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Neurosteroids: Pregnenolone in human sciatic nerves

(dehydroepiandrosterone/mass spectrometry/steroid sulfates/steroid fatty acid esters)

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ABSTRACT **The characterization and quantification of pregnenolone in human sciatic nerves were undertaken, following previous demonstration of the synthesis of this steroid in rat brain oligodendrocytes, to explore the hypothesis that Schwann cells may demonstrate the same biosynthetic activity. Pregnenolone was definitively identified by mass spectrometry and quantified by specific radioimmunoassay. Its concentration (mean ± SD, 63.9 ± 45.9 ng/g of wet tissue, $n = 12$) was ≥100 times the plasma level and concentration found in tendons and muscle. No correlation was found with sex or age. Free dehydroepiandrosterone as well as sulfate and fatty acid esters of pregnenolone and dehydroepiandrosterone were also measured. Results are discussed in terms of the concept that these "neurosteroids" may be synthesized in the peripheral nervous system.**

Pregnenolone (3β-hydroxy-Δ5-pregnen-20-one) accumulates in the rat brain independently of endocrine gland function, and its synthesis from cholesterol in oligodendrocytes has been demonstrated (1, 2), justifying the term "neurosteroid" (3). Measurements by radioimmunoassay have indicated that pregnenolone is present also in human brain and cranial nerves at levels similar to those in the rat (i.e., ≈0.1 μmol/g of wet tissue) (4–6). Since oligodendrocytes and Schwann cells can share several metabolic characteristics, including the synthesis of myelin, the question arose whether neurosteroids were components of the human peripheral nerve system.

In absence of any previous information which could help to answer this question, we decided to identify pregnenolone rigorously and to measure it by specific radioimmunoassay in sciatic nerves obtained from humans shortly after death. Sulfate and fatty acid esters of pregnenolone were also determined, as well as free and esterified dehydroepiandrosterone (DHEA; 3β-hydroxy-Δ5-androsten-17-one).

MATERIALS AND METHODS

Chemicals. [1, 2, 6, 7-^{3}H]DHEA (79 Ci/mmol; 1 Ci = 37 GBq) and [7-^{3}H]DHEA sulfate ammonium salt (16.3 Ci/mol) were obtained from New England Nuclear. [7-^{3}H]Pregnenolone (12.7 Ci/mmol) was purchased from Amersham. This steroid was used for the microscale synthesis of [7-^{3}H]pregnenolone sulfate (7). The purity of each ^{3}H-labeled steroid was checked by thin-layer chromatography and purification was carried out when necessary with the system previously reported (4, 7). Unlabeled pregnenolone, pregnenolone sulfate, DHEA, DHEA sulfate, and cortisol were generous gifts from Roussel-Uclaf. All solvents were of analytical grade and were purchased either from Merck or Carlo Erba (Milano, Italy).

Tissues. Tissues were obtained <48 hr after death from cadavers of both sexes bequeathed to medical research. Corpses were kept refrigerated at 4°C. Ages ranged from 44 to 90 years. Causes of death were not recorded. Portions of sciatic nerves, tendons, and muscle were taken close to the knee. Samples of spleen were obtained in one case. The collected samples (0.675–4.870 g) were first weighed and then frozen in liquid nitrogen until processed further.

Extraction and Purification Procedures. Frozen samples in liquid nitrogen were first pulverized in a mortar and then homogenized in phosphate-buffered saline at 4°C. The procedure used for all tissue samples included extraction of free and fatty acid-esterified steroids with isooctane/ethyl acetate, 1:1 (vol/vol) (4) and defatting of the extracts by partition between isooctane and 90% methanol (8). The fatty acid esters of the steroids were present in isooctane. Chromatography of the recovered 90% methanol material, once dried and redissolved in fresh 90% methanol, was done on C_{18} Amprep reverse-phase cartridges (Amersham). From the latter, elution with 90% methanol yielded the free steroids. Saponification was performed under nitrogen in 1 ml of a 95:5 (vol/vol) mixture of 95% (vol/vol) ethanol and 40% (wt/vol) KOH for 1 hr at 80°C followed by 12 hr at room temperature. To measure the sulfated steroids present in the aqueous phase after isooctane/ethyl acetate extraction, solvolysis was performed in three cases as follows: the aqueous phase, once brought to pH 1 with sulfuric acid and made 20% NaCl, was extracted three times with ethyl acetate and the pooled extracts were incubated at 37°C for 16 hr. Further processing was carried out according to Burstein and Lieberman (9). Free steroids recovered in each of the free, saponified, and solvolyzed fractions were separated by partition chromatography on celite microcolumns before radioimmunoassay (4, 10). The addition of ^{3}H-labeled steroids allowed appropriate corrections to be made for losses during the isolation procedures.

