Cardiovascular Effects of Antihypertensive Polar and Neutral Renomedullary Lipids

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SUMMARY Two antihypertensive lipids can be extracted from fresh renal medulla. One is polar (the antihypertensive polar renomedullary lipid, or APRL) and the other is nonpolar (the antihypertensive neutral renomedullary lipid, or ANRL). APRL and ANRL differ in their biologic activities: APRL in bolus intravenous injections causes a very rapid decline in arterial pressure (AP) while ANRL, after a lag of 2 minutes, causes a slower decline in AP. APRL increases heart rate and sympathetic activity. ANRL decreases heart rate and sympathetic activity. ANRL appears to convert to APRL, under certain in vitro circumstances, suggesting that the structure of the two molecules is related. ANRL and APRL appear in the renal venous effluent after unclipping; biologically, ANRL seems dominant. The venous effluent of the unclipped isolated kidney lowers the HR and sympathetic activity of the normal rat. Unclipping degranulates the renomedullary interstitial cells (RIC). The antihypertensive effect of unclipping appears due to the secretion of ANRL and APRL by the kidney. It is concluded that ANRL seems to be the antihypertensive hormone of the RIC. (Hypertension 5 (supp I): I-112–I-118, 1983)

KEY WORDS • antihypertensive polar renomedullary lipids • heart rate • antihypertensive neutral renomedullary lipids • sympathetic activity • unclipping • renomedullary interstitial cells

TWO groups of antihypertensive lipids have been derived from fresh renal medulla. One is termed the "antihypertensive polar renomedullary lipid," or APRL, and the other, the "antihypertensive neutral renomedullary lipid," or ANRL.1 APRL is a conglomerate of 1-0-alkyl ethers of phosphatidylcholine (the main alkyl ethers are the C16:0, C16:1, and C18:1),2 while ANRL is identified as a nonpolar entity by chromatographic and solubility studies. This report investigates the relationship of these two lipids.

Materials and Methods

Derivation of Antihypertensive Polar Renomedullary Lipid (APRL)

Fresh rabbit renal papilla (125 g) was homogenized in Sorensen’s phosphate buffer (125 ml, pH 7.5, 0.268 M), incubated at 37°C for 30 minutes, freeze-dried overnight, subjected to total lipid extraction, and evaporated to an oil (4.7 g), as previously described.3 This oil (11 g) was dissolved in 77 ml of ether:benzene (80:20 vol/vol), and 15 ml of Vitride (70% solution of sodium-bis-(2 methoxyethoxy) aluminum hydride in benzene) was added to this solution slowly and with care. After gentle swirling at 37°C for 30 minutes, 4% of acetic acid anhydride (184 ml) was added slowly. The white slurry was extracted four times with 150 ml of ether and two times with chloroform (CHCl3). Rotary evaporation yielded 8.3 g of oil. Acetylation was carried out with pyridine (13 ml), acetic acid anhydride (52 ml), and incubation at 65° to 70°C for 45 minutes (frequent swirling). Rotary evaporation yielded 8.6 g of oil. This oil was dissolved in 25 ml of the lower phase of the mixture of chloroform:methanol:acetic acid:water (CHCl3:MeOH:HOAc:H2O; 100:98:2:100 vol/vol) and placed on a CHCl3 washed Unicel (activated silica) column (2.54 × 73.7 cm). The column was eluted with a mixture of 50:25:8:4 (vol/vol) of the above solvents (flow, 2 drops/sec). A major band of APRL-type antihypertensive activity was eluted. Further purification was attained by two thin-layer chromatography (TLC) systems.
In the first system, 15 mg of Unisil column fraction was dissolved in 700 \( \mu l \) of CHCl₃, a 50 \( \mu l \) fraction was placed on a 5 \( \times \) 20 cm Kiesegel G glass TLC plate as a spot (marker plate), and the remainder streaked across the width of a 20 \( \times \) 20 cm plate (preparative plate). The plates were dried at room temperature and placed in a tank containing 200 ml of CHCl₃:MeOH:HOAc:H₂O (50:25:8:3, vol/vol). After reaching the 10 cm level, the marker plate was sprayed lightly with concentrated H₂SO₄ and heated on a hot plate to char organic material. The preparative plate was cut according to the spots on the marker plate, and the powder was scraped off. The lipid was eluted as previously described.³ The antihypertensive fraction of the first dimension (fraction 2) was chromatographed on marker and preparative plates except that the solvent system was CHCl₃:MeOH:NH₄OH (75:25:4, vol/vol). This second dimension material was eluted in the same manner. The first dimension active product (8.5 mg) yielded 5.8 mg of the second dimension product (fraction 2). This was the APRL activity used in the present study (fig. 1).

