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Communications to the Editor

Sustained Brain-Specific Delivery of Estradiol Causes Long-Term Suppression of Luteinizing Hormone Secretion

Sir:

We have recently demonstrated that drugs can be specifically delivered to the central nervous system (CNS) with use of a dihydropyridine ⇌ pyridinium salt redox carrier system.[1-3] Application of this drug delivery system to drugs that do not readily cross the blood–brain barrier (BBB), such as simple amines, catecholamines, and more complex ionized molecules, has resulted in selective and sustained delivery of these drugs to the brain.[1,2] While most steroid hormones readily pass into and out of the brain due to their high lipophilicity, the necessary blood–brain equilibrium has limited their usefulness in treating brain-specific, steroid-deprivation syndromes, such as hot flushes.[4] Also, the use of steroidal hormones to achieve reduction in gonadotropin secretion for contraception and the treatment of steroid-dependent diseases such as endometriosis[5] and prostatic hypertrophy[6] is limited by the unwanted peripheral side-effects of these hormones.[7]

For these reasons, we have evaluated this redox system for the specific delivery of steroids to the CNS. Application of the redox system to testosterone resulted in the accumulation of the ionized form of the chemical delivery system in the brain and persistent, local release of the active steroid.[3] We presently report that estradiol can be selectively delivered to the brain and causes sustained suppression of gonadotropin secretion in rats, without an elevation in peripheral circulating concentrations of estradiol.

3-Hydroxy-17β-[[(1-methyl-1,4-dihydropyridin-3-yl)-carbonyl]oxy]estra-1,3,5(10)-triene (estradiol 17-(1,4-dihydrotrigonellinate), compound **2**, Figure 1) was synthesized by a modification[8] of the method described previously for testosterone.[3] This lipid-soluble drug–carrier combination when administered in vivo readily crosses the blood–brain barrier and is converted to the corresponding hydrophilic quaternary pyridinium salt (compound **3**, Figure 1), thus dramatically reducing its rate of egress from the CNS. Subsequent hydrolysis of **3** results in the release of the steroid (compound **1**) from the "locked-in" delivery system, providing elevated and sustained brain concentration of estradiol (compound **1**).

Successful delivery and subsequent sequestering of the delivery system into the brain require that the oxidation of the dihydropyridine moiety in compound **2** to the quaternary pyridinium ion as in compound **3** occurs faster in brain and other tissues than potential hydrolysis of compound **2**. Thus we determined the rate of oxidation of **2** in the plasma and in homogenates of liver and brain tissue. Pseudo-first-order rate constants for oxidation of **2** were observed for all tissues.[9] The rate of oxidation in liver ($k = (3.87 \pm 0.4) \times 10^{-4}$ s^{-1}) and brain ($k = (4.03 \pm$

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(8) The 3, 17β-dinicotinate ester of estradiol was made by refluxing 17β-estradiol with nicotinoyl chloride or nicotinic anhydride in pyridine. This derivative was selectively hydrolyzed to the 17-monoester of estradiol with potassium bicarbonate in 95% methanol. The monoester of estradiol was then quaternized with methyl iodide. The delivery system, **2**, was then prepared by reduction of the obtained **3** with $Na_2S_2O_4$. The structure of each intermediate and the final product (compound **2**, Figure 1) was confirmed by nuclear magnetic resonance and elemental analysis: mp 115–130 °C dec; NMR ($CDCl_3$) δ 7.0–6.8 (m, 2 H, C-1 E_2 proton + C-2 pyridine H), 6.7–6.4 (m, 2 H, C-2, 4 E_2 protons), 5.7–5.4 (br d, 1 H, C-6 pyridine H), 5.0–4.5 (m, 3 H, C-17α E_2 + C-5 pyridine + phenolic OH (exchangeable), 3.2–3.0 (m, 2 H, C-4 pyridine protons), 3.0–2.9 (s, 3 H, NCH_3), 2.8–1.1 (m, 15 H, E_2 skeletal H's), 1.0–0.9 (s, 3 H, C-18 E_2 protons). The yields at each synthetic step were 64–94%.