Radioimmunoassays. Pregnenolone and DHEA and their respective sulfate and fatty acid esters were measured by radioimmunoassays using specific antibodies (5, 11). Measurements were made in triplicate on three dilutions of the fractions, thus allowing the construction of regression lines (measured picograms versus dilution) whose slopes gave the concentrations of steroids in the samples. Results were expressed as nanograms of steroid (free or released from conjugates) per gram of tissue, and standard deviations were calculated.

Gas Chromatography–Mass Spectrometry (GC–MS). Unconjugated pregnenolone from sciatic nerves was identified by GC–MS. After individual measurements by radioimmunoassay of the fractions from the celite partition chromatography, the remaining unconjugated pregnenolone from three

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Abbreviation: DHEA, dehydroepiandrosterone.

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sciatic nerves was pooled. The amounts of pregnenolone indicated by radioimmunoassay were 8.05, 8.93, and 8.27 ng in the free, solvolyzed, and saponified fractions, respectively. To each of the three samples was added 40,000 dpm of [7-^{3}H]pregnenolone (0.158 ng). The dried samples were dissolved in aqueous 95% methanol and passed through a small bed of the anion exchanger TEAP-LH-20 in OH^- form to remove acids (12). The neutral fractions were taken to dryness and converted to trimethylsilyl ether derivatives (12). The reagents were removed under a stream of nitrogen and the trimethylsilyl derivatives were dissolved in 30 μl of hexane.

GC–MS was carried out using a VG 7070E double-focusing mass spectrometer, a Dani 3800 gas chromatograph, and a VG 11-250 data system (VG Analytical, Manchester, U.K.). The column (25 m × 0.32 mm internal diameter), a fused silica capillary coated with cross-linked methyl silicone (film thickness, 0.25 μm; Quadrex, New Haven, CT), was directly connected and extended into the ion source. Samples were injected using an all-glass falling-needle system. The ion source was kept at 250°C, the accelerating voltage was 6 keV, the ionization energy 70 eV, and the trap current 200 μA. Spectra were taken by repetitive magnetic scanning of the range m/z 400–100 at a scan rate of 2 sec per decade (0.5-sec interscan time) and a resolution of 1000 (5% valley). Suitable conditions were established with the trimethylsilyl ether of authentic pregnenolone. The retention time was about 10 min at 230°C. Identification of pregnenolone trimethylsilyl ether from the complete mass spectrum and the ion chromatograms of m/z 388 (M), 332 (M − 56), 298 (M − 90), 259 (M − 129), and 129 was achieved by injection of 0.44 ng, whereas 0.15 ng was identified by the retention times of the peaks in the chromatograms of m/z 298, 259, and 129.

RESULTS

Characterization of Pregnenolone. The preliminary identification of pregnenolone (either free or released from its sulfate or fatty acid esters) was based on (*i*) partition chromatography on celite columns and isolation of eluates containing pregnenolone and (*ii*) radioimmunoassay using a specific antiserum having minimal cross-reaction with other steroids. Following the group separation by partition and ion-exchange chromatography, the fractions containing pregnenolone included large amounts of neutral compounds, which interfered in the GC–MS analysis. However, pregnenolone could be definitively identified in the unconjugated form. Peaks having the retention time of pregnenolone trimethylsilyl ether appeared in all the chromatograms of ions characteristic of pregnenolone trimethylsilyl ether and its 3β-trimethylsiloxy-5-ene structure. Except for a slight increase of m/z 129 due to an adjacent contaminant, the relative intensities of the diagnostically significant peaks were essentially the same for the unconjugated steroid from sciatic nerves and the reference compound (Table 1). Approximative quantitation yielded a value of 10 ng as compared with the value of 8.05 ng by radioimmunoassay. Thus the GC–MS value was consistent with the radioimmunoassay value. The data prove the identity of unconjugated pregnenolone.

Table 1. Relative intensities (% of base peak m/z 129) of diagnostically important mass spectral peaks given by the trimethylsilyl ethers of pregnenolone and of the compound isolated from human sciatic nerves

m/z	Reference pregnenolone	Compound from sciatic nerve
388 (M^+)	9.4	7.5
332 (M − 56)	9.3	8.8
298 (M − 90)	30.9	19.7
259 (M − 129)	22.1	18.7
129	100.0	100.0*

*Intensity slightly increased by an adjacent contaminant.

In contrast, GC–MS evidence for pregnenolone released from its sulfate or fatty acid esters could not be obtained. While interference by contaminants was an important reason, peaks with the retention time of pregnenolone trimethylsilyl ether were at least 5–10 times less intense in the analysis of compounds released from the sulfate and fatty acid esters than in the analysis of unconjugated pregnenolone. Thus, the evidence for the presence of esterified pregnenolone rests on the partition chromatographic behavior on celite and the specificity of the radioimmunoassay. Interference by other steroids is unlikely but nonspecific binding of neutral contaminants may influence the assay.

