Adrenic Acid Metabolites as Endogenous Endothelium-Derived and Zona Glomerulosa-Derived Hyperpolarizing Factors

Phillip G. Kopf, David X. Zhang, Kathryn M. Gauthier, Kasem Nithipatikom, Xiu-Yu Yi, John R. Falck, William B. Campbell

Abstract—Adrenic acid (docosatetraenoic acid), an abundant fatty acid in the adrenal gland, is identical to arachidonic acid except for 2 additional carbons on the carboxyl end. Adrenic acid is metabolized by cyclooxygenases, cytochrome P450s, and lipoxygenases; however, little is known regarding the role of adrenic acid and its metabolites in vascular tone. Because of its abundance in the adrenal gland, we investigated the role of adrenic acid in vascular tone of bovine adrenal cortical arteries and its metabolism by bovine adrenal zona glomerulosa cells. In adrenal cortical arteries, adrenic acid caused concentration-dependent relaxations, which were inhibited by the epoxyeicosatrienoic acid antagonist 14,15-epoxyeicos-5(Z)-enoic acid and the cytochrome P450 inhibitor SKF-525A. The large-conductance calcium-activated potassium channel blocker iberiotoxin or removal of the endothelium abolished these relaxations. Reverse-phase high-pressure liquid chromatography and liquid chromatography/mass spectrometry isolated and identified numerous adrenic acid metabolites from zona glomerulosa cells, including dihomo-epoxyeicosatrienoic acids and dihomo-prostaglandins. In denuded adrenal cortical arteries, adrenic acid caused concentration-dependent relaxations in the presence of zona glomerulosa cells but not in their absence. These relaxations were inhibited by SKF-525A, 14,15-epoxyeicos-5(Z)-enoic acid, and iberiotoxin. Dihomo-16,17-epoxyeicosatrienoic acid caused concentration-dependent relaxations of adrenal cortical arteries, which were inhibited by 14,15-epoxyeicos-5(Z)-enoic acid and high potassium. Our results suggest that adrenic acid relaxations of bovine adrenal cortical arteries are mediated by endothelial and zona glomerulosa cell cytochrome P450 metabolites. Thus, adrenic acid metabolites could function as endogenous endothelium-derived and zona glomerulosa-derived hyperpolarizing factors in the adrenal cortex and contribute to the regulation of adrenal blood flow. (Hypertension. 2010;55[part 2]:547-554.)

Key Words: adrenal cortex ■ cyclooxygenase ■ cytochrome P450 ■ endothelium-dependent relaxation ■ endothelium-derived hyperpolarizing factor ■ epoxygenase ■ potassium channels

Arachidonic acid (20:4; ω-6) is the precursor to eicosanoids, a group of signaling molecules with diverse physiological action, including the regulation of vascular tone. Arachidonic acid (7,10,13,16-docosatetraenoic acid, 22:4; ω-6) is an abundant polyunsaturated fatty acid in the adrenal gland, brain, kidney, and vasculature that is identical to arachidonic acid except for 2 additional carbons on the carboxyl end.1-5 Adrenic acid is formed by arachidonic acid chain elongation or elongation and desaturation of the essential fatty acid linoleic acid (18:2; ω-6), the precursor to arachidonic acid.1,6 Adrenic acid can also be converted to arachidonic acid by β-oxidation.1,3,6,7

As with arachidonic acid, adrenic acid is metabolized by cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450s (CYP450s) to dihomo (DH) eicosanoids. Adrenic acid is metabolized in the renal medulla to DH-thromboxane and DH-prostaglandins (PG),1 and in platelets to DH-thromboxane and DH-hydroxyeicosatetraenoic acids (HETE).8 Our laboratory has demonstrated that adrenic acid is metabolized in human vascular endothelial cells to DH-prostacyclin (PGI2), which inhibited thrombin-induced platelet aggregation.9 We have also demonstrated that adrenic acid induces concentration-dependent relaxations of bovine coronary arteries, which were endothelium-dependent, blocked by K+ channel inhibition, and inhibited by COX and CYP450 inhibition.10 Several adrenic acid metabolites of coronary arteries were isolated and identified, including DH-PG and DH-epoxyeicosatrienoic acids (EET). In particular, DH-16,17-EET, the dihomo analog of 14,15-EET, induced concentration-dependent relaxations by activation of vascular smooth muscle K+ channels and hyperpolarization.10 These observations are consistent with another study examining the effect of DH-EETs in porcine coronary arteries.11