(9) Freshly obtained human plasma was diluted with an equal volume of phosphate buffer. Two grams of fresh rat liver or brain tissue was homogenized in phosphate buffer and diluted with the same buffer to a final concentration (tissue weight/buffer volume) of 2% or 4%, respectively. A solution of compound **2** (5×10^{-5} M) was added to the homogenates, maintained at 37 °C and pH 7.4 in a UV cell, and the rate of disappearance of the dihydronicotinate absorbance peak at 359 nm was determined. The half-lives (minutes) and correlation coefficients for the oxidation of **2** were 156.6 and 0.983 for plasma, 29.9 and 0.991 for liver, and 29.2 and 0.997 for brain. For all tissues, the rate constant for oxidation of compound **2** was determined from the slope of the log of the disappearance curve.

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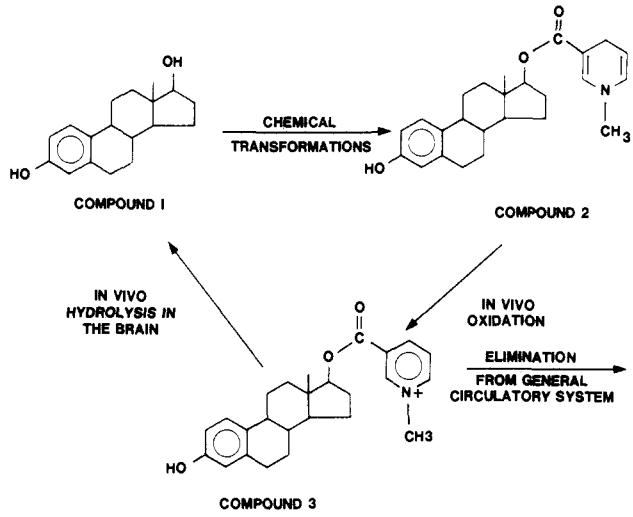

Figure 1. The brain-specific delivery of estradiol (compound 1). Compound 2 is the chemical delivery system, and compound 3 is the oxidized form locked into the brain and quickly eliminated from the rest of the body. The trigonelline (*N*-methylnicotinic acid) formed upon the hydrolysis of compound 3 is nontoxic and is easily cleared from the brain. Thus following iv administration of the simple ethyl dihydrotrigonellinate [1-methyl-3-(ethoxycarbonyl)-1,4-dihydropyridine, MECDP] at a 50 mg/kg dose, the corresponding ethyl trigonellinate ($MECP^+$) is found in the brain at 46 μg/g at 30 min, but due to its hydrolysis to trigonelline (T) and its direct elimination, it disappears from the brain by 6–8 h. The trigonelline formed in situ from $MECP^+$ (peak level 20.4 μg/g at 30 min) has also disappeared from the brain between 12 and 24 h. None of the components of this simple test system can be detected after 24 h. (N. Bodor, E. Shek, and M. El-Kommos, unpublished observation.) Compound 2 was synthesized by a modification[8] of the method described for the synthesis of a redox delivery system for testosterone.[3]

0.59) × 10^{-4} s^{-1}) tissues was 5.2- and 5.5-fold higher, respectively, than in plasma (k = (0.738 ± 0.09) × 10^{-4} s^{-1}). No significant hydrolysis of 2 to 1 to took place under these conditions. This and the observed higher oxidation rate of 2 in the brain than in plasma would contribute to the accumulation of the charged pyridinium salt of estradiol (compound 3) in brain tissue following its intravenous administration of the delivery form 2.

Compound 2 was then administered (60 mg/kg body weight, iv) to male rats,[10] and the distribution of the quaternary pyridinium salt derivative of estradiol (compound 3) in various tissues was determined. While 3 could not be detected in the blood at any sampling time, its concentrations in the brain peaked at 28 ± 1.5 μg/g by 60 min and remained elevated above 14 ± 1.2 μg/g through the last sampling time at 24 h (Figure 2). The calculated half-life of 3 in the brain was 23 h as compared to 0.77, 5.5, and 7 h for 3 in the liver, lung, and kidney, respectively.[11] Hence, 3 was essentially cleared from the liver

