Plasma Phospholipase A_2 Activity in Nephrectomized Rats and the Question of Renin Inhibition[1,2] (37722)

D. H. OSMOND, P. A. MCFADZEAN, AND L. J. ROSS
(Introduced by B. Cinader)

Departments of Physiology and Medicine, Medical Sciences Building, University of Toronto, Toronto, Canada M5S 1A8

The long-standing concept of an "endocrine" antihypertensive function of normal renal tissue implies loss of this capacity following total nephrectomy. Among the recently proposed antihypertensive factors is a circulating renin-inhibiting system involving a phospholipid "preinhibitor," a lysophospholipid "inhibitor," and a phospholipase that catalyzes deacylation of the first to produce the second (1–3). Circulating "preinhibitor" was originally thought to be supplied by the kidney, but other sources must exist because its concentration in blood does not fall in anephric rats (4) and even rises in dogs (5) and humans (6). Consequently, attention turned to a possible renal influence on phospholipase activity in the blood (3, 5) with the view that if the kidney secretes this enzyme or influences its activity, nephrectomy would curtail the formation of "inhibitor" and produce a deficiency of renin-inhibiting capacity.

A central role of phospholipase activity in the blood was implied by rat experiments involving intravenous infusions of "preinhibitor" (7). Within a few minutes, pressor responses to renin, but not to angiotensin, were blocked, suggesting rapid conversion of infused "preinhibitor" to active renin "inhibitor" in the blood. After stopping the infusion, renin blockade quickly disappeared, implying that the newly formed "inhibitor" had a very short half-life. Such evanescence of exogenous "inhibitor" suggests that endogenous "inhibitor" is normally present in much lower concentrations and might be difficult to determine in normal or nephrectomized rats by conventional methods (4). Another obstacle to direct determination of "inhibitor" is that like other lysophospholipids, it may continue to be formed while a blood sample is being centrifuged and prepared for analysis (8, 9), making it difficult to distinguish original from newly formed compound. These considerations prompted an alternate approach to the assessment of renal influence upon phospholipase activity, which we report here. Fresh plasma from nephrectomized and control rats was incubated with radioactive "preinhibitor", and the rates of conversion to "inhibitor" were compared. The plasmas were minimally diluted with additives so they would closely reflect the electrolyte, acid–base, and other metabolic characteristics of the donor animals that might influence the plasma phospholipase. Enough evidence now favors the view (4, 10–13) that "preinhibitor" is a phosphatidylethanolamine (PE) and "inhibitor" is its lyso derivative (LPE) to justify usage of the corresponding terms interchangeably in this report.

Materials and Methods. Male rats of the strains indicated in the *Results* section generally weighed 300–400 g. They were nephrectomized or sham nephrectomized bilaterally under ether anesthesia using the dorsolateral approach and given free access to a standard laboratory diet and water. Forty eight hours later, after an overnight fast, the rats were anesthetized with ether and opened ventrally. The renal blood vessels were

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clamped on both sides and blood drawn into a heparinized syringe (sodium heparin, Connaught, 5000 USP U/ml, about 0.3 ml/10 ml blood) by direct aortic puncture. After centrifugation at 2075*g* for 20 min at 4°, plasma was separated, kept on ice, and usually incubated within 1 hr of blood collection.

