Acta neuropath. (Berl.) 39, 97 – 100 (1977)

Acta Neuropathologica

Effects of Zuclomiphene in Combination with Triparanol and AY-9944 on Developing Rat CNS Morphology and Biochemistry

Robert B. Ramsey and Vernon W. Fischer

Departments of Neurology and Anatomy, St. Louis University School of Medicine, St. Louis, Missouri 63104, U.S.A.

Summary. Developing rats were injected intraperitoneally twice weekly with a combination of three hypocholesterolemic agents: Zuclomiphene (formerly called *trans*-clomiphene; dosage, 30 mg/kg body weight), Triparanol (30 mg/kg body weight) and AY-9944 (3 mg/kg body weight). Treatment was initiated at 4 days of age. Biochemical and electron microscopic examination was conducted on animals sacrificed at 20 days of age. Cytoplasmic inclusion bodies were not seen in the CNS. Isolated edematous changes were seen in myelinated axons. Analysis of the sterol content of the brain and spinal cords of drug-treated animals indicated the presence of abnormal concentrations of five sterols, desmosterol, 5α-cholesta-7,24-dien-3β-ol, zymosterol (5α-cholesta-8,24-dien-3β-ol), 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) and 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol). Zymosterol and 5α-cholesta-7,24-dien-3β-ol were minor constituents (5 – 7% and 1 – 1.5% of total sterol, respectively). The 7-dehydrosterols represented approximately one-half (44 – 52%) of the total CNS sterol.

Key words: Brain – Spinal cord – Hypocholesterolemic agents – Cholesterol – Desmosterol – Lipids – Cytoplasmic inclusion bodies – Zuclomiphene – AY-9944 – Triparanol.

Introduction

It has been suggested that a correlation exists between a high concentration of Δ^7 sterols and the presence of cytoplasmic inclusion bodies in the developing CNS (Suzuki et al., 1974). For this reason, a series of experiments has been conducted to further probe the possibility that these phenomena are related (Ramsey et al., 1975, 1976a, b, c). The findings thus far indicate that the presence of large quantities of Δ^7 sterols is not consistently associated with the demonstration of a large number of cytoplasmic inclusion bodies within the CNS. In the present study, another hypocholesterolemic agent, Triparanol, which also causes accumulation of Δ^7 sterols and cytoplasmic inclusion bodies in the developing CNS (Suzuki et al., 1974), has been tested in combination with two previously utilized hypocholesterolemic agents, zuclomiphene and AY-9944.

Materials and Methods

Animal Experiments. Wistar rats of both sexes were used. Nursing rats were left with their mothers until weaned. Both mothers and weaned rats were maintained on standard laboratory diet.

A combination of 30 mg zuclomiphene per kg of body weight, 30 mg of Triparanol per kg of body weight and 3 mg of AY-9944 per kg body weight, dissolved in propylene glycol-water (2:1, v/v), was injected twice weekly beginning at 4 days postnatally. Controls were given a corresponding quantity of propylene glycol-water only. A total of 5 injections were administered before the animals were sacrificed at 20 days of age. The CNS of four controls and six experimental animals was examined microscopically. Three control and three experimental rats were used for biochemical analysis.

Microscopic Studies. All animals were perfused via the left cardiac ventricle under Diabutal anesthesia, with 3% glutaraldehyde in Sorensen's buffer at pH 7.2. Representative 1-mm cubes of medulla oblongata, cerebellum and cerebrum were post-fixed in Millonig's osmium tetroxide and subsequently embedded in Epon-araldite. Ultrathin sections, stained with uranyl acetate and lead citrate, were viewed in a Phillips 300 electron microscope. For orientation, Epon sections, 1.5 μm thick, were stained with 1% Methylene Blue.

Adjacent tissue blocks were placed in 10% neutral buffered formalin, embedded in paraffin, and prepared for conventional light microscopic examination.

Biochemical Studies. Lipids of the brain and spinal cords were extracted by the method of Folch et al. (1957). Extracts were taken to dryness under nitrogen and the sterols re-dissolved in ethyl acetate. Measured

amounts of cholestane were added to each sample. Sterol content was determined by means of gas-liquid chromatography (GLC). Samples were analyzed on a 6 foot glass column having a 4 mm inside diameter. The column was packed with 3% OV-17 on Gas ChromQ (100/120 mesh). The phase was purchased from Applied Science Laboratories, Inc., State College, Pa. Operating temperature of the column bath was 265° C. Identification of sterol peaks was made by comparison to standard sterol retention times previously derived (Ramsey et al., 1971, 1972).

