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INHIBITION OF RENAL, CARDIAC AND CORNEAL (Na^+-K^+)ATPase BY 12(*R*)-HYDROXYEICOSATETRAENOIC ACID

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Abstract—12(*R*)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(*R*)-HETE] is one of the major arachidonate metabolites of the corneal epithelial cytochrome P450 system. We studied its inhibitory effect on different preparations of (Na^+-K^+)ATPase. 12(*R*)-HETE had no effect on ATPase activity in the absence of Na^+ and K^+. However, it inhibited ouabain-sensitive (Na^+-K^+)ATPase obtained from bovine corneal epithelium, rat kidney and rat heart ventricle in a concentration-dependent manner with an IC_{50} of 10^{-6} M. Its enantiomer, 12(*S*)-HETE, was inactive at 10^{-7} M and 10^{-6} M, but it inhibited (Na^+-K^+)ATPase at higher doses, an effect also seen with arachidonic acid. 12(*R*)-HETE as an endogenous metabolite of arachidonic acid may modulate physiological and pathophysiological processes by affecting (Na^+-K^+)ATPase activities *in vivo*.

Cytochrome P450 enzymes of human and bovine corneal epithelium oxidize arachidonic acid at carbon 12 to yield specifically the 12(*R*)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(*R*)-HETE] enantiomer as the major metabolite [1]. The liver and psoriatic skin lesions are other tissues capable of generating 12(*R*)-HETE from arachidonic acid [2, 3]. 12(*R*)-HETE has quantitatively different biological activities when compared to its stereoisomer 12(*S*)-HETE, the metabolite of arachidonic acid derived through the 12-lipoxygenase activity in many tissues [4, 5]. For example, 12(*R*)-HETE is a more potent chemotactic and chemokinetic factor than 12(*S*)-HETE [6]. Moreover, 12(*R*)-HETE inhibits (Na^+-K^+)ATPase activity in the cornea and modulates vascular tone, properties not shared by the 12(*S*) enantiomer [1, 7]. Furthermore, Takahashi *et al.* [8] demonstrated that nanomolar concentrations of 12(*R*)-HETE, injected into the renal artery of the rat, result in natriuresis accompanied by renal vasodilatation, suggesting a possible role for this and other endogenously generated eicosanoids in the regulation of renal function under physiological and/or pathophysiological conditions.

In this study, we tested and compared the inhibitory effect of 12(*R*)-HETE on the activity of several partially purified (Na^+-K^+)ATPase preparations, namely (Na^+-K^+)ATPase isolated from kidney and heart of the rat, dog kidney and bovine cornea. The effects of 12(*R*)-HETE were compared to those of 12(*S*)-HETE and arachidonic acid.

MATERIALS AND METHODS

Materials. The dog kidney (Na^+-K^+)ATPase enzyme was a lyophilized commercial preparation (ATP phosphohydrolase, EC 3.6 1.3, Grade IV, Sigma Chemical Co., St. Louis, MO). 12(*R*)-HETE and 12(*S*)-HETE were obtained from the Cayman Chemical Co. (Ann Arbor, MI). The purities of these compounds were >99% as assessed by reverse-phase HPLC separation using a chiral column and ultraviolet absorption at 237 nm. Arachidonic acid was from Nucheck.

Purification of (Na^+-K^+)*ATPase activity.* Partially purified (Na^+-K^+)ATPase enzymes from Wistar–Kyoto (WKY) rat renal cortex, Sprague–Dawley heart ventricle and from bovine corneal epithelium were prepared as described by Jorgensen [9]. Briefly, microsomes were solubilized with sodium dodecyl sulfate (SDS) by incubating 1.4 mg/mL of microsomal protein with SDS (0.56 mg/mL) in 2 mM EDTA, 50 mM imidazole, 3 mM ATP, pH 7.5, for 45 min at room temperature with continuous stirring. The solubilized microsomes were then applied on discontinuous density gradient and centrifuged at 110,000 *g* for 90 min. The gradient consisted of three successive layers of sucrose 29.4%, 15%, and 10% (w/v). The pellet was resuspended in 25 mM imidazole, 1 mM EDTA, pH 7.5, and stored at −70°.