Our incubation conditions were designed to provide an excess of substrate ("preinhibitor") of which only a small proportion would be deacylated to form "inhibitor", in order to conform with requirements for linear kinetics (10). The underlying rationale was that plasma phospholipase activity would determine the proportions of "preinhibitor" and "inhibitor" existing at the end of the incubation period. All incubations were in 2.5 × 5 cm flat-bottomed vials in a shaker bath for 8–72 min at 37°. In order to retain closely the original composition of plasma reflecting the physiological condition of the donor rats, no buffer solution was added. The only additive was ^{32}P-labeled "preinhibitor" isolated from rat liver (11) and dissolved either in anhydrous diethyl ether (Fisher Reagent) or aqueous sodium deoxycholate (British Drug Houses, Toronto) of a concentration giving 5 mg/ml in the incubate. A known weight of "preinhibitor", *e.g.*, 1–1.5 mg in 0.2 ml of vehicle, was added to each vial, on ice, followed by 1 ml fresh plasma. The weight of "preinhibitor", and its radioactivity, was constant within, but not necessarily between, experiments. Vials were mixed gently and then transferred to the shaker bath for incubation, after which the reaction was stopped by dipping them about ¾ in. deep in an acetone–dry ice mixture. Cracking of vials was prevented by rapid predipping in acetone at room temperature to remove surface water. Triplicate unincubated control vials were prepared by standing them in acetone–dry ice, adding the "preinhibitor" solution, and then slowly adding representative plasma from control or experimental rats so that it froze on contact.

Extraction of total incubates was essentially as for whole plasma (4, 5, 10). Briefly, deep-frozen incubates were allowed to thaw until the frozen layer was loosened enough to transfer to a semimicro container of a blender (Eberbach Corp., P. O. Box 1024, Ann Arbor, MI 48106) with rinsings of distilled water and methanol. After extraction for 3 min with chloroform:methanol (80:40 ml), the contents were transferred through a filter funnel (Whatman No. 1) to a 500-ml separatory funnel. After twice washing with ⅕ volumes of distilled water, the extract was concentrated in a flash evaporator under reduced pressure, quantitatively transferred to a thin layer silica gel chromatography (TLC) plate, and developed in chloroform:methanol: H_2O (95:35:6, v/v/v) (4, 10, 11). After drying, the "preinhibitor" (PE) and "inhibitor" (LPE) spots were visualized with ninhydrin reagent, scraped off into counting vials, liquifluor added, and radioactivity determined (10, 11). Baseline counts in the PE and LPE zones were derived from the control unincubated samples in each experiment; with these we compared the proportions of counts obtained after incubating labeled PE with plasma from sham-operated or nephrectomized rats, thus estimating relative phospholipase activities.

Results. Experiments 1 and 2. In these preliminary experiments, we used male albino rats from Rolfsmeyer Laboratory Animals, 4300 Sintz Rd., Springfield, OH. In Expt. 1, four binephrectomized (2NX) and three sham-nephrectomized (control) rats averaging 412 g were bled 48 hr after operation, and 1 ml plasma incubated in triplicate for 8 min with radiolabeled PE added in ether. In Expt. 2, the same procedure was followed with five 2NX and four control animals of similar body weight. Representative blood urea nitrogen (BUN) values, determined by an autoanalyzer technique in a hospital laboratory, averaged 336 mg% for 2NX rats and 17 mg% for controls. Typical plasma protein values averaged 5.9 and 6.2 g%, respectively.

In both experiments, the rates of conversion of PE to LPE were substantial, and little changed by nephrectomy. "Inhibitor" counts expressed as percent of unincubated control values in Expt. 1 were 252 and 233 for sham control and nephrectomized animals, respectively. In Expt. 2, the corresponding values

TABLE I. Experiment 3: Conversion of PE to LPE in Plasma from Sham-Nephrectomized and Nephrectomized Wistar Rats 48 hr After Operation.[a]

Group (no. of rats)	PE cpm	LPE cpm	Total cpm	p	$\frac{\text{LPE cpm}}{\text{Total cpm}} \times 100$	p
Control, unincubated[c]	25917 ± 370	493 ± 73	26410 ± 298	N.S.[b]	1.91 ± 0.31	<0.01
Sham control (6)	23367 ± 407	1846 ± 181	25213 ± 334	—	7.37 ± 0.75	—
Nephrex (6)	23459 ± 333	2110 ± 205	25568 ± 276	N.S.[b]	8.26 ± 0.81	N.S.[b]

[a] Rounded averages ± SEM of net cpm of triplicate determinations.
[b] All comparisons with sham control group. N.S. = not significant.
[c] Triplicate determinations on pooled control rat plasma.

were 160 and 148%. These increments in LPE counts due to plasma phospholipase activity were not consistently matched by proportional losses of counts from the PE fraction in both groups, making further statistical evaluation difficult. In the experiments below, counts in all fractions were considered, *i.e.*, PE, LPE, and total (PE and LPE), in comparing sham control and nephrectomized rats.

