2$ resulted in a significant 10-fold increase in Evans blue extravasation, whereas sham injection or NaCl injection had no effect. Pretreatment with either MK-801 or U-74006F significantly reduced the $FeCl_2$-induced increase in capillary permeability by 43 and 63%, respectively. Combined treatment with MK-801 and U-74006F resulted in a 65% reduction in vascular leakage that was not significantly greater than pretreatment with either drug alone. These results show that both excitatory amino acids and free radicals can damage the cerebral microvasculature and that an excitatory amino acid antagonist can partially protect the BBB after free radical-induced injury (Zuccarello and Anderson, 1993).

Relative to the mechanism of action of lazaroids at the subcellular level, it should be noted that endrin, a polyhalogenated cyclic hydrocarbon, induces hepatic lipid peroxidation, modulates calcium homeostasis, decreases membrane fluidity, and increases nuclear DNA damage. The effects of endrin were assessed in rat brain and liver 24 h following an acute oral dose of 4.5 mg/kg (Bagchi et al., 1995). Lipid peroxidation associated with whole brain mitochondria increased 2.4-fold, whereas microsomal lipid peroxidation increased 2.8-fold following endrin administration. Catalase activity decreased 24% in the *hypothalamus*, 23% in the cortex, 38% in the *cerebellum* and 11% in the brain stem in response to endrin. Pretreatment of rats intraperitoneally with the lazaroid U-74389F (16-desmethyl tirilazad) (10 mg/kg in two

doses) attenuated the biochemical consequences of endrin-induced oxidative stress (Bagchi et al., 1995).

The antioxidant enzymatic system in the ischemia/reperfusion-induced brain injury in rats after U-74389G administration has been evaluated. Ischemia/reperfusion caused a decrease, also of total and free sulphydryl groups, whereas TBARS became elevated. Administration of U-74389G led to restoration to normal value of all the above parameters (Farbiszewski et al., 1994). A protective effect of the drug in ischemia/reperfusion-induced brain injury has been suggested by these authors.

From the standpoint of relevance to Alzheimer's disease, amyloid protein has been reported to exacerbate excitotoxic damage to cortical neurons (Koh et al., 1990). Taken together, these studies suggest that amyloid, excitotoxicity (i.e., glutamate-induced) and iron-catalyzed, oxygen radical-induced lipid peroxidation may be interactive neurodegenerative mechanisms. In any case, lipid antioxidant therapy may be capable of interrupting both excitotoxic and lipid peroxidative degeneration relative to Alzheimer's pathogenesis.

As part of an ongoing investigation of the role of oxygen free radicals in Alzheimer's disease, the formation of peroxidation products, the activities of free radical defense enzymes and the level of total iron were determined in autopsy brain tissue from donors with Alzheimer's disease and from age-matched nondemented donors (Richardson, 1993). Calcium uptake was also investigated in mitochondria harvested from fibroblasts grown in tissue culture from skin samples taken from brain donors. Compared with controls, homogenates of Alzheimer's disease frontal cortex produced elevated levels of peroxidation products, and this difference was amplified in a dose-dependent manner by iron (1 to 200 μM). Peroxidation produced by 200 μM iron was reduced dose-dependently by the lazaroid U-74500A. The IC_{50} was 10 μM in Alzheimer's disease cortex and 2.5 μM in controls. SOD, one of the free radical defensive enzymes, was reduced by 25 to 35% in Alzheimer's disease frontal cortex, *hippocampus* and *cerebellum,* whereas in other brain areas, SOD did not differ between Alzheimer's disease patients and control. The activities of catalase and glutathione peroxidase were the same in Alzheimer's disease and in control samples. Endogenous iron levels were higher in Alzheimer's disease frontal cortex (2.5 nmol/mg protein) than in controls (1.5 nmol/mg protein). Calcium uptake by Alzheimer's disease fibroblast mitochondria is 50% lower than in controls under basal conditions. Following exposure to 200 μM iron, mitochondrial calcium uptake is increased by 58% in Alzheimer's disease and by 38% in controls. Pretreatment with 200 μM U-74500A or 1 mM deferoxamine, before exposure to 200 μM iron, gave complete protection to Alzheimer's disease mitochondria.

