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DETECTION OF 20-HYDROXYEICOSATETRENOIC ACID IN RAT URINE

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20-Hydroxyeicosatetraenoic acid (20-HETE), an arachidonate metabolite of the cytochrome P450 ω hydroxylase, was detected in rat urine by gas chromatography-mass spectrometric techniques. The concentration of 20-HETE in urine from 7-week-old hypertensive and normotensive rats was 2.1 and 1.3 nM, respectively. This is the first demonstration of 20-HETE urinary excretion and thus calls attention to the possibility that 20-HETE participates in the regulation of renal function via its effect on vascular tone and ion transport processes.

20- Hydroxyeicosatetraenoic acid (20-HETE) is a metabolite of arachidonic acid formed by a cytochrome P450 ω-hydroxylase activity in human kidney, liver, neutrophils and bone marrow (1-3). It is a potent vasoconstrictor of isolated rat aorta, rabbit mesenteric, carotid and renal arteries, and dog renal microvessels (4,5). 20-HETE has also been shown to cause natriuresis when administered to anesthetized euvolemic rats (6), and to inhibit $^{86}Rb^{+}$ uptake in the isolated medullary thick ascending limb cells from the rabbit (7). In addition to its biological activities it is also an excellent substrate for cyclooxygenase (8). Hence, it can compete and interfere with arachidonic acid metabolism to prostanoids that are important in the modulation of cellular functions. Recently, we demonstrated that 20-HETE is the major cytochrome P450 arachidonate metabolite formed in cortical microsomes from kidneys of SHR and WKY rats where its formation is drastically increased at 5-7-week of age. Its production by SHR renal microsomes is 2 to 4-fold higher than by WKY renal

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microsomes (9). An abrupt increase of 30-fold over the fetal and newborn levels occurs between 3-weeks and 5-weeks of age. 20-HETE production in renal microsomes is sustained during the period of rapid elevation of blood pressure in the SHR (5 to 9 weeks) followed by a decrease to the newborn level (9). It is, therefore, important to assess whether 20-HETE is an endogenous metabolite of arachidonic acid. In the present study , we measured the level of 20-HETE in urine samples that were collected from 6-7-week-old SHR and WKY rats.

Materials and Methods

Materials. Pentafluorobenzyl (PFB) bromide and N,N-diisopropylethylamine were obtained from Sigma Chemical Co. (St. Louis, MO). N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide was from Pierce Chemical Co. (Rockford, IL). Synthetic 20-HETE and [20,20-$^{2}H_{2}$] 20-HETE were prepared by modification of a repoted procedure (10). [5,6,8,9,10,11,14,15-^{3}H]-20-HETE (240 Ci/mmol) was a gift from New England Nuclear (Boston, MA).

Urine extraction. Urine was collected for 7 days from 6-week-old SHR and WKY rats. ^{3}H-20-HETE (50,000 cpm) and [20,20-$^{2}H_{2}$] 20-HETE (100 ng) were added to 20 ml of the pooled urine. The sample was adjusted to pH 8.0 with 1N NaOH and allowed to stand at room temperature for 15 min. Following acidification to pH 3.0 with 0.1N HCl, the sample was applied onto a Tox Elut column (Analytical International, Harbor City, CA) and eluted 3 times with 20 ml dichloromethane. The combined organic extracts was washed with 0.05N sodium borate buffer (pH 8.0) and evaporate to dryness. The residue was resuspended in 200 µl of methanol and lipids were separated by thin layer chromatography on silica gel G using the upper phase of ethyl acetate:water:iso-octane:acetic acid (110:100:50:20, v/v/v/v). Radioactivity was monitored by a radioactive scanner and the zone corresponding to 20-HETE standard was scraped and extracted with ethyl acetate. The extract was evaporate to dryness, resuspended in 100 µl acetonitrile and converted to the PFB ester by adding 10 µl of pentafluorobenzyl bromide and 10 µl of N,N-diisopropylethylamine and incubating it at room temperature for 30 min. The PFB ester derivative was further purified by thin layer chromatography on silica gel G using hexane:ethyl acetate (3:1 v/v) as the solvent. The radioactive zone which corresponded to the 20-HETE-PFB standard was scraped and extracted with ethyl acetate. The final extract was evaporated and incubated with 100 µl of N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide for 30 min at 65^{o}C to obtain the PFB ester, *tert*-butyldimethylsilyl (TBDMS) ether derivative of 20-HETE. The samples were concentrated to dryness and dissolved in 20 µl of octane for GC/MS analysis.

