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EFFECT OF 21-AMINOSTEROID U74500A (PREGNA-1,4,9 (11)-TRIENE-3,20-DIONE, 21-(4-(5,6-BIS(DIETHYLAMINO)-2-PYRIDINYL)-1-PIPERAZINYL)-16-METHYL-, HCl (16α)) ON RAT BRAIN CORTEX LIPID PEROXIDATION INDUCED "*IN VIVO*" BY IRON–CARBOHYDRATE

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Abstract—Compounds derived from glucocorticoids, 21-aminosteroids, were reported to inhibit *in vitro* lipid peroxidation in CNS tissue. In order to evaluate the possible scavenging and/or iron chelating activities *in vivo* of the 21-aminosteroid U74500A (pregna-1,4,9(11)-triene-3,20-dione, 21-(4-(5,6-bisdiethylamino)-2-pyridinyl)-1-piperazinyl)-16-methyl-, HCl (16α)), the drug was administered for seven days to rats. These rats had been induced by iron–saccharate complex injection a slow process of lipid peroxidation into their right brain hemicortex. The drug was injected also to intact rats (normal rats). Seven days after the operation the extent of iron-induced lipid peroxidation in both the hemicortices and the effect of the drug, were assessed by the evaluation of lipid-soluble fluorescence and of conjugated diene formation. The assessment was performed both in vehicle (control) and in U74500A-treated rats. In the iron-injected rat groups the drug induced a significant dose-related reduction of fluorescence values. Formation of conjugated dienes showed a significant decrease when U74500A (48 mg/kg every 48 hr) was administered to cortico-cerebrally iron-injected animals. The lipid peroxidation of cortices in normal rats was evaluated as thiobarbituric acid reactant substances in both the drug-treated and the control animals. In normal rats, U74500A (48 mg/kg every 48 hr) caused a significant decrease of TBARS values, as compared to those observed in the control group. The iron content in the iron-injected hemicortices, which was evaluated by the ferrozine method, was not modified by drug treatment. U74500A appears to have *in vivo* antioxidant properties and not to affect the iron content in the neural tissue. An interaction of this drug with the metal, however, cannot be excluded.

Key words: 21-aminosteroid; brain; lipid peroxidation; iron; fluorescence; conjugated diene

A considerable body of experimental evidence indicates that lipid peroxidation [1, 2], the presence of iron [3, 4] and the depletion of natural antioxidants [5] seem to be a common epiphenomena of some pathologies in the CNS†. The role of iron (either free or complexed) in catalysing oxygen derived free radical production and consequently in the peroxidative process [6–8], is well known, even though the involvement of this biochemical pathway with the pathogenesis of neuro-pathologies still remains unclear.

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 [9–12], but only some of them seem to be efficient for the activity *in vivo* [13, 14].

Recently a family of steroid compounds, 21-aminosteroids, was developed and although it derived from glucocorticoids, it lacked glucocorticoid and mineralcorticoid activities [9]. These compounds were shown to scavenge lipid peroxyl radicals and to inhibit iron-dependent lipid peroxidation [15, 16]; moreover they were observed to improve survival, to preserve neurons and to reduce cerebral oedema in animal models of focal cerebral ischemia [17, 18].

Among these, U74500A is able to decrease TBARS formation in rat brain homogenates which are incubated in the presence of iron [15].

This aminosteroid was reported to inhibit the cytotoxicity and lipid peroxidation of iron-loaded cultured endothelial cells which had been submitted to an exogenous or endogenous oxidant attack [19]. Moreover this compound reduces hydrogen peroxide generation by stimulated human polymorphonuclear leukocytes and decreases both, chemiluminescence and hydrogen peroxide, produced by monocytes that are harvested from the blood of patients affected by multiple sclerosis [20, 21].

No reports about the activity of U74500A *in vivo* are yet available. The aim of our study was to assess a possible inhibitory activity of U74500A on a slow lipid peroxidation process induced *in vivo* in the rat brain cortex by iron-saccharate [22]. Lipid soluble

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† Abbreviations: CNS, central nervous system; TBARS, thiobarbituric acid reactant substances; PEG, polyethyleneglycol; MDA, malondialdehyde; U74500A, pregna-1,4,9(11)-triene-3,20.-dione, 21-(4-(5,6-bis(diethylamino)-2-pyridinyl)-1-piperazinyl)-16-methyl-, HCl (16α)).