These studies indicate that in Alzheimer's disease, both CNS and peripheral cells show increased sensitivity to oxygen free radicals. The source of this increased sensitivity has not yet been identified but could reflect either reduced free radical defenses or increased free radical formation, or both. Work is controlled using electron paramagnetic resonance spectrometry to determine in vivo, *premortem* free radical activity in Alzheimer's disease patients (Richardson, 1993). The formation of thiobarbituric acid-reactive products was measured as an index of peroxidation by oxygen free radicals in homogenates of frontal cerebral cortex and *cerebellum* from brains taken at autopsy and verified histologically as being Alzheimer's ($n = 6$) or normal ($n = 6$). Compared with controls, basal peroxidation is significantly higher in Alzheimer's cortex, and this difference is also evident in the presence of exogenous iron. Peroxidation in *cerebellum* and levels of total glutathione, ribonucleic acid and DNA in cortex and *cerebellum* do not differ significantly between Alzheimer's brain and controls. Iron-induced peroxidation in cortex is reduced by the lazaroid U-74500A, with calculated IC_{50} values that are significantly higher in Alzheimer's samples (10 μM) than in controls (2.5 μM).

All these observations suggest that cerebral cortex from Alzheimer's patients differs from controls with respect to in vitro peroxidation (Subbarao et al., 1990).

VII. Aging

A. Background

A possible mechanism for the decrease in membrane fluidity with age is an increased peroxidation of membrane lipids, and the evidence that this happens in old age is quite convincing. The flux of electrons down the respiratory chain gives rise to the generation of superoxide free radicals ($\cdot O_2$) via the single-electron reduction of O_2 (Boveris et al., 1972; Chance et al., 1979). The main site of superoxide generation appears to be ubiquinone (Boveris et al., 1976), although cytochrome b_{-566} also has been implicated (Nohl et al., 1981). In addition, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase was recognized as a source of superoxide free radicals, albeit a more minor one (Turrens and Boveris, 1980). By the presence of an Mn-requiring superoxide dismutase (Fridovich, 1975) within the mitochondrial matrix (Nohl and Hegner, 1978), superoxide free radicals are converted to H_2O_2 and mitochondria are protected against this by the presence of catalase and of glutathione peroxidase. It has been assumed that catalase is quantitatively more important in the removal of H_2O_2 in prokaryotes and glutathione peroxidase in eukaryotes (Nohl and Jordan, 1980).

The relationship between lipid peroxidation and aging has been put by Harman (1972, 1968) and by Tappel (1973), who have drawn attention not only to the metabolic state that could be wrought by the cross-linking of membrane enzyme proteins, but also to the likelihood of this happening in the mitochondrion, with both (poly)-unsaturated fatty acid and heme iron present to poten-

tiate lipid peroxidation. In the context of membrane fluidity, these processes act via two mechanisms (Munkres, 1979). The peroxidative attack on unsaturated fatty acids lowers their content in the membrane directly, and the cross-linking of both phospholipid and protein molecules also introduces an increased rigidity. A direct demonstration of loss of fluidity with nonenzymic peroxidation has been made by Dobretsov et al. (1977) using three different fluorescent probe systems.

Aging affects both the rate of production of superoxide free radical by the mitochondrial respiratory chain and the rate of removal of such superoxide by SOD and of hydrogen peroxide by the action of catalase and glutathione peroxidase. Thus, Nohl and Hegner (1978) showed that coupled heart mitochondria from old rats produce superoxide free radicals more actively than those from young controls. Mitochondrial SOD was found to be unchanged with age in heart mitochondria (Nohl et al., 1979), but the capacity for the removal of H_2O_2 was found to be increased. Thus, Nohl et al. (1979) report increases in the activity of both catalase and the selenium-dependent glutathione peroxidase in rat heart mitochondria in old age. The authors interpret these as adaptive responses to the raised content of peroxidized lipids, which they identified in senescence (Nohl and Hegner, 1978), and implicate catalase as the major mechanism for the removal H_2O_2 and glutathione peroxidase as catalyzing the reduction of lipid peroxides (Nohl and Jordan, 1980; Nohl et al., 1979).