Characterization of Antihypertensive Polar Renomedullary Lipid (APRL)

Enzymolysis

Phospholipase A₂ (Rattlesnake Venom). This enzyme (60-150 units) was added to 1 mg of APRL in 0.5 ml ether and 5 ml Tris buffer (pH 7.3) plus 0.001 M CaCl₂ and 12 mM NaCl, and subjected to tumbling at 25°C for 8 hours. This enzyme hydrolyzed acyl groups on the carbon 2 position of acylglycerylphosphorylcholine. Lipids were recovered from the digestion mixture by CHCl₃:MeOH (2:1, vol/vol) and assayed for antihypertensive activity. The assay was negative. Upon reacetylation the assay became positive. These findings support an acetyl in the second position.

Phospholipase A₁ (Rhizopus arrhizus). The specificity of this enzyme for a primary ester on carbon 1 is virtually absolute.⁴,⁵ The lipase (5500 units) was added to 1 mg of APRL in 0.5 ml ether plus 5 ml MOPS buffer (3-morpholinopropanesulfonic acid, sodium salt) 0.1 M, pH 7.5, containing 12 mM NaCl and subjected to 8 hours of tumbling at 25°C. Lipids were extracted as above and assayed for antihypertensive activity. The latter was positive, indicating the presence of a nonacyl group in the first position.

Purification of APRL. APRL was treated with phospholipase A₁, as above, and the reaction product was subjected to TLC (CHCl₃:MeOH:HOAc:H₂O, 50:25:8:4, vol/vol), and the fraction having antihypertensive activity was identified. This fraction was analyzed by field desorption (FD) mass spectrometry (MS) and the fast atomic bombardment (FAB)-MS. A synthetic phospholipid, lysopalmitoylglycerylphos-
phorylcholine, was analyzed by FD-MS and FAB-MS for comparison.

FD-MS data of phospholipids displayed peaks in the protonated molecular ion (M + H)⁺ region plus a peak at (M + 2H + choline)⁺ for 1-0-hexadecenyl-(622), 1-0-hexadecyl-(628) and 1-0-octadecenyl-2-acetoyl-sn-glyceryl-3-phosphorylcholine (654), corresponding to the most abundant glycerylethers present (C16:1, C16:0, C18:1).

FD-MS spectra were examined for the presence of one of the ether analogs of phospholipids, namely, 1-0-hexadecyl-2-acetoyl-sn-glyceryl-3-phosphorylcholine (C26H40NO7P, M⁺ = 523.3638). The presence of a compound having this accurate mass was verified by peak matching M⁺ at 523.3638 under FD conditions with an appropriate perfluorokerosene mass marker peak under EI conditions.