Concentration of Pregnenolone in Human Sciatic Nerve. Triplicate measurements of pregnenolone were carried out by radioimmunoassay on four sets of dilutions of each of the three isolated fractions (unconjugated fraction, solvolyzed sulfate fraction, saponified fatty acid fraction). Free pregnenolone ranged from 20.4 to 159.9 ng/g with no significant relation to sex or age of the corpses (Table 2). Pregnenolone sulfate in three cases ranged from 13.2 to 39.8 ng/g. Pregnenolone in the saponified fatty acid ester fraction ranged from 13.0 to 45.5 ng/g.

Concentration of DHEA in Human Sciatic Nerve. Free DHEA and DHEA released from sulfate and fatty acid esters were chromatographed on celite microcolumns. The DHEA-containing fraction, completely separated from pregnenolone, was used for radioimmunological measurements of DHEA. Concentrations of DHEA sulfate were much higher than those of free and fatty acid-esterified DHEA (Table 3).

Concentration of Pregnenolone in Other Human Tissues. Tendon, muscle, and spleen tissues derived from the corpse of a 44-year-old woman were collected and processed as sciatic nerve samples. Tendon from a 61-year-old man was also studied. Results were compared with those obtained for the sciatic nerves (Table 4). In tendons and muscle, concentrations of pregnenolone, pregnenolone sulfate, and DHEA sulfate were markedly lower than in sciatic nerves. In contrast, spleen contained much pregnenolone, pregnenolone sulfate, and DHEA sulfate, actually several times more pregnenolone and pregnenolone sulfate than in sciatic nerve. However, the concentration of pregnenolone esterified by fatty acids was of the same order of magnitude in all tissues.

Table 2. Concentrations of free pregnenolone, pregnenolone sulfate, and pregnenolone fatty acid esters in human sciatic nerves

		Pregnenolone, ng/g		
Age	Sex	Free	Sulfate	Fatty acid esters
59	M	20.4 ± 0.4	ND	31.2 ± 0.4
75	M	54.3 ± 7.3	ND	43.4 ± 1.5
64	M	106.7 ± 8.6	ND	ND
75	M	25.9 ± 0.7	ND	13.0 ± 0.6
51	M	159.9 ± 9.3	ND	ND
58	M	44.5 ± 2.2	21.5 ± 0.8	34.1 ± 1.3
54	M	41.8 ± 1.8	39.8 ± 1.0	38.2 ± 1.2
88	F	48.0 ± 2.0	ND	24.7 ± 0.7
86	F	42.9 ± 0.6	ND	30.2 ± 1.2
51	F	150.9 ± 10.1	ND	ND
61	M	28.1 ± 1.0	ND	45.5 ± 1.3
44	F	43.9 ± 2.7	13.2 ± 0.7	20.6 ± 0.7
		63.9 ± 45.9	24.8 ± 11.1	31.2 ± 10.0

Triplicate measurements were carried out by radioimmunoassay. Values are given as nanograms of pregnenolone per gram of fresh sciatic nerve (mean ± SD). M, male; F, female; ND, not determined.

Table 3. Concentrations of free DHEA, DHEA sulfate, and DHEA fatty acid esters in human sciatic nerves

		DHEA, ng/g		
Age	Sex	Free	Sulfate	Fatty acid esters
59	M	9.2 ± 0.3	88.3 ± 1.1	0.4 ± 0.1
75	M	3.6 ± 0.2	150.5 ± 4.2	0.1 ± 0.1
44	F	ND	171.5 ± 5.2	ND

Triplicate measurements were carried out by radioimmunoassay. Values are given as nanograms of DHEA per gram of fresh sciatic nerve (mean ± SD). ND, not determined.

DISCUSSION

Since pregnenolone and DHEA and their sulfate and fatty acid conjugates have been characterized in the rat brain and measured in human brain, it was of interest to see whether they were also present in human peripheral nerves. Schwann cells share several developmental and metabolic characteristics with oligodendrocytes, and in the rat these myelin-forming cells can synthetize pregnenolone from cholesterol (1).

In this work, pregnenolone was definitively identified in human sciatic nerves obtained from corpses within 48 hr after death. Pregnenolone was measured by specific radioimmunoassay as a free compound, and also in fractions obtained after solvolysis and saponification, containing sulfate and fatty acid ester conjugates, respectively. In addition, DHEA and its conjugates were measured in a few sciatic nerves. Steroids in other tissues were also measured in two cases, for tentative quantitative comparison.