Whereas adrenic acid metabolites play a potential role in the regulation of vascular tone in the coronary circulation,
litter is known concerning the role of adrenic acid in other vascular beds. Because of its abundance in the adrenal gland, we investigated the role of adrenic acid in the regulation of vascular tone of adrenal cortical arteries. A complex interaction occurs between the adrenal zona glomerulosa (ZG) cells and its vasculature in the regulation of adrenal arterial tone. Therefore, we additionally investigated the metabolism of adrenic acid by ZG cells and the vascular effect of ZG cell-derived docosanoids on adrenal cortical arteries.

**Materials and Methods**

**Vascular Reactivity**

Fresh bovine adrenals were obtained from a local slaughterhouse. Small adrenal cortical arteries closely attached to the adrenal surface (200–300 μm) were dissected and cleaned of connective tissues. Isolated arterial segments were threaded on 2 stainless steel wires (40 μm diameter) and mounted on a 4-chamber wire myograph (model 610M; Danish Myo Technology) in physiological saline solution (PSS) containing 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 24 mM NaHCO₃, 1.18 mM KH₂PO₄, 26 μM EDTA, and 5.5 mM glucose, bubbled with 95% O₂–5% CO₂ at 37°C for 30 minutes, as previously described. Arteries were gradually stretched to a resting tension of 1 millinewton (mN) and stimulated with KCl (60 mmol/L) plus U-46619 (100 nmol/L) 3 times gradually stretched to a resting tension of 1 mN and stimulated with KCl (60 mM) plus U-46619 (100 μM) 3 times at 10-minute intervals. Arteries were then allowed to equilibrate for another 30 minutes before the initiation of experimental protocols.

The arteries were preconstricted to 50% to 75% of maximal KCl contraction by addition of the thromboxane A2 mimetic U-46619. Contractions were performed in the presence of ZG cells (5×10⁶) and pretreated with the CYP450 inhibitor, SKF-525A (10 μM) for 10 minutes at 10-minute intervals. Arteries were then allowed to equilibrate for another 30 minutes before the initiation of experimental protocols.

**Adrenal ZG Cell Isolation**

ZG cells were prepared by enzymatic dissociation of adrenal cortical slices as previously described. Cells were 95% to 98% ZG cells and 2% to 5% zona fasciculata cells. Adrenal fibroblasts were isolated and cultured as previously described.

**Metabolism of Adrenic Acid by ZG Cells**

ZG cells (5×10⁶) were incubated in 5 mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at 37°C with [1-¹⁴C] adrenic acid (0.8 μCi, 10⁻⁷ M). After 15 minutes, calcium ionophore A-23187 was added to final concentration of 5 μg/mL and the incubation continued for 10 minutes. Incubations were stopped by adding ethanol to 15% and buffer and cells were separated by centrifugation. The buffer was removed and extracted using C18 Bond Elut solid phase extraction columns (Varian) as previously described. The samples were evaporated to dryness under a stream of N₂ and stored at −80°C until analysis of high-pressure liquid chromatography (HPLC). For liquid chromatography–mass spectrometry, incubations were repeated with using adrenic acid (10⁻⁷ M) and ZG cells (5×10⁶) under similar conditions.

**Reverse-Phase HPLC**

Adrenic acid metabolites were resolved by reverse-phase HPLC (Nucleosil; C18 column, 5 μm, 4.6×250 mm) using solvent system I as previously described. Solvent A was water and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program was a 35-minute linear gradient from 50% solvent B to 94% solvent B and flow rate of 1 mL/min. Column eluates were collected in 0.2-mL fractions and radioactivity of each fraction was determined by liquid scintillation spectrometry.

For liquid chromatography–mass spectrometry, column eluates were collected as 4 major peaks corresponding to the reported major adrenic acid metabolites. The retention time range of the 4 peaks are: 3 to 6.8 minutes, 15.6 to 20.6 minutes, 21 to 24.6 minutes, and 24.6 to 30 minutes. These fractions were acidified with glacial acetic acid and extracted with cyclohexane/ethyl acetate (50:50). The combined organic extracts were dried under a stream of N₂ and stored at −40°C until liquid chromatography–tandem mass spectrometry analysis.