FAB-MS data of the intact phospholipid mixture are shown in figure 2. Accurate masses and corresponding elemental compositions were given at 525 and 539. The following experiment was performed. The FAB-MS of a mixture of synthetic phospholipid ethers with C15, C16, and C18 sidechains (11:50:50, wt/wt) was studied. In this mixture, peak-matching between the (M + H)⁺ of C16 ether vs C18 ether was performed. The two masses, 525.3795 for C26H40NO7P (C16 ether standard) and 553.4043 (endogenous C18 ether), were found with an error of 0.006 mass units. Second, a biologic extract sample was analyzed, and its mass spectrum is shown in figure 2. The two masses at 525 and 539 (endogenous C16 ester) were peak-matched with 525 (C16 ether standard). Mass 539 was shown to be 539.3577 and differed by 0.001 mass unit from that of a mass of a C16 ester (C26H32NO8P = 539.3587). Third, a mixture of the synthetic ethers and biologic sample was analyzed, and two significant features were illustrated. First, the C16 ether, at a mass resolution of 10,000, was shown to be a singlet, incontrovertibly demonstrating that the peak at 525 in the biologic extract was a C16 ether. Second, with this known ether mass as the internal standard, the peak at 539 was peak-matched and showed a mass of 539.3577, or a mass difference of 0.005 for an eight-oxygen species, again demonstrating that the biologic peak at 539 was an ester. A combination of these enzymatic, chromatographic, chemical, and spectrometric (CI, FD, and FAB) data unambiguously assign an ether to the peak at 525 and an ester to the peak at 539.

Synthesis of 1-0-Alkyl Ether. Curt Roos (Larodan Lipids, Malmo, Sweden) synthesized 1-0-hexadecyl-rac-glyceryl-3-phosphorylcholine for us. The lyso compound was racemic. Acetylation, followed by treatment with phospholipase A₂, showed that half of the material was a lyso compound (L form). The enzyme hydrolyzed only the natural isomer, which was separated from unhydrolyzed D form by TLC (ChCl₃:MeOH:HOAc:H₂O, 50:25:8:4, vol/vol). Re-acetylation of the L fraction produced a very active antihypertensive compound (dose < 500 ng). The molecular weight of this compound was confirmed by FD-MS.

Antihypertensive activity of synthetic 1-0-alkyl ethers was indistinguishable from the activity of compounds derived from fresh renal medulla (fig. 1). Mass spectral data cannot discern whether the ether group is in the first or second position. Enzymic data indicated that the ether linkage was in the first position and that the second position was occupied by an acyl group.

The above observations indicate the complete structural elucidation of APRL. Chromatography, enzymolysis, chemical derivation, mass spectrometry (CI, GC/MS, FD, FAB), and chemical synthesis tech-
niques were combined to assign unambiguously the structure 1-0-alkyl-2-acetoyl-3-glyceryl-phosphorylcholine to the semisynthetic native material.

FAB and FD mass spectra demonstrated the presence of 0-alkyl ethers. High resolution FD- and FAB-MS accurate mass measurement and corresponding elemental composition data unambiguously distinguished the presence of an ether (seven oxygens) vis-à-vis an ester (eight oxygens) at m/z 525. The low resolution FD and FAB spectra demonstrated the presence of residual esters after equilibrium was established, while unambiguous GD/MS data illustrated that phospholipase A, treatment does remove C-1 esters.

Derivation of ANRL

The derivation of ANRL was previously described in detail.3

Conversion of ANRL to APRL

Three different systems have been used in the apparent conversion of ANRL to APRL. The ANRL was derived as previously described3 from either fresh renal papilla or renal venous effluent after unclipping the one-kidney, one clip hypertensive animal.

ANRL was further purified beyond the previous method3 by a third TLC system consisting of ether:HOAc:H₂O (100:0.5:0.5, vol/vol). ANRL had an Rf of 0.57 (TLC system, upper panel, fig. 3). Use of a fourth system of acetonitrile:MeOH:8%H₃PO₄ (130:5:1.5, vol/vol) on a silicic acid plate converted much of the ANRL to APRL. APRL had an Rf of 0.24 (TLC system, lower panel, fig. 3).