Gas-chromatography/ mass-spectrometry (GC/MS). Negative chemical ionization (NCI)-GC/MS was performed on a HP 5989A (Hewlett Packard, Palo Alta, CA) mass spectrometer interfaced with a SP-2330 capillary column (15 m x 0.25mm inside diameter, 0.2 µm

film thickness, Supelco, Bellefonte, PA) programmed from 100-250°C at 25°C/min using helium as carrier gas. The mass spectrometer was scanned from 200-600 mass units in one second or single ions, *m/z* 433 for the endogenous derivatized 20-HETE (PFB ester TBDMS ether) and *m/z* 435 for the derivatized internal standard, were monitored. An estimation of the total 20-HETE content in the purified biological sample was made by comparison of the ion intensities at *m/z* 433:435 vs. a standard curve of 20-HETE-PFB-TBDMS/$^{2}H_{2}$-20-HETE-PFB-TBDMS molar ratio constructed by NCI-GC/MS analysis.

Results and Discussion

The presence of 20-HETE in urine from SHR and WKY rats was established by comparing, under identical conditions, the chromatographic and mass spectral properties of esterified, silylated urine extract with similarly treated synthetic 20-HETE. NCI-GC/MS analysis of the PFB ester TBDMS ether of synthetic 20-HETE demonstrated the elution of *m/z* 433 (M-181, M-PFB) at 7.49 min. Although the thin layer chromatography steps included in the extraction procedure do not separate 19-HETE from 20-HETE, the capillary GC column does separate these similar molecules. The retention time of the PFB ester TBDMS ether derivative of the synthetic 19-HETE was 7.2 min (Figure 1). The PFB ester TBDMS ether derivative of the internal standard, $^{2}H_{2}$-20-HETE, *m/z* 435, eluted slightly after the derivatized undeuterated 20-HETE at 7.51 min (Figure 1). Analysis of the relative abundance of *m/z* 433, *m/z* 434, *m/z* 435 and *m/z* 436 in the internal standard showed a ratio of 1:0:22:8.

The elution profile of *m/z* 433 in urinary extracts from SHR and WKY demonstrated a single peak which co-eluted with the *m/z* 433 of the authentic 20-HETE derivatized standard at 7.47 min (Figure 2A and 2B). The mass fragmentation pattern of the peak at 7.47 min derived from the urine sample was similar to that of authentic 20-HETE-PFB-TBDMS analyzed under identical conditions (Figure 2C and 2D). These results further confirmed that the urinary peak at 7.47 min is 20-HETE. The concentration of 20-HETE in SHR urine was estimated by measuring the ion intensities at *m/z* 433:435 (areas under the corresponding fragmentograms) and comparing them with a standard curve of ion intensities vs. molar ratio. Based on the yield of radiolabeled recovery standard, it was calculated that the concentration of 20-HETE in urine from 6-7-week-old SHR was 2.1±0.3 nM whereas the concentration in age-matched WKY was 1.3±0.4 nM (mean±SE, n=3).