GLC analysis of total extracts resulted in incomplete resolution of a number of sterol peaks. To further characterize the sterol fraction, the free sterols were applied to $AgNO_3$ thin-layer chromatographic (TLC) plates, TLC plates were impregnated with $AgNO_3$ and developed by a modification of the method described by Suzuki et al. (1974). Silica gel-G plates were first developed in a 7% aqueous solution of $AgNO_3$. The plates were then air-dried for 30 min and activated at 105° C for 1 h. After samples were spotted, plates were developed in chloroform-acetone (95:5, v/v). Sterol standards were visualized by exposure to iodine. Areas of interest were scraped and eluted with ethyl ether. Sterols purified in this manner were then re-examined by GLC.

Results

Electron Microscopy. Examination of the central nervous system of drug-exposed rats revealed only sparse alterations, limited in extent to neuronal processes. Isolated lesions, in the medulla oblongata, consisted primarily of a dilatation, or ballooning, of axons, associated with distention and fragmentation of the covering myelinated sheath (Fig. 1 a, b, c). Occasionally the expansion of the neuronal process progressed to such an extent that it produced compression and distortion of the adjacent nervous tissue (Fig. 1 c). No other alterations were noted in the CNS of these rats. The brains of untreated (control) animals were free from abnormalities.

Biochemistry. CNS sterol content of drug-treated animals was depressed in comparison to the controls. A more outstanding observation was the profusion of sterols other than cholesterol present in substantial quantities in the brain and spinal cord of drug-treated animals (Table 1). Cholesterol was present in the treated animals' CNS, but constituted less than one-third of the total sterol. Desmosterol, a normal component of the developing CNS, was increased in the treated CNS. Four other sterols not normally present in the developing CNS of the rat, were found in the CNS of the animals treated with the mixture of three hypocholesterolemic agents. Two of these sterols, 7-dehydrocholesterol and 7-dehydrodesmosterol, accounted for approximately 50% of the CNS sterol content. Another sterol containing a Δ^7 double bond but not possessing a conjugated $\Delta^{5,7}$ double bond system, 5α-cholesta-7,24-dien-3β-ol, was also found to be present, but in a relatively small concentration. A fourth sterol, probably a metabolic precursor of the other sterols mentioned above, zymosterol (5α-cholesta-8,24-dien,3β-ol), was also shown to be present in appreciable amounts.

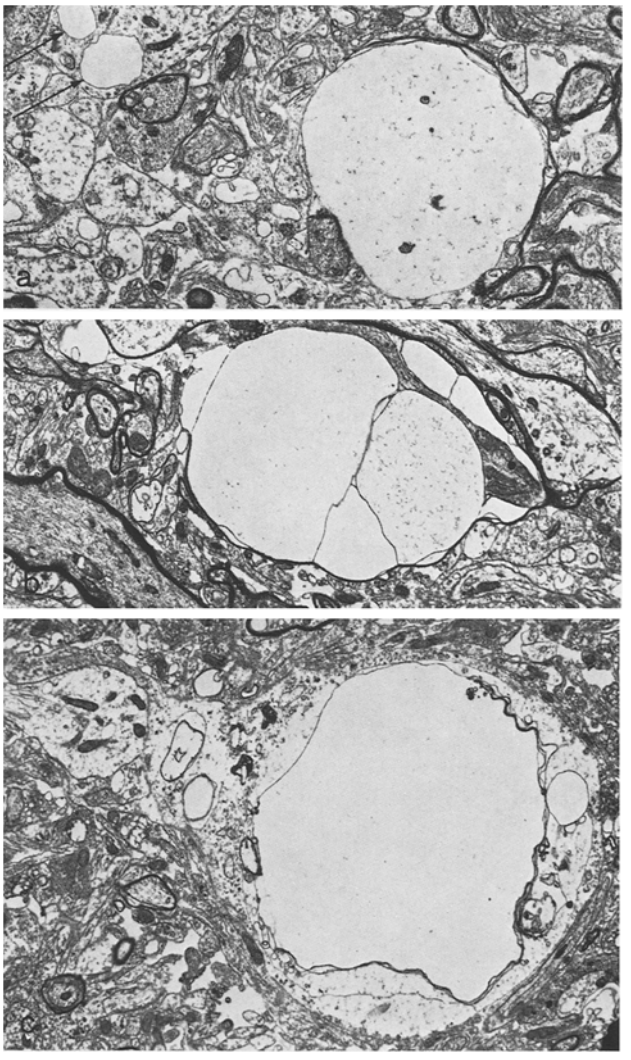

Fig. 1a–c. **a** Inferior medulla oblongata of 20 day old rat treated with zuclomiphene, Triparanol and AY-9944. Note lesions within neuronal processes. Small lesions (arrows) may represent initial changes. ×2920. **b** Inferior medulla oblongata of 20 day old rat treated with zuclomiphene, Triparanol and AY-9944. Axonal "ballooning" and loss of axoplasm is accompanied by splitting of the myelin sheath. ×2920. **c** Inferior medulla oblongata of 20 day old rat treated with zuclomiphene, Triparanol and AY-9944. Severe axonal damage and compression of adjacent tissue is evident. ×2920