High performance liquid chromatography (HPLC) using a system of methanol:1mM K₂HPO₄ buffer (pH 7.5), 9.5:0.5, vol/vol, yielded no activity. When the column was washed out by flushing with five 150 µl pulses of dimethylsulfoxide, APRL was derived. In other words, ANRL went on the column, APRL came off the column (HPLC system, fig. 3).

The total lipid extract was extracted further with heptane and 95% methanol (3:1 vol). When the nonpolar phase (heptane) was chromatographed on a silica plate with CHCl₃:MeOH:H₂O (100:54:9, vol/vol), a material with the same Rf (0.54) and biologic activity as APRL was derived (solvent partition-TLC system, fig. 3).

Biologic Techniques

Indwelling catheters in the abdominal aorta and inferior vena cava, prepared and inserted as previously described,6 were used to measure the arterial pressure (AP) and inject the lipids i.v. Wistar rats, weighing about 200 g, were made hypertensive by the one-kidney, one clip technique, as previously described.7 The rats were used 3 or more months later. The assay of APRL and ANRL consisted of injecting the lipid as a bolus dose i.v. into the hypertensive recipient and monitoring the AP. APRL caused a sudden decrease in

![Figure 3](http://hyper.ahajournals.org/)

*Figure 3*. Three systems used in the conversion of antihypertensive neutral renomedullary lipid (ANRL, upper panels) to APRL (lower panels). For the TLC system, the ANRL was derived from fresh renal medulla. The conversion to APRL took place on a silica acid plate. The ANRL of the high performance liquid chromatography (HPLC) system was derived from the renal venous effluent after unclipping. The conversion to APRL took place on a C18 reverse phase column. The ANRL of the solvent partition-TLC system was virtually inactive with the dose used. The conversion took place on a silica acid plate.
AP. ANRL, after a lag period of 2 minutes, caused a slower depression of the AP.

Tonic renal nerve sympathetic activity, heart rate, and AP in another group of rats (SHR and WKY) were obtained as described by Ricksten and Thorén. Under chloralose anesthesia and through a midline incision, the left renal pedicle was isolated and the renal nerve separated from the renal vein and artery. A thin bipolar silver electrode was placed around a segment of the nerve and isolated carefully with silicone rubber (Wacker Sil Gel 604). The nerve activity was recorded through a cable. The nerve signal was amplified (Grass p 511) and rectified. The mean nerve activity was displayed on a Grass polygraph (Model 7). The mean arterial pressure was measured from the tail artery via a catheter. The transducer was connected to a tachygraph for determining heart rate. The animals were maintained under light anesthesia as indicated by response (jerk) and increased sympathetic activity following pinching of the skin.

Normotensive WKY rats (weight, 300 g) were used when ANRL was injected as an i.v. bolus dose. The preparation was purified but still contained a great deal of additional material. From mass spectrometry signals, it appeared to contain no more than 10% of the active principle (ANRL). A dose of 1 mg was injected amounting to about 100 μg of ANRL. The APRL (either extracted or synthetic) was infused i.v. in increasing amounts varying between 1 and 10 ng/100 g/min over 10 minutes. The dose was adjusted so that the drop in pressure was comparable to that following ANRL. Results with the extracted APRL were the same as with the synthetic compound. In addition, APRL was injected in a like manner in six hypertensive SHR.

Results

Acute Vasodepressor Effect

APRL causes a rapid depression of the AP when injected as a bolus i.v. (fig. 4). ANRL, under similar conditions, causes a slower decline of the AP after a lag period of about two minutes (fig. 4). The magnitude of the APRL effects is dose dependent. The limited amount of ANRL available for bioassay prevented a clear demonstration of a dose effect. The existence of a threshold dose, low grade effects, and high grade effects with different doses suggests but does not demonstrate a dose effect.

Conversion of ANRL to APRL

Figure 3 depicts the results of the conversion of ANRL to APRL by the three systems described in Materials and Methods.