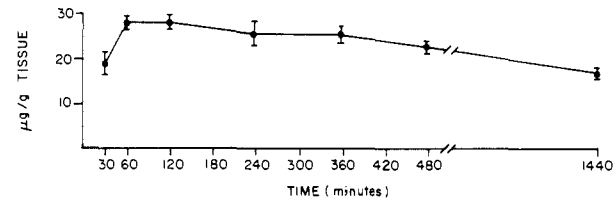

Figure 2. Brain concentrations of the quaternary pyridinium salt containing ester of estradiol (compound 3) following a single intravenous injection of the estradiol chemical delivery system (compound 2). Compound 2 was injected at a dose of 60 mg/kg in a vehicle of 100% dimethyl sulfoxide, and groups of male Sprague–Dawley rats (200–250-g body weight) were killed at various times thereafter. The brains were homogenized in 1 mL of water and extracted with 4 mL of acetonitrile. A 50-μL aliquot of supernatant was injected onto an Altex Ultrasphere C_{18} reversed-phase column. The mobile phase consisted of 70% acetonitrile/30% water with 0.2% of the sodium salt of 1-pentane sulfonic acid as the ion-pairing reagent. At a flow rate of 2 mL/min, the retention time of compound 3 was 4 min. Blood, lung, liver, and kidneys were processed similarly.[20] Each point represents the mean and the vertical bars depict ±1 SEM (five or six animals per data point).

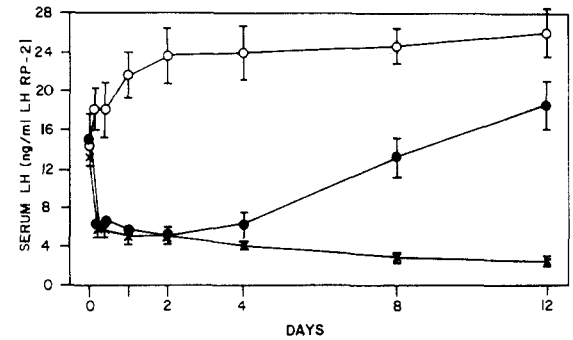

Figure 3. Effects of estradiol chemical delivery system (compound 2) on serum LH concentrations in orchidectomized rats. At 2 weeks after orchidectomy, animals were administered intravenously compound 2 (3.0 mg/kg, x), an equimolar dose of estradiol (2.1 mg/kg, ●), or the dimethyl sulfoxide vehicle (0.5 mL/kg, ○). Blood samples (0.4–0.5 mL) were obtained by cardiac puncture while rats were under light ether anesthesia. Depicted are the mean ± SEM for four to eight animals. LH concentrations are expressed in terms of the reference preparation LH-RP-2 provide with the radioimmunoassay kit from the Pituitary Hormone Distribution Program of the NIADDK.

by 6 h and from the lung and kidney by 24 h, while brain levels of the ionized form of the delivery system remained elevated. These data indicate that compound 2 readily penetrates the BBB and the charged moiety 3 is "locked-in", thus providing the basis for the long half-life of 3 in the brain in association with its rapid clearance from peripheral tissues.

To evaluate the response of luteinizing hormone (LH) to this chemical delivery system, we administered, intravenously, compound 2 (3.0 mg/kg), an equimolar dose of estradiol (2.1 mg/kg), or the dimethyl sulfoxide (Me_2SO) vehicle to male rats at 2 weeks after orchidectomy. Both estradiol and compound 2 reduced serum LH concentrations equivalently by 68–79% from 4 to 48 h (Figure 3). From 4 to 12 days after drug administration, the LH levels in estraiol-treated animals increased progressively to levels equivalent to those in Me_2SO-treated rats. By contrast, LH concentrations in animals treated with 2 continued to be suppressed by 82%, 88%, and 90% when compared to control (Me_2SO) values at 4, 8, and 12 days after treatment, respectively.