Table 1. CNS sterol content of animals injected with zuclomiphene, Triparanol and AY-9944 and corresponding controls

Sterol	Brain				Spinal cord			
	control		drug treated		control		drug treated	
	mg/g dry wt tissue	% of total sterol	mg/g dry wt tissue	% of total sterol	mg/g dry wt tissue	% of total sterol	mg/g dry wt tissue	% of total sterol
Total sterol	41.7 ± 2.67	–	33.30 ± 2.59*	–	57.50 ± 3.55	–	45.60 ± 4.22*	–
Cholesterol	40.0 ± 2.68	95.90 ± 0.15	9.94 ± 0.36***	30.00 ± 1.25	54.80 ± 3.57	95.30 ± 0.76	12.20 ± 1.18***	26.70 ± 0.78
Desmosterol	1.7 ± 0.10	4.06 ± 0.15	6.62 ± 1.80**	19.70 ± 4.07	2.72 ± 0.39	4.73 ± 0.75	5.76 ± 0.23***	12.70 ± 0.94
7-Dehydrocholesterol	–	–	4.97 ± 0.13	15.00 ± 0.81	–	–	10.00 ± 1.74	21.90 ± 1.96
7-Dehydrodesmosterol	–	–	9.65 ± 0.38	29.00 ± 1.32	–	–	13.70 ± 1.01	30.10 ± 0.86
Zymosterol	–	–	1.72 ± 0.28	5.13 ± 0.47	–	–	3.26 ± 0.14	7.13 ± 0.42
5α-Cholesta-7,24-dien-3β-ol	–	–	0.38 ± 0.33	1.20 ± 1.12	–	–	0.70 ± 0.17	1.50 ± 0.30
7-Dehydrosterol content as % of total sterol	–	–	–	44.00	–	–	–	52.00

Values are means ± standard deviations
* $P < 0.02$ ** $P < 0.01$ *** $P < 0.001$

Discussion

Only minimal morphological alteration in the CNS was observed when developing rats were treated with a combination of hypocholesterolemic agents. Cytoplasmic inclusion bodies were not seen, but occasionally edematous changes of axons were observed. In contrast, the hypocholesterolemic agents did cause an abnormal accumulation of sterols containing a Δ^7 double bond, mainly $\Delta^{5,7}$ sterols, in the CNS. Previous proposals had suggested that the presence of large amounts of Δ^7 sterols in the developing CNS might bring about the formation of cytoplasmic inclusion bodies (Suzuki et al., 1974). Indeed, two of the three hypocholesterolemic agents used, AY-9944 and Triparanol, in addition to effecting a build-up of Δ^7 sterols, caused cytoplasmic inclusion body formation when given individually to developing rats (Suzuki et al., 1973, 1974).

The ability to induce cytoplasmic inclusion bodies in the CNS and other tissues is, however, not restricted solely to hypocholesterolemic compounds (Lullmann et al., 1973, 1975). Actually, composition studies of the cytoplasmic inclusion bodies formed by a whole series of compounds have emphasized the accumulation of phospholipid in the bodies rather than the sterol content (Yates et al., 1967; Gray et al., 1971; Hruban et al., 1972; Thys et al., 1973; Schmien et al., 1974; Seiler and Wassermann, 1975). That is not to say that the sterol composition and content may not be important for their evolution; at the moment, however, this question remains unresolved.

The present work would suggest that, in any case, the Δ^7 sterols do not by themselves cause the formation of cytoplasmic inclusion bodies. The bodies seen in the CNS following treatment of developing rats with AY-9944 or Triparanol must result from an action of the drug other than its ability to alter sterol formation. The abnormal accumulation of cholesterol-precursor sterols may add to or accentuate the formation of cytoplasmic inclusion bodies or it may play no active role at all. The actual involvement of sterols in the formation of these bodies remains to be proven.

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Received December 27, 1976 / Accepted February 4, 1977