APRL and ANRL in the Renal Venous Effluent Following Unclipping of the Goldblatt Hypertensive Rat

Earlier, an antihypertensive lipid was derived from the renal venous effluent as the AP fell following unclipping the one-kidney, one clip hypertensive rat. This lipid had the chromatographic characteristics of ANRL on first dimension TLC. At that time, we were...
not convinced that ANRL converted to APRL. Now, we believe that the end product of that experiment, from the second dimension TLC, contained APRL.

At this time, the demonstration of both APRL and ANRL in the renal venous effluent after unclipping the Goldblatt hypertensive rat is recorded (fig. 5). It is not known whether the APRL is a conversion product of ANRL under these conditions or whether the impact of the outstanding stimulation of the kidney after unclipping liberates both lipids. The latter appears more likely.

Effect of APRL and ANRL on Sympathetic Activity and Heart Rate (HR)

The i.v. bolus dose of ANRL in WKY (normotensive) rats caused the AP to drop slowly over 10 minutes from 113 ± 6 mm Hg by 10%, 20%, and 30% (n = 8, figs. 6 and 7). At the same time, the HR was reduced from 399 ± 11 to 383 ± 10 bpm, and the renal nerve sympathetic activity was reduced by 33% ± 8% (p < 0.01).

An i.v. infusion of APRL in the same rats was adjusted to between 1 and 10 ng/100 g over 10 minutes so as to cause the same decrease in AP as the ANRL bolus dose. This infusion caused increases in HR from 400 ± 11 to 432 ± 15 bpm and in renal nerve sympathetic activity of 58% ± 11% (n = 7, p < 0.001).

The infusion of APRL in the hypertensive SHR was also adjusted to drop the AP by 10%, 20%, and 30% from 163 ± 10 mm Hg over 10 minutes. Under these conditions, the HR rose from 361 ± 5 to 389 ± 7 bpm, and the sympathetic tone to the renal nerve increased by 32% ± 10% (p < 0.05). Thus, the effects of APRL on the hypertensive animal were similar to those on the normotensive animal.

Discussion

APRL is a potent vasodilator agent that acts on resistance vessels.10 It appears to induce reflex tachycardia and to increase sympathetic activity, since the increase in sympathetic tone and tachycardia parallels the increase in dilatation of resistance vessels and the drop in AP. ANRL, on the other hand, lowers the sympathetic tone in a major way despite a substantial decrease in AP. At the same time a mild bradycardia occurs. These agents, therefore, have certain opposing biologic effects.

It is of great interest that unclipping either the one-kidney, one-clip or the two-kidney, one-clip hypertensive rat is associated with the presence of both ANRL and APRL in the renal venous effluent. Importantly, the renal venous effluent after unclipping causes a decrease in renal nerve sympathetic activity and bradycardia.11 Five separate observations can now be linked. These are: 1) unclipping of the Goldblatt hypertensive rat lowers the AP; 2) the unclipping is associated with the presence of ANRL and APRL in the renal venous effluent; 3) the renal venous effluent of the unclipped isolated kidney lowers the HR and sympathetic tone; 4) the observations following unclipping are mimicked by i.v. infused ANRL extracted from the renal papilla and purified; and 5) the RIC degranulate following unclipping.12 These five observations support the view that the combined release of ANRL and APRL or closely related compounds from the renal papilla and its RIC mediates the lowering of the AP after unclipping. These compounds (or related compounds) are true antihypertensive hormones. In view of the decrease in sympathetic tone and bradycardia following unclipping, ANRL appears as the dominant agent.
The seeming conversion of ANRL to APRL, a non-polar to a polar entity, raises important issues. It suggests similarities as well as differences in structure. The differences appear to relate to the phosphorylcholine portion of the molecules. The opposite effects of APRL and ANRL on sympathetic tone appear to dispel the notion that ANRL must be converted to APRL in order to be biologically active. Thus, ANRL appears to be the main antihypertensive hormone of the renal papilla and its RIC.

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