There is evidence that despite an increased activity of the enzymes that protect mitochondria from the attack of free radicals, peroxidative damage does accumulate in old age (Nohl and Hegner, 1978). Antioxidants also have the capability of limiting peroxidative reactions (Leibovitz and Siegel, 1980), and dietary antioxidants have been investigated for possible effects on longevity, with varied results. For example, Grinna (1976) found no evidence that dietary alpha-TC prevented either age-dependent changes in structure, i.e., the degree of unsaturation of microsomal fatty acids, or mitochondrial functions, i.e., succinate-cytochrome *c* reductase and 3-hydroxybutyrate dehydrogenase activity. However, the conclusions are clouded by the fact that it probably was not possible to generate a true vitamin E deficiency state in older animals (Hansford, 1983). There is evidence, however, for a decreased lipid peroxidation in response to dietary antioxidant, as shown by a lesser accumulation of age-pigment (Tappel et al., 1973). In any case, damage to the mitochondria leads to impairment of ATP production and loss of cellular homeostasis, especially noting the fact that peroxide generation takes place in the inner membrane of mitochondria, possibly causing damage to the mitochondrial DNA also.

B. Selected Experimental Data

As previously reported, physiological changes, such as decreased cardiac output, blunted homeostatic mechanisms and diminished hepatic and renal functions, occur with increasing age (Dawling and Crome, 1989). Liver size and liver blood flow also decrease with age in humans (Woodhouse and Wynne, 1988). These physiological changes in the elderly result in altered pharmacokinetic properties of several drugs, among which tirilazad mesylate has been shown in this setting. It is likely that, based on its pharmacokinetic properties in young volunteers, tirilazad mesylate will exhibit altered pharmacokinetics in the elderly (Hulst et al., 1994).

VIII. Comment

The release of neuronal energy for the aerobic metabolism is a mechanism that uses oxygen as the terminal acceptor of electrons that flow into the mitochondrial "respiratory" (transfer) chain. Thus, oxygen is an essential molecule for the survival of the majority of living organisms. There is evidence to suggest that the increase in energy metabolism by aerobic pathways enhances the intracellular concentration of oxygen free radicals, which in turn enhance the rate of the autocatalytic process of lipid peroxidation, possibly inducing damage in brain structures, especially when physiological defenses became insufficient. Thus, the mechanisms of both the aerobic energy transduction and the release of partially reduced oxygen intermediates, i.e., the oxygen free radicals, are related to the same biophysical system: the mitochondrial electron transfer chain. To use oxygen as the terminal acceptor of electrons in the mitochondrial electron transfer chain, the various potential substrates, i.e., carbohydrates, proteins and lipids, undergo a series of metabolic breakdowns whose products are transported into the tricarboxylic acid cycle (Kreb's cycle). Here, the so-called electron donors (NADH and succinate) are produced for the mitochondrial respiratory chain, where electrons are transferred to molecular oxygen with release of energy, through an ordered and sequential series of oxidation-reduction reactions.