Table 1. Effect of U74500A administration on the fluorescence of lipid-soluble extracts in rat brain cortex seven days after iron-injection

	U74500A administration schedule (mg/kg) Time after iron–saccharate injection			
	First day		Following six days	
Rat group	Time 0	After 30 min	Every 24 hr	Every 48 hr
A	2.5(a)	9.5(b)		12(b)
B	5(a)	19(b)		24(b)
C	10(a)	38(b)		48(b)
D	10(a)	38(b)	48(b)	

	Results Fluorescence evaluation (U/g wet weight/mL)			
Rat group	Iron-injected (control) Ipsilateral	Contralateral	Iron-injected + U74500A Ipsilateral	Contralateral
A	68 ± 3.3	61 ± 2.8	59 ± 1.8*	55 ± 2.7
B	67 ± 3.0	60 ± 2.7	55 ± 1.9†	41 ± 3.9‖
C	49 ± 3.5	37 ± 3.7	37 ± 3.4‡	28 ± 2.2¶
D	60 ± 3.1	55 ± 3.2	35 ± 3.1†	38 ± 4.3‖

(a) Citrate buffered saline solution pH 3, s.c. administration; and (b) Retard preparation (1.4% polyethyleneglycol 4000 aqueous sol.), s.c. administration.
* $P < 0.05$; † $P < 0.005$; and ‡ $P < 0.001$ significant versus ipsilateral iron-injected values.
‖ $P < 0.02$; and ¶ $P < 0.01$ significant versus contralateral iron-injected values.
The values are means ± SEM.

fluorescence compounds, conjugated diene formation, TBARS and the iron content were measured in order to investigate the scavenging and/or chelating activities of this drug in our model.

MATERIALS AND METHODS

Materials. Iron–saccharate (Haussman, CH); 21-aminosteroid U74500A (kindly supplied by Upjohn, U.S.A.); 1.1.3.3. tetramethoxypropane, quinine sulfate, bovine serum albumin, (the Sigma Chemical Co., MO, U.S.A.); ferrozine (Boehringer, F.R.G.). All the reagents were of the highest analytical grade commercially available.

Experimental conditions. Wistar rat group (250–300 g), anesthetized with 20 mg/100 g ketamine HCl i.p., placed in a small animal stereotaxic apparatus, were subjected to exposure of brain dura by drilling the right calvarium 1 mm posterior and 3 mm lateral to the bregma. Ten microliters of solution containing 3.3 mg iron–saccharate (200 μg of Fe^{3+}) were injected 1.6 mm below the exposed dura [22]. Each iron-injected rat was randomly assigned to either the vehicle treatment (control group) or U74500A treatment groups. Then at least five animals were used for each type of experiment.

Soon after injury a dose of U74500A, that had been dissolved in citrate buffer saline vehicle (0.02 M citric acid monohydrate, 0.0032 M sodium citrate dihydrate, 0.077 M NaCl, pH 3), was administered i.p. or s.c. to rat groups (1st group: 12 mg/kg every 24 hr i.p.; 2nd and 3rd group: 48 mg/kg every 48 hr, respectively i.p. and s.c.).

Other rat groups received s.c. one fifth of the total dose (loading dose) of 21-aminosteroid dissolved in the citrate vehicle. Half an hour later, they received the remaining part of the drug, now dissolved in PEG 4000 (1.4%) aqueous solution (retard preparation). The drug treatment was repeated every 48 hr for the following 6 days by s.c. injections (the last 24 hr before sacrifice) with a total dose of U74500A retard preparation (groups A, B and C in Table 1). A group of rats received 48 mg/kg every 24 hr as described (Group D in Table 1). In order to evaluate the conjugated diene formation another group was treated like the group C.

Normal rats (i.e. not cortico-cerebrally injected rats) were treated with 48 mg/kg of PEG dissolved aminosteroid every 48 hr for 7 days.