**Liquid Chromatography–Electrospray Ionization Mass Spectrometry**

The chemical identity of the major metabolites isolated by reverse-phase HPLC was determined by liquid chromatography–electrospray ionization mass spectrometry. HPLC was performed using a reverse-phase C18 column (Kromasil; 250×2 mm) and a Waters 2695 liquid chromatograph (Waters Corporation). Samples were dissolved in acetonitrile and the volume of injection was 10 μL. The mobile phase consisted of solvent A, water containing 0.01% glacial acetic acid, and solvent B, acetonitrile containing 0.01% glacial acetic acid. The program was a 40-minute linear gradient from 50% solvent B to 100% solvent B with a flow rate of 0.2 mL/min. Mass spectrometry was performed using a Micromass Quattro Micro API mass spectrometer (Waters Corporation) equipped with an electrospray ionization source. The mass spectrometer was operated in the negative ion mode. Product ion spectra were generated by collision-induced decomposition of the precursor ions (m/z 381 for DH-PGF₂α; m/z 379 for DH-PGE₂; m/z 363 for DH-dihydroxyeicosatetraenoic acids; m/z 365 for DH-dihydroxyeicosatrienoic acid; m/z 307 for 14,15-dihydroxy-7,10,12-triadecatrienoic acid; and m/z 347 for DH-HETE and DH-EET, respectively). Only the precursor ion was allowed to pass through the first quadrupole, and the ion was activated by collision with argon in the second quadrupole. Product ion spectra were recorded for the m/z range of 50 to 420. Results were processed using Masslynx version 4.0 software (Micromass).

**Whole-Cell Patch Clamp**

Bovine adrenal cortical artery smooth muscle cells were freshly isolated and whole-cell recordings of K⁺ currents were obtained as previously described.

**Statistics**

Vascular reactivity and patch-clamp data are expressed as mean±SEM. Significant differences between mean values were evaluated by ANOVA, followed by the Student-Newman-Keuls multiple comparison test. Significance was accepted at a value of P<0.05.

**Results**

**Vascular Response to Adrenic Acid**

Adrenic acid caused concentration-dependent relaxations of the preconstricted adrenal cortical arteries with a maximal response of 70±2% at 10⁻⁴ M (Figure 1). The CYP450 inhibitor SKF-525A (10 μmol/L) and the EET antagonist 14,15-epoxyeicosa-5(Z)-enonic acid (10 μmol/L) attenuated the adrenic acid relaxations (maximal relaxations of 28±8%...
and 17±4%, respectively, at 10⁻⁴ M). Treatment of arteries with the BKCa channel blocker, iberiotoxin (100 nmol/L), or the removal of endothelium abolished the relaxations. Indomethacin did not alter adrenic acid relaxations. These findings suggest that endothelial CYP450 metabolites mediate the relaxations to adrenic acid and the relaxations are dependent on K⁺ channel activation.

**Vascular Response to ZG Cell Metabolites of Adrenic Acid**

Steroid-producing cells produce diffusible vasorelaxing factors. To examine if ZG cells metabolize adrenic acid to a vasorelaxant, adrenic acid concentration responses were performed with denuded adrenal cortical arteries in the presence of ZG cells (Figure 2). Denuded arteries do not relax in response to adrenic acid alone; therefore, any observed relaxations of denuded vessels to adrenic acid in the presence of ZG cells can be attributed to adrenic acid metabolites produced by ZG cells.

For consistency with previous experiments, all denuded vessels were pretreated with the endothelial nitric oxide synthase inhibitor, 6-nitro-arginine (30 μmol/L). Indomethacin (10 μmol/L) did not alter the response to adrenic acid (data not shown) and was included in all further preparations. The addition of ZG cells (5×10⁵–1×10⁶) to preconstricted adrenal cortical arteries resulted in a slight relaxation (27±2%), consistent with previous observations. Adrenic acid caused a slight contraction at 10⁻⁷ M (15±3%) but concentration-dependent relaxations at higher concentrations (10⁻⁶–10⁻⁴ M; maximal response of 89±3% at 10⁻⁴ M). These relaxations were abolished by SKF-525A (10 μmol/L), a CYP450 inhibitor that does not inhibit aldosterone synthesis. The EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (10 μmol/L) also abolished these relaxations, as did high K⁺ (60 mmol/L) and iberiotoxin (100 nmol/L). Inhibition of lipooxygenases with cinnamyl-3,4-dihydroxy-cyanocinnamate (10 μmol/L) did not alter the relaxations to adrenic acid but inhibited the contractions observed with 10⁻⁷ M adrenic acid. When adrenal fibroblasts were substituted for ZG cells, no significant vascular responses to adrenic acid were detected. These results suggest that ZG cells produce transferrable CYP450 metabolites of adrenic acid, presumably a DH-EET that induces relaxations by activation of smooth muscle cell K⁺ channels.