To better define the time-course of the suppression of LH by 2, we repeated the aforementioned study and sam-

(10) Male rats of the Sprague–Dawley strain were used in these studies since low circulating estradiol concentrations are found in males [Nisnihara, M.; Takahagni, M. *Biol. Reprod.* **1983**, *29*, 1092. Saksena, S. K.; Lau, I. F. *Exp. Aging Res.* **1979**, *5*, 179. Keel, B. A.; Abney, T. O. *Endocrinology (Baltimore)* **1980**, *107*. 1226. Ewing, L. L.; Desjardins, C.; Irby, D. C.; Robaire, B. *Nature (London)* **1979**, *269*, 409] and this steroid has been shown to be potent in reducing LH secretion in males [Kalra, P. S.; Kalra, S. P. *Endocrinology (Baltimore)* **1980**, *106*, 390. Nisnihara, M.; Takahagni, M. *Biol. Reprod.* **1983**, *29*, 1092].

(11) The half-lives of compound 3 in brain, liver, lung, and kidney were determined by calculating the negative slope of the log of the disappearance rate of 3 following an intravenous dose of 60 mg/kg of compound 2.

Table I. Serum Concentrations of LH and Estradiol after Intravenous Administration of Me_2SO, Estradiol (E_2), or Compound 2 in Orchidectomized Rats[a]

drug	days after drug administration 12	18	24
	serum LH (ng/mL)		
Me_2SO	6.8 ± 0.8 (7)[b]	12.4 ± 2.5 (7)	8.6 ± 1.7 (7)
estradiol (2.1 mg/kg)	12.9 ± 1.7 (6)*[d]	12.1 ± 0.9 (6)	11.6 ± 1.4 (7)
2 (3.0 mg/kg)	0.8 ± 0.7 (7)*	1.7 ± 0.7 (7)*	2.9 ± 1.1 (7)*
	serum E_2 (pg/mL)		
Me_2SO	27.5 ± 3.8 (4/7)[c]	<20 (7/7)	24.4 ± 3.3 (4/7)
estradiol (2.1 mg/kg)	29.3 ± 5.9 (4/6)	<20 (6/6)	31.2 ± 8.6
2 (3.0 mg/kg)	25.9 ± 3.9 (5/7)	<20 (7/7)	24.1 ± 3.2 (5/7)

[a] Animals were killed by decapitation, and serum was assayed for LH by the methods described in Figure 3, while estradiol was assayed by the method described by Lindberg et al.[21] as modified by Schille et al.[22] All samples were assayed in duplicate. [b] Mean ± SEM (number of animals per group). [c] Mean ± SEM (number of samples that were below the sensitivity limits of the assay (20 pg/mL)/number of animals per group). [d] Significantly ($p < 0.05$) different from both other groups.

pled animals for serum LH and estradiol concentrations at 12–24 days after administration of 2, estradiol, or Me_2SO. While estradiol did not suppress serum LH from 12 to 24 days after a single intravenous injection, compound 2 reduced LH concentrations by 88%, 86%, and 66% relative to the Me_2SO controls at 12, 18, and 24 days, respectively (Table I). Serum concentrations of estradiol were not different among treatment groups and were undetectable in most samples (Table I). Thus a persistent elevation in circulating concentrations of estradiol subsequent to the administration of compound 2 was not responsible for the observed, chronic suppression of LH release. Rather, local release of estradiol in the brain, and in particular the hypothalamus, would appear to be responsible for the sustained suppression of LH release.[12]

Elevated circulating estradiol levels reduce serum LH by effecting the release of luteinizing hormone-releasing hormone (LHRH) from nerve terminals in the median eminence of the hypothalamus[13] and the responsiveness of the anterior pituitary to the decapeptide.[14,15] In the present experiments we could not dissociate the effect of the delivered estradiol on LHRH-containing neurons directly from effects that may be mediated by an action of estradiol on the anterior pituitary gland subsequent to estradiol release from median eminence into the hypophyseal portal system. However, our observation of (i) the accumulation in the brain of the ionized, hydrophilic quaternary pyridinium salt type ester of estradiol (compound 3), (ii) chronic (at least 24 days) suppression of LH secretion in rats following a single intravenous injection of 2, and (iii) low circulating concentrations of estradiol at 12–24 days after 2 administration strongly supports a local action of the drug in the CNS, presumably on hypothalamic LHRH-containing neurons. This proposal is consistent with the observation that estradiol implanted into the basal hypothalamus can reduce LHRH release and, as a result, chronically suppresses circulating concentration of LH.[16] Evaluation of LHRH release into the portal vessel, as well as the response of the anterior pituitary to LHRH, will be needed to establish firmly the site of action of this estradiol delivery system. While the lipophilic character of the dihydropyridine derivative 2 is facilitating the brain delivery, this alone is not enough for the prolonged action. Thus, the "lock-in" of the positively charged form 3 is the major, conceptual difference from the usual lipophilic "prodrug" approach. The difference can easily be demonstrated by comparing the effect of the similarly lipophilic 17β-valerate ester (4) of estradiol to 2 on the serum LH.[16]