Experiment 3. Male Wistar Rats averaging 350 g body wt were obtained from Woodlyn Farms Ltd., R. R. #3, Guelph, Ontario. Six were sham nephrectomized and six binephrectomized. After 48 hr, triplicate 1-ml aliquots of plasma from each rat were incubated for 8 min with labeled PE added in ether as in Expts. 1 and 2. The incubates were extracted, chromatographed, and PE and LPE fractions counted. The data (Table I) indicate losses of counts from the PE fraction and gains in the LPE fraction that are comparable in nephrectomized and sham control rats. The ratios of LPE to total counts were not statistically different, suggesting unchanged plasma phospholipase activity in the nephrectomized rats.

Experiment 4. One-milliliter samples of plasma from three sham-nephrectomized and three nephrectomized male rats (Wistar, Woodlyn Farms) were incubated for 72 min in duplicate, with the PE added in ether. Another 1-ml duplicate sample was incubated with sodium deoxycholate. Unincubated control vials were prepared in triplicate. The data in Table II indicate comparable recovery of total counts in the incubated groups, with the unexplained exception of group D. As shown previously (10), the activity of this phospholipase is enhanced in the presence of deoxycholate. Statistical comparison of corresponding nephrectomized and sham control groups revealed no impairment of phospholipase activity in nephrectomized rats (Table II, B vs C and D vs E).

Discussion. There is evidence to suggest that if a renin inhibitor is produced, or its activity influenced by the kidney, a deficiency should express itself clearly within 48 hr of nephrectomy. An exaggerated pressor response to injected renin was noted in rats by 18 hr (14) and certainly by 24–48 hr (1, 3, 15, 16). Such high renin responses could be due to an increased substrate concentration and also to disappearance of a renin inhibitor. In dogs, between 24 and 48 hr after nephrectomy, plasma substrate elevation leveled off while angiotensin-generating capacity continued to rise, suggesting progressive loss of renin-inhibiting influence (2). In terms of the phospholipid theory (1–3), such loss could be due to disappearance of either "preinhibitor" or "inhibitor" from the blood. "Preinhibitor" is now known not to diminish in rats (4) or dogs (5) within 48 hr of nephrectomy. This leaves the question of what happens to "inhibitor", the answer to which can be sought by direct determination of it in the blood or by estimating the capacity to form it from circulating "preinhibitor". For reasons given before, we elected the latter option and incubated plasma that was minimally altered by additives, so as to reflect the metabolic status of the nephrectomized and sham-nephrectomized donor rats.

The substrate for the plasma phospholipase was a ^{32}P-labeled phosphatidylethanolamine

(PE) isolated from rat liver (4, 10, 11). Considerable evidence now indicates that "preinhibitor" is a PE (4, 10–13), and its continued presence in blood after nephrectomy (4, 5) makes a hepatic source very likely, since the liver is a major supplier of blood phospholipids (17). It seemed appropriate, therefore, to use hepatic PE as a substrate for the plasma phospholipase, which is now known to deacylate actively the beta position (10). Unpublished experiments indicate that, if anything, liver PE is deacylated more rapidly than kidney PE by plasma in our incubation system.

Our incubation conditions were kept deliberately simple. The only additive, apart from heparin, was PE dissolved in diethyl ether or sodium deoxycholate solution. The volume of PE solution was 0.2 ml/ml plasma, and where ether was the solvent, most would evaporate during incubation in open vials at 37°, leaving plasma substantially unchanged. This consideration probably increases the chances that our *in vitro* results, with ether at least, using homologous liver PE as the substrate, has considerable physiological validity.