**Metabolism of Adrenic Acid by Bovine ZG Cells**

Because CYP450 metabolites appear to contribute to adrenic acid-induced relaxation, we investigated the metabolism of adrenic acid by ZG cells. Cells were incubated with 14C-labeled adrenic acid and the medium was extracted and purified by HPLC. Adrenic acid was metabolized to 4 major peaks (Figure 3). The 4 peaks were separately collected and analyzed by liquid chromatography–electrospray ionization mass spectrometry (Table). Peak 1 (fractions 3–6.8 minutes) metabolites comigrated with the DH-PG. The metabolite eluting at 6.20 minutes (peak 1A) comigrated with the DH-PG. The metabolite eluting at 8.4 minutes (peak 1B) comigrated with the DH-PG. The metabolite eluting at 11.6 minutes (peak 1C) comigrated with the DH-PG. The metabolite eluting at 14.4 minutes (peak 1D) comigrated with the DH-PG.
Metabolites in peak 2 (fractions 15.6–20.6 minutes) eluted liquid chromatography–electrospray ionization mass spectrometry between 20 to 24 minutes (Table). There were 3 groups of metabolites in peak 2. The molecular weight of the first group (2A–2E) was 364, which exceeds the molecular weight of DiHETE (molecular weight=336) by 28 (2CH₂). The metabolites eluted at 20.60, 21.80, 22.25, 23.15, and 23.89 minutes. The fragmentation patterns of these 5 metabolites correspond to DH-dihydroxyeicosatetraenoic acid and were identified as DH-10,17-DiHETE, DH-13,15-DiHETE, DH-13,17-DiHETE, DH-10,14-DiHETE, and DH-7,14-DiHETE, respectively. The molecular weight of the second group of metabolites from peak 2 (2F–2I) was 366, which exceeds the molecular weight of DHET (molecular weight=338) by 28 (2CH₂). They eluted at 20.10, 21.25, 21.9, and 22.57 minutes. For the peak with a retention time of 20.10 minutes (peak 2F), major ions were 365 [M-H]⁻, 347 [M-H-H₂O]⁻, and 235 (cleavage between C15 and C16), indicating DH-16,17-DHET (Table, Figure 4B). The spectra of the peaks at 21.25, 21.9, and 22.57 minutes (2G, 2H, and 2I) showed similar ions at 365 [M-H]⁻ and 347 [M-H-H₂O]⁻, with fragmentation patterns indicating DH-13,14-DHET, DH-10,11-DHET, and DH-7,8-DHET, respectively.

A third metabolite in peak 2 (2J) eluted at 21.6 minutes. It had a molecular weight of 308 and its major ions corresponded to 14-hydroxy-7,10,12-nonadecatrienoic acid.

Metabolites eluting in peak 3 (fractions 21–24.6 minutes) were resolved by liquid chromatography–electrospray ionization mass spectrometry and had elution times of 27.7, 28.6, 29.2, 29.5, and 29.87 minutes (Table). These 5 compounds had identical molecular weights of 348, which exceeds the molecular weight of 340 by 8 (2CH₂). They were identified as DH-17-HETE, DH-13-HETE, DH-14-HETE, DH-7-HETE, and DH-11-HETE, respectively.

A fourth metabolite in peak 3 (4A) eluted at 33.34 minutes. It had a molecular weight of 347 and its major ions corresponded to 14-hydroxy-7,10,12-nonadecatrienoic acid.
molecular weight of the HETE (molecular weight = 320) by 28 (2CH₂), suggesting DH-HETE. Analysis of the peak with a retention time of 27.7 minutes (3A) indicated DH-17-HETE. The other 4 compounds in peak 3 (3B–3E), showed similar ions at 347 [M-H]⁻ and 329 [M-H-H₂O]⁻. Collisional dissociation produced fragmentation patterns indicating DH-13-HETE, DH-14-HETE (Figure 4C), DH-7-HETE, and DH-11-HETE, respectively.