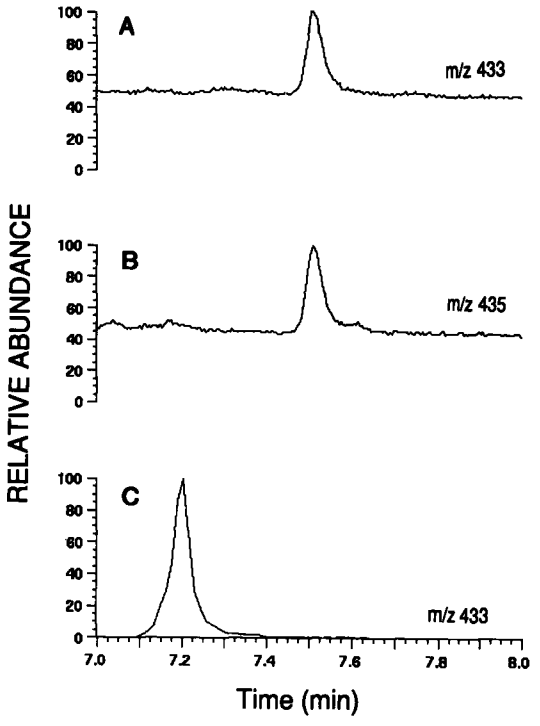

Figure 1. NCI/GC-MS analysis of selected PFB-TBDMS-derived ions present in synthetic standards. Derivatized standards were injected onto the capillary GC column and column eluents were monitored by mass fragmentography at either *m/z* 433 or *m/z* 435. (A) 20-HETE; (B) $^{2}H_{2}$-20-HETE; and (C) 19-HETE.

We have previously demonstrated that total renal microsomal cytochrome P450-dependent metabolism of AA is increased in SHR and that renal cortical ω and ω-1 hydroxylases, but not epoxygenase activities, were significantly higher in SHR than in WKY (9). In accordance with our previous reports, the concentration of 20-HETE in the urine was significantely higher in SHR than in WKY. The production rate of the vasoconstrictor 20-HETE increased drastically at the developmental phase of hypertension in SHR. 20-HETE may participate in blood pressure elevation by contributing as a vasoconstrictor to the increased total peripheral resistance which has been postulated as an important factor for maintenance of hypertension in SHR.

The present study establishes for the first time the presence of 20-HETE in rat urine and documents differential levels in SHR and WKY excretion. These findings indicate the endogenous origin of 20-HETE and further implicate the physiological significance of this biologically active compound.

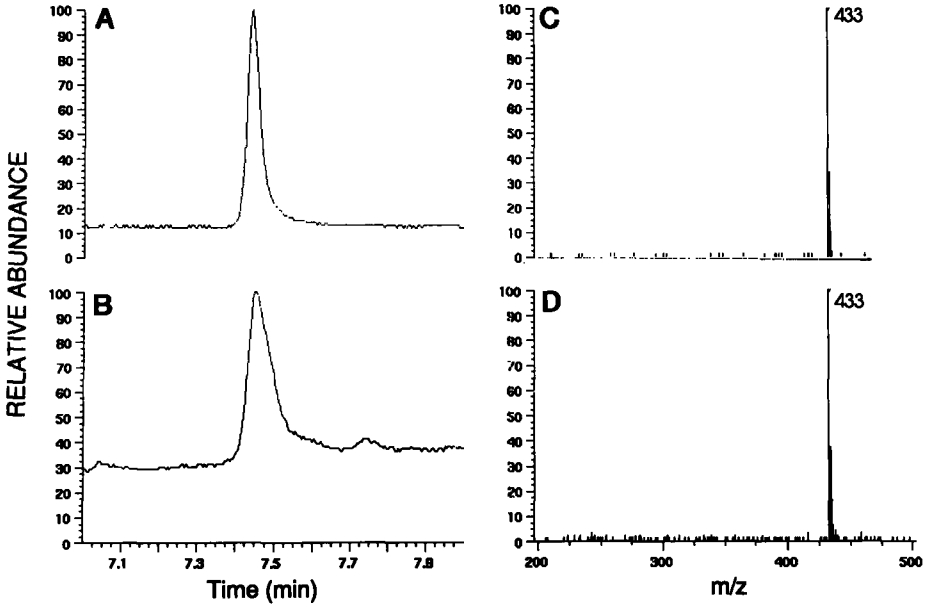

Figure 2. NCI/GC-MS analysis of the purified urine sample. Urine extract was derivatized to the PFB ester TBDMS ether and injected onto the capillary GC column. The eluent was monitored by NCI-MS. (A) Selected ion monitoring of *m/z* 433 in the synthetic 20-HETE standard and (B) in the urine sample; (C) Mass spectrum of 20-HETE standard; and (D) Mass spectrum of the peak in urine sample eluting at 7.47 min.

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