(Na^+-K^+)*ATPase assay.* The activity of (Na^+-K^+)ATPase was measured as the rate of release of inorganic phosphate (P_i) as previously described [10]. The incubation mixture contained 30 mM histidine, 20 mM KCl, 130 mM NaCl, 3 mM $MgCl_2$, and 3 mM ATP, pH 7.5, in a final volume of 0.5 mL. Enzyme concentration ranged between 0.25 and 5 μg per incubation. The reaction was started by addition of ATP and was allowed to proceed for 15 or 30 min at 37°. During these periods no more than 10% of the substrate was utilized by the enzyme. Aliquots of the compounds to be tested were dried under nitrogen and resuspended in reaction buffer without ATP. Following a 10 min preincubation, ATP was added to start the reaction. Ouabain was dissolved in warm distilled water. The reaction was terminated by placing the tubes on ice and adding 0.8 mL of color reagent containing ammonium molybdate, malachite green, and sterox as described by Candida and coworkers [11, 12]. After 1 min, 100 μL of 34%

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Table 1. Purification of (Na^+-K^+)ATPase activity from bovine corneal epithelium

Fraction	Enzyme activity (μmol P_i/hr/mg)		% Ouabain-sensitive (Na^+-K^+)ATPase
	Total	+ Ouabain (1 mM)	
8000 *g* Supernatant	7.43	6.84	8
100,000 *g* Pellet	17.60	12.80	27
Sucrose pellet	37.10	12.60	66

Bovine corneal epithelial cells were homogenized with phosphate-buffered saline, pH 7.4, and microsomes were prepared by centrifugation at 8000 *g* and 100,000 *g*, solubilized with SDS, and submitted to discontinuous sucrose gradient. (Na^+-K^+)ATPase activity in each fraction was assayed as described in Materials and Methods. Results are means of three different determinations; SE was less than 10%.

Table 2. Total ATPase and (Na^+-K^+)ATPase activities obtained from partially purified enzymes of different sources

Source	Activity (μmol P_i/hr/mg)		% Ouabain-sensitive (Na^+-K^+)ATPase	Ouabain IC_{50} (μM)
	Without ouabain	With ouabain (1 mM)		
WKY rat renal cortex	118.9 ± 12.3	27.4 ± 3.8	77	200
Sprague–Dawley heart ventricle	49.3 ± 4.3	26.6 ± 3.5	46	400–600
Dog kidney (Sigma)	73.6 ± 3.8	2.9 ± 0.4	96	1.7
Bovine corneal epithelium	37.1 ± 3.1	12.6 ± 2.9	66	300

Partially purified (Na^+-K^+)ATPase was prepared by solubilization and discontinuous sucrose gradient of microsomes from WKY rat renal cortex, Sprague–Dawley heart ventrical and bovine corneal epithelium and assayed as described in Materials and Methods. The dog kidney enzyme was obtained from Sigma. Values are means ± SE, N = 6–24.

(w/v) sodium citrate solution was added and mixed. The final solution was then analyzed at 660 nm in a Beckman DB spectrophotometer using a reference blank containing boiled enzyme with all the reagents. A standard curve was constructed using P_i. Enzyme activity was expressed as micromoles of P_i released per hour per milligram of protein.

Statistical analysis. Statistical analysis was performed by a one-way analysis of variance. If this indicated significance, comparisons between groups were analyzed by a Bonferroni modification of Student's *t*-test. Significance was accepted at $P < 0.05$.

RESULTS

The activity of partially purified (Na^+-K^+)ATPase was studied using microsomal preparations isolated from rat kidney, rat heart and bovine corneal epithelium by sucrose gradient centrifugation as described in Materials and Methods. Fractions before and after purification were analyzed for their enzymatic activity. As shown in Table 1 for the bovine corneal enzyme, specific activity of the ouabain-sensitive (Na^+-K^+)ATPase (the activity in the presence of Na^+,K^+ and ouabain subtracted from total activity) at the end of the purification was 8-fold higher than in the microsomal fraction, and 42-fold higher than in the original cell-free homogenate (8000 *g* supernatant). For all the preparations tested, (Na^+-K^+)ATPase activity was defined as the ATPase activity that could be inhibited with 1 mM ouabain during a 10-min preincubation. Using this parameter, we found that for the purified bovine corneal enzyme, 66% of the total ATPase activity was inhibited by ouabain and thus corresponded to (Na^+-K^+)ATPase The specific activities of several (Na^+-K^+)ATPase enzymes, obtained from different tissues, are shown in Table 2. The highest activity of the ouabain-inhibited portion was observed for the enzyme purified from WKY rat renal cortex. For comparison, the table includes the properties of the commercially available dog kidney ATPase (Sigma), which is a highly ouabain-sensitive preparation ($IC_{50} = 1.7$ μM).

The effects of 12(*R*)-HETE, 12(*S*)-HETE and arachidonic acid on the (Na^+-K^+)ATPase activities are shown in Table 3. 12(*R*)-HETE at 10^{-6} M inhibited all (Na^+-K^+)ATPase enzymes by 52–57%, the only exception being dog kidney ATPase which was inhibited by 21%. In contrast, no specific pattern of inhibition was seen with either 12(*S*)-HETE or arachidonic acid at 10^{-6} M, i.e. in response to the latter, fluctuations between 10% inhibition and 14% stimulation were observed.