The change in standard free energy (DG°) that is transduced when two redox couples interact with each other at a specific standard potential of oxidation-reduction, is given by reaction 16:

$$DG^\circ = -nFDx'_o \qquad [16]$$

where: n = number of electrons transferred; F = Faraday constant (23,062 cal × volt $^{-1}$ × equiv $^{-1}$); $Dx'_o = x'_o$ of the couple that receives the electrons *minus* x'_o of the couple that yields the electrons; x'_o = standard redox potential at pH = 7. The oxidation-reduction reactions, i.e., redox reactions, are characterized by the transfer of electrons from an electron donor (the reducing agent) to an electron acceptor (the oxidizing agent), sometimes by means of the transfer of hydrogen. Oxidizing and reducing agents can thus act only when they form "redox couples" or "conjugate redox pairs," i.e., an electron

donor and its conjugated electron acceptor: the NADH/NAD^+, flavin adenine dinucleotide (reduced form)/flavin adenine dinucleotide, succinate/fumarate or oxygen/water couples. The various reducing agents (electron donors) behave differently when they release electrons, and this electron-releasing tendency is described by the standard redox potential, which is indicated as x_o when calculated at a pH of 0 and as x'_o when calculated at a pH of 7.

The change in standard free energy, which occurs in mitochondria when a pair of electrons is transferred from NADH ($x'_o = -0.320$ V) to molecular oxygen ($x'_o = +0.816$ V) may therefore be calculated as in reaction 17:

$$DG° = -52.6 \text{ kcal} \times \text{mol}^{-1} \quad [17]$$

which is independent of the pathway followed by electrons and needs no identification of the intermediate molecules involved in electron transfer. The energy in NADH oxidation is not, however, freed gradually or in a continuous way from a potential of -0.320 V to a potential of +0.816 V, but in a discontinuous way, in a series of jumps in potential. We can calculate the minimum change in potential needed to obtain the synthesis of one mole of ATP using the general equation 18:

$$W = q \times (p_a - p_r) \quad [18]$$

where W = work done by the system; q = charge transferred; (p_a - p_r) = difference in potential; W is equal to twice the electron charge (2 × 96,487) multiplied by the difference of potential (+1.14 V).

To express the variation of free energy in calories, it is sufficient to divide the work done (W) by the mechanical equivalent of the calorie, i.e., 4.18 joules/calorie. Applying the above-quoted formula, (p_a - p_r) is equal to 0.15 V. Thus, for one mole of ATP to be formed, a difference must exist in redox potential which is found in:

- complex I, between NADH/NAD^+ ($x'_o = -0.300$ V) and ubiquinone/ubiquinol ($x'_o = +0.0045$ V);
- complex III, between ubiquinone/ubiquinol and cytochromes c_{ox}/c_{red} ($x'_o = +0.254$ V);
- complex IV, between cytochromes c_{ox}/c_{red} and O_2/H_2O ($x'_o = +0.816$ V).

The redox enzymes, coenzymes and cytochromes involved in the mitochondrial electron transfer are complex both in their structure and mechanism of action and are located within the mitochondrial inner membranes. In cerebral tissue, during aerobic metabolism, the electron transfer from donors (NADH and succinate) to molecular oxygen causes the release of considerable amounts of energy for ATP synthesis, ion translocation, protein synthesis and so on. Electron transfer arises with the vectorial translocation of protons in a series of molecular complexes, consisting of various equipotential subunits located in the mitochondrial inner membrane, which consists of 70% proteins and 30% lipids, providing both the transduction of oxidative energy in protonmotive force and use of proton energy in ATP synthesis. The electron transfer is conditioned by the integrity of the phospholipid structures and catalytic activities of enzymes. The mitochondrial complexes are made up of more than 60 polypeptides. However, only 13 are known to be encoded by mitochondrial DNA. The electron transferring molecules of the respiratory chain may be grouped into three *quasi*-equipotential regions, characterized by both the fall in their half-reduction potential (xm), which is less that 0.1 V with minimal energy loss and maximum energy conservation. These regions, the complexes I, III and IV, are separated by three intervals, characterized by a fall in (xm) of 0.15 V. All the electron transfer system is reversible, and an electron flow can be generated against the current. However, the final stage of electron transfer, complex IV to oxygen, is irreversible, so that the equilibrium in the system is shifted toward ATP synthesis. The activity of complex IV is labeled as cytochrome *c* oxidase.