Seven days after the brain iron injection, the rats were killed by decapitation and the brain was removed and placed on an ice-cold platform for dissection. Cortical samples (100–150 mg) were obtained from both, ipsilateral side (this included the iron-injection site) and contralateral side of the brain. The measurement of the lipid soluble fluorescence, the conjugated diene formation and the iron content were performed. TBARS were evaluated in the cerebral cortices of normal rats 7 days after the first drug treatment. Because of the variability of absolute lipid peroxidation levels that were previously found among the rat groups derived from different lots [12, 22], each experimental group was compared with its matched control group.

Lipid soluble fluorescence measurement. The method of Fletcher *et al.* [23] was followed with minor modifications. In order to extract lipid soluble fluorescent materials the cortical samples were

weighed and homogenized (10% w/v) at 0° for 20 sec with a chloroform:methanol mixture (2:1 v/v). After the addition of distilled water to the homogenate (1:1 v/v), each test tube was vortexed, placed in ice for 20 min and then centrifuged at 1900 *g*, at 0° for 5 min. The chloroform layer was separated and 1 mL was placed into a quarz cuvette and 0.1 mL of methanol was added. The fluorescence of the organic extracts was determined in a Perkin-Elmer 650-10s apparatus, calibrated to read 11–16 fluorescence units (excitation 370 nm; emission 430 nm) against a quinine sulfate standard solution (0.1 μg/mL 0.05 M H_2SO_4). Data were expressed as units of fluorescence/g wet weight/mL extract.

In order to evaluate the possible influence of the drug on the fluorescence determination, an analysis was performed in the presence of 1 mM U74500A.

Measurement of conjugated dienes. Cortical brain samples, homogenized at 0° in 4% NaCl (10% w/v) were extracted with 8 volumes of chloroform: methanol (2:1) mixture [24]. Aliquots of chloroform extracts (obtained by centrifugation at 0° for 10 min at 1900 *g*) equivalent to 10 mg of cortical brain were evaporated under an Argon stream. The lipid fraction was redissolved in 1.5 mL spectrograde cyclohexane.

The spectrum between 220 and 350 nm (against cyclohexane blank) was scanned by Perkin-Elmer Spectrophotometer apparatus and the values at 232 nm were considered. The digitized raw spectra were corrected in order to eliminate the background due to Rayleigh scattering [25]. A molar absorption coefficient of 2.52×10^{-4} M was applied [26]. Conjugated-diene formation was evaluated in the iron saccharate-injected hemicortices and in the contralateral parts of U74500A treated rats and of controls (drug-untreated) animals. Moreover, the analysis was performed on both the hemicortices of sham-operated (saccharose injected) rats. The values of dienes in iron-injected rats were calculated by subtraction at 232 nm, respectively, of the average values obtained from ipsilateral and contralateral specimens of sham-operated animals. Conjugated-dienes were expressed in μmol/g wet weight of brain tissues.

An analysis was performed after addition of 1 mM U74500A to the cerebral homogenate in order to verify the possible influence of the drug on conjugated diene determination.

Measurement of TBARS. Evaluation of TBARS was performed according to Aruoma *et al.* [27] with minor modifications. 0.2 mL brain cortex homogenate (10% in phosphate buffer pH 7.4) were placed in test tubes with 0.5 mL of TBA (1% w/v 0.05 N NaOH) and 0.5 mL HCl (25% v/v). The tubes were sealed with screw caps and boiled in a water bath for 10 min. The pink chromogen that developed was extracted with 3 mL of *n*-butanol and then centrifuged at 2000 *g* for 10 min. The absorbance of the supernatant was read at 532 nm and the values were determined by a standard curve of 1,1,3,3-tetramethoxypropane. The values were expressed as nmol MDA/mg protein.

Protein assay. The protein concentrations were determined according to Lowry *et al.* [28]. Bovine serum albumin was used as standard reference.

Iron measurement. The iron evaluation was performed using the ferrozine method [3]. Cortical samples (about 50 mg) were homogenized in 1 mL 0.003 N HCl containing 0.1% pepsine and 50 μL 8 mM ferrozine. Granulated ascorbic acid (20 mg) was added for reduction of iron Fe^{3+} to Fe^{2+}. The mixture was incubated for 30 min at 37° and centrifuged at 10,000 *g* at 4° for 15 min. The absorbance of the supernatant was read at 578 nm against a blank sample after 30 min. The values were calculated by an iron standard curve and expressed as μg/g wet weight.