The question whether 12(*R*)-HETE inhibits total ATPase or specifically the ouabain-sensitive (Na^+-K^+)ATPase was addressed by incubating the enzyme (either WKY rat renal cortex or bovine corneal epithelium) in the presence of 12(*R*)-HETE with or without 1 mM ouabain and by measuring their effect in the absence of K^+ and Na^+. It was assumed that, if the inhibitory effect of 12(*R*)-HETE in the presence of ouabain was greater than that found with ouabain alone, 12(*R*)-HETE had an effect on the non-Na^+-K^+-dependent phosphatase activity

Table 3. Effects of 12(R)-HETE, 12(S)-HETE and arachidonic acid (AA) on (Na⁺-K⁺)ATPase activity

Source	(Na⁺-K⁺)ATPase activity (μmol P_i/hr/mg)			
	Ouabain-sensitive (Na⁺-K⁺)ATPase	12(R)-HETE	12(S)-HETE	AA
WKY rat renal cortex	91.9 ± 10.2	39.4 ± 4.7 (57%)	91.3 ± 8.7 (0%)	96.2 ± 7.6 (+5%)
Sprague–Dawley heart ventricle	22.7 ± 3.5	10.0 ± 2.3 (56%)	20.5 ± 1.9 (9.0%)	22.9 ± 3.6 (0%)
Dog kidney (Sigma)	70.7 ± 4.1	56.0 ± 6.4 (21%)	77.1 ± 10.4 (+1.1%)	80.6 ± 9.7 (+14%)
Bovine corneal epithelium	24.5 ± 2.9	11.6 ± 2.4 (53%)	23.7 ± 4.6 (3.2%)	24.5 ± 4.8 (0%)

Test compounds (final concentration = 10^{-6} M) were added to the assay mixture 10 min before starting the reaction with the addition of ATP as described in Materials and Methods. Values are means ± SE; N = 6–24. The values in parentheses indicate the per cent inhibition, where 100% indicates complete inhibition of ouabain-sensitive activity; (+) indicate per cent stimulation over control.

Table 4. Effects of 12(R)-HETE on (Na⁺-K⁺)ATPase in the presence and absence of ouabain

Source	(Na⁺-K⁺)ATPase activity (μmol P_i/hr/mg)					
	Total ATPase activity	12(R)-HETE		12(R)-HETE + Ouabain (1 mM)		Ouabain (1 mM)
		10^{-7} M	10^{-6} M	10^{-7} M	10^{-6} M	
WKY rat renal cortex	118.9 ± 12.3	74.1 ± 6.4	66.8 ± 8.3	25.8 ± 3.4	26.3 ± 3.6	27.4 ± 3.8
Bovine corneal epithelium	37.1 ± 3.1	25.8 ± 4.2	24.1 ± 3.9	11.8 ± 2.6	11.8 ± 3.1	12.6 ± 2.9

(Na⁺-K⁺)ATPase activities were measured in the presence of 12(R)-HETE (10^{-7} M and 10^{-6} M) with or without ouabain (1 mM) as described in Materials and Methods. Results are means ± SE, N = 6.

present in the preparations. As seen in Table 4, 12(R)-HETE inhibited the WKY rat and the bovine corneal enzymes by 38–30% at 10^{-7} M and by 44 and 35% at 10^{-6} M respectively. 12(R)-HETE, in the presence of ouabain, inhibited both enzymes by 68–78%. The extent of this inhibition was similar in magnitude to that seen with ouabain alone (66–67%). In the absence of Na^+ and K^+ in the incubation medium, the ATPase activity of the corneal and renal fractions corresponding to 9.8 ± 3.1 and 21.5 ± 3.2 μmol P_i/hr/mg respectively. Addition of either 12(R)- or 12(S)-HETE (10^{-6} to 10^{-5} M) did not cause any significant changes in these values (data not shown).

The inhibitory effect of 12(R)-HETE on (Na⁺-K⁺)ATPase activity was concentration dependent. Figure 1 shows the concentration–response relationship for 12(R)-HETE on the corneal and renal (Na⁺-K⁺)ATPase. The estimated IC_{50} values for the corneal and renal preparations were 0.8×10^{-7} M and 5×10^{-7} M, respectively. 12(S)-HETE at 10^{-6} M inhibited the corneal and renal preparations by 3 and 1%, respectively, and at 10^{-5} M by 8 and 5%, respectively.