The H^+/e^- stoichiometry of proton translocation coupled to electron flow is estimated to be between 1 and 2. In other words, it is believed that in complex I, one to two protons are translocated vectorially for every electron transferred. The most traditional mechanism conceivable envisages vectorial release of protons from complex I toward the extramitochondrial compartment through the mitochondrial inner membrane, during the electron exchange in the redox cycles of respiratory chain. The protons would appear to be "pumped" through the mitochondrial inner membrane by means of pK changes induced by variations in the redox states of some prototrophic protein's residues, with variations of the ionization, as indicated by pK changes.

Oxygen accepts one electron at a time, generating a cascade of partially reduced intermediates (oxygen with one or two or three electrons) until the oxygen itself is completely reduced to water (oxygen with four electrons). Complex IV retains all the partially reduced oxygen intermediates bound to its active sites until complete reduction to water, whereas the other components of the mitochondrial respiratory chain (ubiquinone and cytochrome *b* populations) transfer the electrons directly to oxygen and do not retain the partially reduced oxygen intermediates in their active sites. During aerobic energy transduction, oxygen reduction by means of complex IV reveals electron capturing processes in the formation of three intermediates: (*a*) in the formation of the intermediate I, i.e., acquisition of two electrons for the constitution of a *peroxide* complex; (*b*) in the formation of the intermediate II, i.e., acquisition of a third electron, that leads to the cleavage of the "oxygen-oxygen bond" and the reduction of one atom of oxygen to water; (*c*) in the formation of the intermediate III, i.e., acquisition of a fourth electron, with reduction of the second atom of oxygen.

Complex V or synthetase is the enzyme system responsible for the synthesis of ATP from ADP and inorganic phosphate (P_i), using proton energy derived from the transfer of electrons in complex I, III and IV. Complex V also carries out ATP hydrolysis at the same time as proton translocation from the matrix to the cytosolic side of mitochondrial inner membrane.

In conclusion, oxygen accepts, or rather prefers to accept, one electron at a time. When it acquires the first electron, a superoxide free radical is formed, which may be defined as a radical in that it contains one unpaired electron. Most of these superoxide radicals are formed in the mitochondrial and microsomal electron transfer chain. Whereas cytochrome oxidase retains all the partially reduced oxygen intermediates bound to its active site, it must be recalled that other elements in the mitochondrial respiratory chain, e.g. ubiquinone, transfer the electrons directly to oxygen and do not retain the partially reduced oxygen intermediates in their active sites. Because oxygen accepts only one electron at a time, the superoxide radical is released, and its release increases as the concentration of oxygen increases.

The superoxide anion ($\cdot O_2$) in aqueous environments is in equilibrium with its protonated form ($\cdot HO_2$). When the reduced form of molecular oxygen and the protonated form of the superoxide anion approach equal molar concentrations, spontaneous dismutation occurs, and (H_2O_2) *plus* (O_2) are generated, according to the reactions 2, 13 and 14. Only a limited number of enzymes are able to produce the superoxide free radical directly: xanthine oxidase, tryptophan dioxygenase and indole-amine dioxygenase. In those biological systems in which superoxide free radicals are generated, it is hypothesized that considerable damage will always be found, such as DNA fragmentation and lipid peroxidation of membranes, although the molecular mechanism by which this toxic effect occurs is not fully understood. It should be noted that, when acquiring an electron, the superoxide radicals can be converted into hydrogen peroxide (H_2O_2) by SOD that is present in varying concentrations in eukaryotic and prokaryotic cells and that is capable of dismutating two molecules of ($\cdot O_2$) to form hydrogen peroxide and oxygen. On the internal mitochondrial membrane, the superoxide anion also may be generated by auto-oxidation of semiquinones, rather than as a direct catalytic product. The majority of ($\cdot O_2$) generated by the mitochondrial electron transport is enzymatically dismutated to (H_2O_2). Some reactions catalyzed by several enzymes, such as monoamine oxidase and L-amino acid oxidase, can produce hydrogen peroxide directly.