The identification of free pregnenolone isolated from collected nerves was based upon four criteria: (*i*) identical retention volume on celite partition chromatography, (*ii*) detection and measurement by a specific radioimmunoassay, (*iii*) identical retention time of the trimethylsilyl ether derivative upon capillary column GC, and (*iv*) identical pattern of diagnostically important ions upon GC–MS analysis. In addition, the quantities measured by radioimmunoassay and by GC–MS agreed within 20%. Identification of pregnenolone derived from the sulfate and fatty acid ester fractions obtained from extracts of the sciatic nerves was based only upon the first two criteria.

The concentration of free pregnenolone in 12 sciatic nerves varied from 20.4 to 159.9 ng/g (mean, 63.9 ± 45.9 ng/g). This is much higher than the plasma concentration of free pregnenolone measured by Lacroix *et al.* (5) in subjects of corresponding age, certainly excluding contamination of nerve extract by plasma, and is consistent with either selective accumulation or synthesis in the nerves. However, it is clear that the difference of concentration depends on the basis of the calculation and would be reduced if expressed per gram of fat, for instance. The pregnenolone concentrations in human sciatic nerves are in the same range as those reported in human (5, 6) and rat (4) brain. Myelination in the peripheral nervous system is carried out by Schwann cells in the place of oligodendrocytes, and it is possible that Schwann cells also share with oligodendrocytes the capacity to produce neurosteroids from cholesterol.

For pregnenolone sulfate, the values obtained in three cases are of the same order of magnitude as those of pregnenolone sulfate determined in the plasma in younger adults (13–15). Such data are not in favor of contamination by plasma pregnenolone sulfate, and this is even more so with the very low values found in tendons and muscle.

The concentration of pregnenolone fatty acid esters in nine sciatic nerves (31.2 ± 10.0 ng/g) is not significantly different from that found in other tissues but is much higher than that found in plasma (five determinations in two men and two women between 45 and 65 years of age: 5.2 ± 0.8 ng/ml; C.C., unpublished data). These levels are similar to those previously reported in rat and monkey brain and plasma (10). The mode of biosynthesis and the biological significance of steroid fatty acid esters are still unknown.

Free DHEA and DHEA sulfate concentrations in sciatic nerves average 6.4 ± 2.8 ng/g and 136 ± 35.3 ng/g, respectively. Free DHEA concentration in sciatic nerves is higher than that reported in human plasma (5, 16) and is distinctly lower than that of free pregnenolone in the brain. Whether or not nerve DHEA derives from nerve pregnenolone cannot be deduced from these results, as in the case of brain steroids (4, 17). The very high concentration of DHEA sulfate in human plasma (16) may explain some contamination of nerve extracts. However, the very small amounts of DHEA sulfate found in muscle and tendon tissues suggest that sciatic DHEA sulfate is not a plasma contaminant but is formed or accumulated in the nervous tissue. The very low value of DHEA in the fatty acid esterified fraction is curiously in contrast with the value of pregnenolone fatty acid esters, a result also found in the rat brain (18). A large discrepancy was observed between radioimmunoassays and GC–MS determinations of steroids released from sulfate and fatty acid ester fractions. Full caution should be exerted in interpreting concentrations of these pregnenolone and DHEA derivatives in all types of biological materials.

The preliminary determinations of pregnenolone in other tissues are intriguing. In tendons and muscle, values of "neurosteroids" are very low, with the exception of pregnenolone fatty acid ester. Values found in the spleen, in contrast, indicate high levels of pregnenolone, pregnenolone sulfate, and DHEA sulfate. At least for pregnenolone, the high concentration in spleen cannot be explained by plasma contamination and indeed is also consistent with previous findings in rat spleen (4). The significance of these high levels of pregnenolone is not known.

Table 4. Tissue concentrations of pregnenolone and DHEA

			Pregnenolone, ng/g			
Age	Sex	Tissue	Free	Sulfate	Fatty acid esters	DHEA as sulfate, ng/g
61	M	Tendon	3.7 ± 0.4	1.7 ± 0.4	29.3 ± 1.8	7.5 ± 2.1
		Sciatic	28.1	ND	45.5	ND
44	F	Tendon	8.0 ± 1.1	3.6 ± 0.5	50.8 ± 2.3	6.9 ± 2.0
		Muscle	3.3 ± 0.2	5.2 ± 0.6	17.4 ± 2.1	3.0 ± 0.2
		Spleen	120.0 ± 8.8	83.2 ± 2.7	36.8 ± 2.7	233.9 ± 12.5
		Sciatic	43.9 ± 2.7	13.2 ± 0.7	20.6 ± 0.7	171.5 ± 5.2
Mean		Sciatic*	63.9 ± 45.9	24.8 ± 11.1	31.2 ± 10.0	136.8 ± 35.3

Tissue concentrations are given as nanograms of free steroid per gram of fresh tissue (mean ± SD). ND, not determined.

*Mean concentrations from Tables 2 and 3 are given for comparison.

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