These results indicate that 12(R)-HETE had no effect on the non(Na⁺-K⁺)ATPase activity, an activity which accounts for a substantial portion of the total ATP-hydrolyzing activity (5–40%) in these preparations.

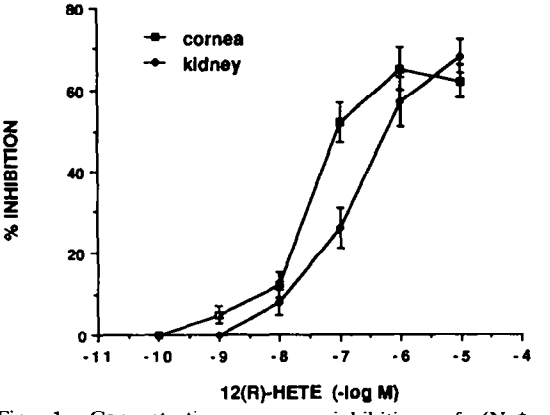

Fig. 1. Concentration–response inhibition of (Na⁺-K⁺)ATPase activity by 12(R)-HETE. Partially purified renal and corneal (Na⁺-K⁺)ATPase were incubated with different concentrations of 12(R)-HETE. 12-(R)-HETE was added to the assay mixture 10 min before starting the reaction with the addition of ATP as described in Materials and Methods. The specific activities of the renal and corneal (Na⁺-K⁺)ATPase were 91.9 ± 10.2 and 24.5 ± 2.9 μmol P_i/hr/mg respectively. Results are means ± SE, N = 4.

DISCUSSION

In the present study, we clearly demonstrated that 12(R)-HETE is a potent inhibitor of (Na⁺-K⁺)ATPase obtained from corneal epithelial, cardiac and renal microsomes. The inhibitory effect of 12(R)-HETE was exclusive for ouabain-sensitive (Na⁺-K⁺)ATPase activity, since this compound had no effect on other ATPases, i.e. the activity in the absence of Na^+ and/or K^+. In contrast, the stereoisomer 12(S)-HETE was inactive at 10^{-7} M and

10^{-6} M, although, like arachidonic acid, it inhibited the (Na^+-K^+)ATPase preparations at higher concentrations. The mechanism for the inhibitory effect of 12(*R*)-HETE on (Na^+-K^+)ATPase is unknown. A steep concentration-response curve has been observed for fatty acids [13, 14], and it is thought that a phase transition of membrane lipids may be triggered when the fatty acid content in the membrane reaches a threshold concentration [15]. However, this effect is seen at much higher concentrations (10^{-5} to 10^{-4} M) than are necessary for 12(*R*)-HETE effects (10^{-7} to 10^{-6} M) furthermore, the inhibition of (Na^+-K^+)ATPase by the 12(*R*)-HETE follows a mass-action relationship, indicating a substrate-reactive site or ligand–receptor interaction rather than membrane perturbation or a non-specific effect on the incubation components (substrates or protein). Relatively high concentrations of 12(*R*)-HETE were required for inhibition of the dog kidney (Na^+-K^+)ATPase obtained from Sigma (IC_{50} ~ 50 μM). This difference in potency may be explained by a low sensitivity of this enzyme to the effect of 12(*R*)-HETE or may be related to the high lipid content of the lyophilized enzyme, approximately 67% lipid,* which might impede access to an active center through non-specific lipid–lipid interactions.

Thus, 12(*R*)-HETE is a potent inhibitor of partially purified (Na^+-K^+)ATPase from different tissues. However, the effectiveness of 12(*R*)-HETE in *in vivo* situations remains uncertain. In a recent study [7], we evaluated the effect of 12(*R*)-HETE on vascular relaxations induced in aortic smooth muscle by exposure to K^+-free buffer. The relaxant response to [K^+], following exposure to zero K^+ buffer, is considered a functional measure of the electrogenic pumping of Na^+ and K^+, and can be attenuated by ouabain [16]. In these tissues bath experiments, 12(*R*)-HETE inhibited K^+-induced relaxations in a concentration-dependent manner, diminishing the peak maximum response and causing a rightward shift of the K^+ concentration–response curve. 12(*S*)-HETE was much weaker in this regard, blocking the maximal effect but not affecting the EC_{50}. Furthermore, Takahashi *et al.* [8] and Quilley and McGiff [17] reported that 12(*R*)-HETE increases urine volume and electrolyte excretion in the isolated perfused rat kidney, effects consistent with inhibition of (Na^+-K^+)ATPase activity. Evidence for a link between pump inhibition by 12(*R*)-HETE and the function of specialized transport epithelia has also been found in the cornea. Infusion of 12(*R*)-HETE into the endothelial side of the isolated perfused rabbit cornea produced a dose-dependent increase in corneal thickness, an effect characteristic of ouabain and other inhibitors of corneal endothelial (Na^+-K^+)ATPase activity.†

The present results and previous evidence, including enantiomer specific biologic and enzymatic effects, strongly suggest that endogenous levels of 12(*R*)-HETE may serve a variety of physiological and or pathophysiological functions *in vivo*.