The "lock-in" to the brain of the pyridinium salt, compound 3, and the sustained local release of estradiol may provide a useful tool for the determination of the central vs. peripheral site of action of this gonadal steroid in behavioral and physiological processes for which the locus of estradiol's site of action is uncertain.[13-18] This chemical delivery system for estradiol may be useful clinically (i) in the treatment of vasomotor instability associated with ovariectomy or the menopause particularly in women for whom peripheral estrogen activity is contraindicated[4] and (ii) in the chronic reduction of gonadotropin secretion for fertility regulation or for the treatment of gonadal-steroid-dependent disease, such as endometriosis[5] and prostatic hypertrophy.[6] Since gonadal steroids are believed to influence a variety of functions of the CNS,[19] the presently described steroid delivery system may have uses beyond those related to regulation of gonadotropin secretion. Finally, this brain-specific redox delivery system is under investigation currently for other naturally occurring gonadal steroids and their synthetic analogues.

(12) Our attempts to measure, by radioimmunoassay, free estradiol in brain homogenates from rats treated with 2 have been unsuccessful, largely because of the high concentrations of 3 in the brain and our observation that 3 cross-reacts with the antisera used in our assay. Procedures for the separation of 3 from free estradiol or the use of antisera that does not recognize 3 will be required to determine levels of estradiol in brain tissues.

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(16) It was found that estradiol 17-valerate (4) does not have any effect on serum LH at 12 (11.3 ± 1.7 ng/mL) or 18 (12 ± 2.2 ng/mL) days following administration of 0.45 mg of 4/kg to ovariectomized rats. Equivalent doses of 2 (0.5 mg/kg) showed profound effects (2.7 ± 0.8 and 2.3 ± 0.6 ng/mL of LH at 12 and 18 days, respectively). Seven animals were used in each group. Blood samples were collected by heart puncture at day 12 and trunk blood was collected on day 18 posttreatment.

(17) Kalra, P. S.; Kalra, S. P. Endocrinology (*Baltimore*) **1980**, *106*, 390.

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(19) McEwen, B.; Biegon, P.; Davis, P.; Krey, L. C.; Luine, V. N.; McGinnis, M. Y.; Paden, C. M.; Parson, B.; Rainbow, T. C. *Recent Prog. Horm. Res.* **1982**, *38*, 41–92.

(20) Approximately 1.6 g of lung, 2.6 g of liver, or 1.7 g of kidney tissue was homogenized in 1 mL of water and extracted with 4 mL of acetonitrile. One milliliter of blood was also extracted with 4 mL of acetonitrile. Analysis of the supernatant for concentrations of the quaternary pyridinium salt of estradiol is the same as described in Figure 2.

(21) Lindberg, B. S.; et al. *Acta Obstet. Gynecol. Scand., Suppl.* **1974**, *62*, 5.

(22) Shille, V. M.; Thatcher, M. J.; Simmons, K. J. *J. Am. Vet. Med. Assoc.* **1984**, 184, 1469.

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Registry No. 1, 50-28-2; 2, 103562-82-9; 3, 103562-83-0; 4, 979-32-8; LH, 9002-67-9; estradiol 3,17β-dinicotinate ester, 4248-62-8; estradiol 17β-mononicotinate ester, 4248-63-9.