In order to evaluate the possible influence of the drug on iron-ferrozine chromogen, 1 mM U74500A was added to the homogenizing mixture and iron determination was performed as described.

Statistical method. Data are presented as mean values ± SEM. Student's *t*-test was used and a probability value of $P < 0.05$ was regarded as significant.

RESULTS

When the buffer citrate-soluted drug (12 mg/kg every 24 hr i.p. or 48 mg/kg every 48 hr s.c. or i.p.) was administered to the iron-injected animals, the lipid soluble fluorescence levels in the ipsilateral and contralateral hemicortices (1st group: 43 ± 3 and 34 ± 1.9; 2nd group: 54 ± 3.1 and 47 ± 2.8; 3rd group: 64 ± 5 and 52 ± 5 U/g wet weight/mL, respectively), were not found to be significantly different from those found in the respective controls (1st group: 44 ± 2.6 and 33 ± 2.1; 2nd group: 54 ± 4.1 and 46 ± 3.5; 3rd group: 59 ± 3.6 and 43 ± 4.5 U/g wet weight/mL).

On the contrary 12 mg/kg U74500A in the retard preparation, that had been administered s.c. every 48 hr for seven days, was enough to significantly reduce lipid soluble fluorescent substance formation in the iron-injected hemicortices, without affecting it in contralateral parts (rat group A, Table 1).

When higher doses of 21-aminosteroid were administered (groups B and C), a significant decrease in fluorescence values was found in both ipsilateral and contralateral samples in comparison to the analogous parts of controls (Table 1). Moreover in the same groups a significant difference between ipsilateral and contralateral parts of drug-treated rats ($P < 0.01$ and $P < 0.05$, respectively, in groups B and C) was observed.

When 48 mg/kg of the same preparation was administered more frequently (every 24 hr) an even greater effect was observed (group D, Table 1) with no significant difference between ipsilateral and contralateral samples.

In all experiments the iron-injected drug-untreated rats gave fluorescence values of operated parts that were significantly higher than those of the contralateral parts (P values were always at least less than 0.05).

In a previous study we observed that the fluorescence levels of contralateral hemicortices did not differ significantly between iron-injected and sham-operated rats [22], giving values close to those found in normal animals (unpublished data).

U74500A does not affect the determination of

Table 2. Effect of U74500A (48 mg/kg every 48 hr) on the conjugated diene formation in the rat brain cortex seven days after iron–saccharate injection

	Conjugated diene (μmol/g wet weight tissue)	
	Ipsilateral	Contralateral
Control	0.246 ± 0.067	0.047 ± 0.023‡
Drug-treated	0.012 ± 0.027*	−0.095 ± 0.047†

The values are means ± SEM. of at least six animals.
* $P < 0.01$; and † $P < 0.05$ significant versus control.
‡ $P < 0.01$ significant versus ipsilateral.

fluorescence since when 1 mM drug was added to the homogenizing mixture the fluorescence levels were found not to be significantly modified. In fact, the difference in fluorescence value was not higher than 0.18 ± 0.03 U/g wet weight/mL in four determinations in comparison to the control.

U74500A in retard preparation (48 mg/kg administered every 48 hr for seven days) was also able to inhibit conjugated diene formation in both ipsilateral and contralateral parts of the corticocerebrally iron-injected animals (Table 2). The digitized raw spectra, obtained from sham-operated animals and purified by the removal of background due to Rayleigh scattering, showed at 232 nm, a mean extinction of 0.202 ± 0.012 and of 0.195 ± 0.014 for the ipsilateral and the contralateral hemicortices, respectively. The extinction value obtained in the contralateral part of iron-injected drug-untreated animals was similar (0.207 ± 0.006). Adding U74500A (1 mM) before chloroform extraction gave results close to those obtained in the experimental tests.

Normal rats, subjected to treatment with 48 mg/kg U74500A in the retard form every 48 hr for seven days, showed a significant ($P < 0.001$) decrease of MDA in the TBARS assay in comparison to untreated animals (1.74 ± 0.05 versus 2.12 ± 0.06 nmol MDA/mg protein; N = 6 in each group).