Although hydrogen peroxide cannot be classified as a radical because it contains no unpaired electrons, it is still a potentially dangerous agent in that (*a*) it easily permeates cell membranes directly and can thus migrate to neuronal compartments other than those where it was first formed, in contrast to superoxide anion ($\cdot O_2$), which crosses cell membranes via anion channels; (*b*) it can interact with the reduced forms of some metal ions, generally bivalent iron or monovalent copper, which decomposes into the highly reactive hydroxyl radical and the hydroxyl ion, according to the following reactions (5 and 19):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \text{ (Fenton)} \quad [5]$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + \cdot OH + OH^- \quad [19]$$

Without metal ions, the Haber-Weiss (Fenton) reaction is extremely slow at physiological pH values and thus would require steady-state concentrations of the reaction partners far beyond those found in mitochondria to account for detectable amounts of the highly unstable radical.

In older subjects, during aging, the release of superoxide radical and of the dismutation product, the hydrogen peroxide, could be a process of considerable proportions, but it can take place even in young ones and during the entire life span. Thus, the formation of superoxide free radical could be associated with the normal process of mitochondrial respiration.

The leakage of electrons, as a mandatory side effect of the normal flux of electrons from both NADH and succinate to molecular oxygen, may substantially be due to changes in the function of the components of the mitochondrial energy-transducing systems, as well as of scavenging biological systems (compounds, enzymes). The oxygen free radicals release does not occur only during heavy aerobic metabolism, but also during the recovery phases from many pathological noxious stimuli of the cerebral tissue, assuming that a marked increase in potential mitochondrial energetic activity occurs during recovery, allowing an enhancement in the oxygen free radical generation during the activated electron flux in transfer chain. Mitochondria subjected to high tensions of oxygen accumulate peroxides over the concentrations normally measured in mitochondria respiring at basal oxygen tensions. Therefore, cerebral mitochondria have limited ability to counteract oxygen-induced disorganization if the concentrations of free radicals deviate from the steady state, as may occur during heavy aerobic metabolism.

The unbalance between free radical production and free radical detoxification in cerebral tissue produces damages to (*a*) mitochondria themselves, as inferred by the increase in lipid peroxidation and drop in coupling of energy transduction and oxygen uptake; (*b*) lysosomes, as inferred by the loss of enzyme latency and enhanced lysosomal activity also in the recovery periods and (*c*) antioxidant system, as inferred by the alteration in the glutathione redox state and scavengers content. However, it should be noted that (*a*) less attention has been paid to the changes on enzymic protein closely associated with lipid components of the mitochondrial membranes and (*b*) in any case, a quite unanswered question is whether there are any cumulative effects with regard

to the damage by sequential and long-time repeated release of oxygen free radicals (i.e., aging): the lazaroid's pharmacology should be completed regarding these two aspects.

During and/or following ischemia, or CNS trauma, changes occur that favor the production of oxygen radicals (Braughler and Hall, 1989; Hall and Braughler, 1989). These changes include consumption of ATP and concomitant accumulation of adenosine monophosphate (Marcy and Welsh, 1984), which is degraded to hypoxanthine. After ischemia, xanthine oxidase may catalyze the formation of superoxide anion, in the presence of hypoxanthine (McCord and Fridovich, 1968). In addition, ischemia or stroke may lead to an increase in lactate levels as cells convert to anaerobic glycolysis following the reduction in oxygen delivery (Rohu et al., 1993). These changes lead to tissue acidosis in regions of the CNS with a fall in local pH to values as low as 6.0 (Von Hanwehr et al., 1986). Both ferritin and transferrin can release iron at a pH of 6.0 or less (Braughler and Hall, 1989), and evidence points to the involvement of iron in the initiation and propagation of lipid peroxidation in CNS trauma (Braughler and Hall, 1989), facilitating the production of oxygen free radicals (Chiu and Lubin, 1989) via the so-called Fenton reaction.