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Molecular Design toward Biologically Significant Compounds Based on Platelet Activating Factor: A Highly Selective Agonist as a Potential Antihypertensive Agent

Sir:

Since the identification of platelet activating factor (PAF)[1] and an antihypertensive lipid[2] as alkylacetylglycerophosphocholine, a number of constitutional analogues have been prepared by many laboratories, and we also recently reported that acetyl glyceryl ether phosphorylcholines, their enantiomers, and their analogues were efficiently synthesized in a stereochemically unambiguous manner starting from D- and L-tartaric acids as chiral synthons.[3] The accumulated study on the biological activities of various synthetic phospholipids with acylic structures showed that the irreversible platelet aggregation always parallels the antihypertensive activity,[3b,4] although several selective antagonists have been found recently and are now investigated intensively.[5] It can be said that PAF analogues are structurally simple compounds but are conformationally complex. Therefore, a molecular design

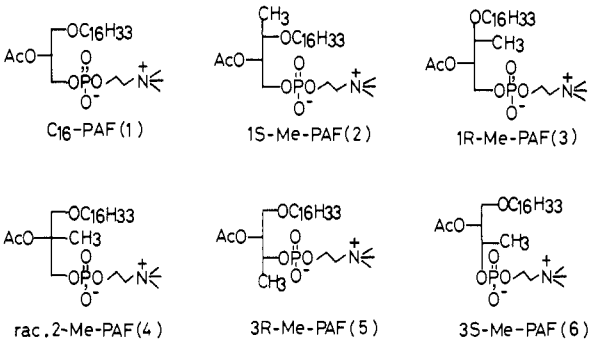

Figure 1. Platelet activating factor and the analogues.

of a PAF analogue has been carried out in such a way as to localize the conformational isomers of PAF by introducing a methyl group[6] in the glycerine moiety, since we assumed that the multiple biological activities of PAF may be mediated by the different stereochemical environment of a receptor or by the multiple receptors[7] with different stereochemical requirements. From this point of view, we attempted to design an analogue with a high selective hypotensive activity but with a limited ability to cause platelet activation. In this paper, we describe a highly selective synthetic agonist with orally potential antihypertensive activity. A methyl group was introduced at C_1 and C_3 diastereoselectively[8] and enantioselectively by our tartaric acid strategy[9] (Figures 1 and 2). For comparison, a methyl group was also introduced at C_2 to afford 2-Me-PAF in racemic form.[9] The simple conformational analysis by using Newman projection formulas shows that the more stable conformers of C_{16}-PAF, 1(*S*)-Me-PAF, 1(*R*)-Me-PAF, 3(*S*)-Me-PAF, and 3(*R*)-Me-PAF are different from one another in the spacial orientation of the methyl, alkyl ether, and phosphocholine groups.[10] Biological activities (platelet activation and antihypertensive) of the synthetic methyl derivatives are described in this paper. The derivatives were assessed for activity to induce platelet activation by measuring the release of [^{14}C]serotonin from rabbit platelets,[11] and the effect of PAF and the methyl derivatives on blood pressure was examined by injecting male Wistar rats with the test compounds intravenously. The results as shown in Table I clearly demonstrate that 1(*S*)-Me-PAF is more important bio-

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(8) Wissner et al. are also proposing such a hypothesis and synthesized some modified compounds at the glycerine backbone, but they obtained such compounds as a mixture of diastereomers in the racemic form and drew a negative conclusion about the biological assay. Wissner, A.; Schaub, R. E.; Sum, P.-E.; Kohler, C. A.; Goldstein, B. M. *J. Med. Chem.* **1985**, *28*, 1181–1187.
(9) Details about the synthetic study of other compounds described here will be published elsewhere. 1(*S*)-Me-C_{16}-PAF (2): mp 222–230 °C; $[\alpha]^{21}_D$ −1.11° (*c* 1.80, $CHCl_3$–MeOH); FAB-MS 538 ($M^+ + 1$).
(10) A study on the relative stability of the conformers is now in progress by computer-assisted calculation.
(11) The details of the biological test of platelet aggregation and serotonin release activities are essentially followed by the method described in the following: Hayashi, H.; Kudo, I.; Inoue, K.; Onozaki, K.; Tsushima, S.; Nomura, H.; Nojima, S. *J. Biochem. (Tokyo)* **1985**, *97*, 1737–1745.

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