The total iron content, measured in both iron-injected and contralateral brain hemicortices, was not significantly different between the drug treated (48 mg/kg every 48 hr for seven days) and control animals (respectively, 24.5 ± 1.4 and 26.1 ± 0.9 μg/g wet weight in the iron-injected part, 25.1 ± 1.2 and 23.6 ± 0.9 μg/g wet weight in the contralateral part), neither was the difference significant between the ipsilateral and the contralateral samples in both groups.

Furthermore, adding 1 mM U74500A to the homogenates did not interfere with the iron content measurement.

DISCUSSION

Using our *in vivo* model, U74500A, following administration in the retard form, showed an inhibitory effect on lipid peroxidation in the iron-injected brain hemicortices. Indeed the levels of fluorescent substances resulted significantly lower in all groups of treated rats in comparison to their respective controls.

Lipid soluble fluorescence substances are considered to be produced by the reactions of degraded lipids (in particular aldehydes) with the amino groups of proteins [29]. Moreover they are reported to be an index of accumulation of stable oxidation end-products, such as, an epiphenomena of an oxygen free radical attack [30, 31].

The effect of U74500A in iron injected hemicortices appears to be dose-related, since fluorescence values were reduced by 13, 18, 25 and 42% as compared to their respective controls at doses of 12, 24, 48 mg/kg every 48 hr and 48 mg/kg every 24 hr, respectively. The dose–response relationship is also arguable due to the fact that differences between ipsilateral and contralateral parts of drug-treated animals showed the same trend. Significant levels were not attained because of unequal effects in A and D groups: the lowest dose (A group) was not enough to affect the basal fluorescence levels in the contralateral hemicortices, whereas the highest dose (D group) markedly decreased lipid soluble fluorescent substances in both iron-loaded and unoperated hemicortices, possibly because of the shorter interval of administration. With middle dosages (B and C groups) significant differences between the hemicortices were observed, as also in the drug-treated animals, as a result of the effects on both parts of the brain cortex.

On the contrary, the aminosteroid dissolved in the buffer 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 (according to J. M. McCall, CNS Research, Upjohn, personal communication). Therefore 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.

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 [32]. In our model, the diene measurement showed a clear decrease in iron-injected drug-treated rats, thus indicating that, in retard form, U74500A inhibits lipid peroxidation at a step prior to diene conjugation.

Thus, the drug also appears to be efficient in iron-unloaded brain tissue, as indicated by the decrease of both lipid soluble fluorescence and diene formation in the contralateral part of iron-injected cortices. This action is analogous to that shown by D-penicillamine [12, 22].

In order to verify whether the 21-aminosteroid has any antioxidant activity even in the undamaged brain tissue, the drug was tested in unoperated rats. In these animals the measurement of TBARS formation gave a significant difference between drug- and vehicle-treated animals: this indicates that in the tissue there is a presence of an antioxidant compound in concentration that is able to inhibit the lipid peroxidation developed during the aerobic procedure of the *in vitro* assay.

It is reported that this drug was as potent as α-tocopherol in inhibiting iron-dependent lipid peroxidation in intact brain phospholipid membranes

[16]. In our model when α-tocopherol was administered to iron injected rats a repeated treatment before operation was necessary in order to prevent lipid peroxidation [22]. Therefore, in the same experimental conditions, U74500A in retard form seems to have a higher inhibitory activity *in vivo* on lipid peroxidation.

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 UV range in the presence of Fe^{2+} [15] and inhibits *in vitro* iron-dependent lipid peroxidation of intact phospholipid membranes [33]. These observations would appear to contrast with our results. However, it is to be considered that in our model, seven days after the operation, the iron injected into the brain has been almost completely reabsorbed, as shown by us in a previous study (no significant difference was found in comparison to normal animals, Ref. 12). Nevertheless, in this model, D-penicillamine clearly showed chelating or complexing properties, since it was able to decrease the iron content in both iron-injected and normal rats [12].

We can argue that U74500A, although not able to decrease the iron levels in the cortical tissue, can interact with the metal, reducing its catalytic activity, in the action sites where the lipid peroxidative process can be induced.

In conclusion, we have reported here the first evidence that U74500A can inhibit *in vivo* a lipid peroxidative process. To exert its effect the drug needs to be administered in a retard form. Thus, also this compound, analogously to what has been proposed for other 21-aminosteroids [17, 34], can be regarded as a possible therapeutical tool in neuropathologies that are characterized by a peroxidative attack.

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