Ion gradients are disrupted by conditions that promote the formation of oxygen radicals. In particular, Ca^{2+} accumulates in damaged cells (Hall et al., 1991b), and increased cytosolic Ca^{2+} may be a final common pathway in cell death (Schanne et al., 1979; Farber, 1990). Rohn et al. (1993) hypothesize that inhibition of both Na^+, K^+-ATPase and Ca^{2+}-ATPase, as caused by oxygen free radicals, leads to the accumulation of intracellular Ca^{2+} to toxic levels. Increased intracellular Ca^{2+} causes or contributes to the cell injury and death in a variety of pathological states. Intracellular Ca^{2+} normally is maintained at extremely low levels in the cytoplasm by one or more processes, including a calmodulin-activated Ca^{2+} pump (Vincenzi et al., 1980; Carafoli, 1991), Na^+/K^+ exchange (Blaustein, 1982), which is energized indirectly by the Na^+, K^+-ATPase and the endoplasmic reticulum Ca^{2+}-ATPase (Ikemoto, 1982).

Preincubation of red blood cell membranes in the presence of ferrous sulfate and ethylenediamine-tetraacetic acid resulted in both a concentration- and time-dependent inhibition of Na^+, K^+-ATPase, basal Ca^{2+}-ATPase and the calmodulin-activated Ca^{2+}-ATPase. The addition to membranes of ferrous iron and ethylenediaminetetraacetic acid in an approximately 1:1 ratio resulted in conversion to the ferric iron form in several minutes. However, inhibition of the ion pump ATPases and cross-linking of membrane proteins occurred over the course of several hours (Rohn et al., 1993). The time course of formation of TBARS closely paralleled inhibition of the ion pump ATPases, prevented by the addition of deferoxamine or SOD, but not by mannitol, ethanol or catalase. Both butylated hydroxytoluene and tirilazad mesylate (U-74006F) prevented the formation of TBARS, limited the inhibition of the ion pump ATPases and reduced cross-linking of membrane proteins. These data may be interpreted to suggest that inhibition of the ion pump ATPases in plasma membranes may occur as a result of iron-promoted formation of superoxide and subsequent lipid peroxidation, which can be prevented by the free radical scavengers, including U-74006F (Rohn et al., 1993).

In many disease states, the nature of the radical species that amplifies the primary damage is unclear, making the design of appropriate antioxidant drugs difficult. Thus, a detailed understanding of the processes leading to the radical-dependent pathology, as well as to the nature and sources of the toxic species, is crucial for the design of effective intervention strategies (Rice-Evans and Diplock, 1993). The essential consideration is the design of the antioxidant drug and appropriate targeting. The need is to deliver the proper scavenger to the affected site within the time frame of maximal tissue damage (Rice-Evans and Diplock, 1993). Concerning whole animal studies, the use of antioxidant therapies now should be addressed. Only when the mechanisms and involvement of free radicals in the pathogenesis of many disorders of CNS are understood will the approaches to antioxidant therapy be designed effectively and targeted successfully (Rice-Evans and Diplock, 1993).

Free radical-induced lipid peroxidation appears to play an important role in CNS injury. The selected and extensively discussed preclinical experimental studies demonstrated that treatment with lazaroids can reduce acute neurological disorders by preventing lipid peroxidation and diminishing free radical generation, but this is not the only molecular mechanism that underlines neuropathology. Whether or not tirilazad will prove to be effective in the treatment of a variety of acute neurological diseases awaits the results of ongoing phase III clinical trials. In fact, although it appears to be highly beneficial in experimental models, the clinical studies to date have failed to confirm this efficacy. The discrepancy in therapeutic efficacy between the various clinical trials using tirilazad reported to date probably reflects differences in the patient populations, with trials with a higher percentage of female patients and anticonvulsant use less likely to obtain therapeutic drug concentration. Again, this failure appears to be largely due to inadequate drug concentration and biological systems having so far been tested (Clark et al., 1995).

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