* Dr. E. Spokas, Searle R & D, personal communication. Cited with permission.

† Dr. H. Edelhauser, Medical College of Wisconsin, personal communication. Cited with permission.

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REFERENCES

1. Schwartzman ML, Balazy M, Masferrer J, Abraham NG, McGiff JC and Murphy RC, 12(*R*)-Hydroxyeicosatetraenoic acid: A cytochrome P450-dependent arachidonate metabolite that inhibits Na^+-K^+-ATPase in the cornea. *Proc Natl Acad Sci USA* **84**: 8125–8129, 1987.
2. Capdevila J, Yadagiri P, Manna S and Falck JR, Absolute configuration of the hydroxyeicosatetraenoic acids (HETEs) formed during catalytic oxygenation of arachidonic acid by microsomal cytochrome P-450. *Biochem Biophys Res Commun* **141**: 1007–1011, 1986.
3. Woolard PM, Stereochemical difference between 12-hydroxy-5,8,10,14-eicosatetraenoic acid in platelets and psoriatic lesions. *Biochem Biophys Res Commun* **136**: 169–172, 1971.
4. Hamberg M and Samuelsson B, Prostaglandin endoperoxides: Novel transformation of arachidonic acid in human platelets. *Proc Natl Acad Sci USA* **71**: 3400–3404, 1974.
5. Hamasaki Y and Tai HH, Calcium stimulation of a novel 12-lipoxygenase from rat basophilic leukemia (RBL-1) cells. *Biochim Biophys Acta* **793**: 393–398, 1984.
6. Cunningham FM and Woollard PM, 12(*R*)-Hydroxyeicosatetraenoic acid is a chemoattractant for human polymorphonuclear leukocytes *in vitro*. *Prostaglandins* **34**: 71–78, 1987.
7. Masferrer J and Mullane KM, Modulation of vascular tone by 12(*R*)-, but not 12(*S*)-hydroxyeicosatetraenoic acid. *Eur J Pharmacol* **151**: 487–490, 1988.
8. Takahashi K, Capdevila J, Falck JR, Jacobsen HR and Badir KF, Effects of cytochrome-P450-derived metabolites of arachidonic acid (AA) on renal hemodynamics and excretory functions in the rat. *FASEB J* **2**: A1262, 1988.
9. Jorgensen PL, Isolation of Na^+-K^+-ATPase. *Methods Enzymol* **32**: 277–290, 1974.
10. Post RL and Sen AK, Sodium and potassium-stimulated ATPase. *Methods Enzymol* **10**: 762–768, 1967.
11. Candida OA, Lanzetta PA, Alvarez JL and Gaines W, Inhibition of ionic transport and ATPase activities by serotonin analogues in the isolated focal lens. *Biochim Biophys Acta* **602**: 389–400, 1980.
12. Lanzetta PA, Alvarez LJ, Reinach PS and Candida OA, An impoverished assay for nanomole amounts of inorganic phosphate. *Anal Biochem* **100**: 95–97, 1979.
13. Ahmed K and Thomas BS, The effects of long chain fatty acids on sodium plus potassium ion-stimulated adenosine triphosphatase of rat brain. *J Biol Chem* **246**: 103–109, 1971.
14. Bidard J-N, Rossi B, Renaud J-F and Lazdunski MA, A search for an 'ouabain-like' substance from the electric organ of *Electrophorus electricus* which led to arachidonic acid and related fatty acids. *Biochim Biophys Acta* **769**: 245–252, 1984.
15. Kelley RA, O'Hara DS, Mitch WE and Smith TW, Identification of Na^+-K^+-ATPase inhibitors in human plasma as nonesterified fatty acids and lysophospholipids. *J Biol Chem* **261**: 11704–11710, 1986.
16. Webb RC and Bohr DF, Potassium-induced relaxation as an indicator of Na^+-K^+-ATPase activity in vascular smooth muscle. *Blood Vessels* **15**: 198–207, 1978.
17. Quilley CP and McGiff JC, Renal effects of cytochrome P450-dependent metabolite of arachidonic acid: 12(*R*)hydroxyeicosatetraenoic acid. *Br J Pharmacol* **96**: 59P, 1989.