In all our experiments, "preinhibitor" was readily converted to "inhibitor" after nephrectomy, indicating high plasma phospholipase activity. Only in preliminary Expts. 1 and 2 was there evidence of slight impairment, and, as noted in *Results*, this was associated with biassed recoveries of counts. It seems very unlikely, therefore, that short-term nephrectomy would deplete the plasma of renin "inhibitor" *in vitro*. A similar conclusion can be reached from observations *in vivo*. "Preinhibitor" infused into nephrectomized rats blocked the pressor response to renin (7), implying that it must have been readily converted to "inhibitor" by phospholipase activity in the blood. Thus, if the kidney is not required to maintain blood levels of "preinhibitor" (4–6) or to sustain "phospholipase" activity by which "inhibitor" is formed, then its proposed central role in renin inhibition is cast into doubt—at least in terms of the phospholipid theory (1–3). The unlikely possibility that the circulating half-life of "inhibitor" is shortened

TABLE II. Experiment 4: Wistar Rats 48 hr After Binephrectomy.[a]

	Group (no. of rats)	PE cpm	LPE cpm	Total cpm	p	$\frac{\text{LPE cpm}}{\text{Total cpm}} \times 100$	p
A	Control, unincubated	5877 ± 323	803 ± 112	6680 ± 431	A vs D, <0.01 A vs E, N.S.	11.9 ± 0.94	A vs D, <0.001 A vs E, <0.001
B	Sham control, ether (3)	3850 ± 183	1820 ± 105	5670 ± 205	B vs C, N.S. B vs D, N.S.	32.2 ± 1.71	B vs C, N.S. B vs D, <0.001
C	2NX ether (3)	4158 ± 231	1671 ± 179	5829 ± 269	C vs D, N.S.	28.6 ± 2.76	C vs E, <0.001
D	Sham control, deoxycholate (3)	2381 ± 133	3142 ± 145	5523 ± 65	D vs E, <0.05	56.9 ± 2.46	D vs E, N.S.
E	2NX, deoxycholate (3)	2565 ± 262	3518 ± 299	6084 ± 207	—	57.7 ± 4.39	—

[a] Incubation with ether or deoxycholate in the medium. Average net cpm ± SEM. Duplicate determinations; control unincubated in triplicate.

by nephrectomy due to heightened lysophospholipase activity seems to be ruled out by the present data showing very similar recoveries of counts in all fractions of all groups. There is nothing to suggest that LPE was degraded more rapidly in plasma from nephrectomized rats.

For several decades, an antihypertensive function of the kidney has been postulated (15, 16). One way in which it could exercise this effect is by neutralizing the pressor effect of renin. The phospholipid theory proposed a lysophospholipid renin "inhibitor" as the agent (1–3), but neither its precursor (4–6) nor the phospholipase activity that catalyzes its formation from precursor in the blood seem to require the kidney in the short term. While altered phospholipase activity after long-term nephrectomy has not been ruled out, the present data coupled with other objections to the phospholipid theory (10–12) suggest there is room for an alternate hypothesis.

Summary. Plasma from 48-hr nephrectomized or sham-nephrectomized rats was incubated with homologous ^{32}P-labeled PE (renin "preinhibitor"). After extraction of total lipids from the incubate and thin layer silica gel chromatography of the extract, radioactivity was recovered as PE or its deacylated derivative LPE (renin "inhibitor"). Nephrectomy did not cause a reduction in the proportion of counts recovered as LPE, suggesting that its rate of formation and degradation is normal. This suggests that plasma from nephrectomized rats should have no lack of renin "inhibitor" if it is formed from what are known to be undiminished circulating levels of "preinhibitor". If the kidney is not the major source of circulating "preinhibitor" and does not affect availability of the "inhibitor", its proposed renin-inhibitory role may be questioned. Alternatively, the phospholipid theory of renin inhibition is untenable in its present form.

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