Metabolites eluting in peak 4 (fractions 24.6–30 minutes) comigrated on liquid chromatography–electrospray ionization mass spectrometry with authentic DH-EET standards. Elution times were 33.34, 34.6, 34.85, and 35.51 minutes. Their molecular weight of 348 was identical to DH-EET. The tandem mass spectrometry analysis spectra of the four metabolites (peaks 4A–4D) were identical to the DH-16,17-, DH-13,14-, DH-10,11-, and DH-7,8-EET standards, respectively.

Vascular Response to DH-16,17-EET

In precontracted coronary arteries, DH-16,17-EET causes concentration-related relaxations with a maximal relaxation at 10⁻⁵ M (96 ± 1%; Figure 5A). 14,15-epoxyeicosa-5(Z)-enoic acid (10 μmol/L) inhibited the relaxation to DH-16,17-EET at lower concentrations but only attenuated the relaxation at 10⁻⁵ M. Relaxations to DH-16,17-EET were abolished by pretreatment with high K⁺ (60 mmol/L). Thus, DH-16,17-EET–induced relaxations are dependent on K⁺ channel activation and are attenuated by 14,15-epoxyeicosa-5(Z)-enoic acid.

Activation of Smooth Muscle Cell K⁺ Currents by DH-16,17-EET

Macroscopic, whole-cell, outward K⁺ currents were generated by 10 mV depolarizing steps from −60 mV to 60 mV in isolated bovine adrenal cortical artery smooth muscle cells (Figure 5B, C). DH-16,17-EET (10⁻⁷–10⁻⁶ M) activated outward K⁺ currents in a concentration-dependent manner. At 60 mV, DH-16,17-EET (10⁻⁶ M) increased current density by 353%. A subsequent addition of iberiotoxin reduced current density to 149% of control current density. Membrane capacitance averaged 51.4 ± 7.8 pF. These results demonstrate that DH-16,17-
EET activates iberiotoxin-sensitive K⁺ channels of isolated bovine adrenal cortical artery smooth muscle cells.

Discussion

This is the first study to our knowledge to examine the role of adrenic acid in the regulation of vascular tone in arteries of the adrenal cortex, a tissue with an abundance of adrenic acid. Adrenic acid relaxations of bovine adrenal cortical arteries are mediated by the production of endothelial and ZG cell CYP450 metabolites. These findings further indicate that a functional and intimate interaction exists between the vasculature and the closely associated steroidogenic cells of the adrenal gland.

Adrenic acid caused concentration-dependent relaxations of preconstricted adrenal cortical arteries, which were endothelium-dependent. COX inhibition had no affect on these relaxations; however, these relaxations were attenuated by CYP450 inhibition or EET antagonism and abolished by BKCa channel blockade. These data suggest that adrenic acid mediates relaxation of adrenal cortical arteries by an endothelium-derived CYP450 metabolite, presumably an EET or DH-EET, which activates the BKCa channels of vascular smooth muscle cells. In bovine coronary arteries, exogenous adrenic acid also caused relaxation, which was mediated by endothelial COX and CYP450 metabolites that activate K⁺ channels with resulting vascular smooth muscle cell hyperpolarization. These vasoactive metabolites were identified as DH-PGI₂ and DH-EET. These similarities suggest that endothelial metabolism of adrenic acid may contribute factors involved in the regulation of vascular tone in multiple vascular beds. Interestingly, COX metabolites contribute to adrenic acid relaxations in the coronary vasculature but not in the adrenal vasculature. With respect to adrenic acid, this difference between vascular beds suggests a greater role of COX metabolism in the bovine coronary vasculature and of CYP450 metabolism in the bovine adrenal vasculature.

Several lines of evidence indicate that ZG cells metabolize adrenic acid into vasorelaxing factors. Adrenic acid caused concentration-dependent relaxations of denuded adrenal cortical arteries when incubated with ZG cells. These relaxations were abolished by CYP450 inhibition, EET antagonism, high K⁺, and BKCa channel blockade. COX inhibition had no effect on these relaxations. As with endothelium-dependent relaxation in response to adrenic acid, the relaxant factor produced by ZG cells is likely a CYP450 metabolite that causes hyperpolarization of vascular smooth muscle cells by activation of BKCa channels. On stimulation by ACTH, ZG cells produce EETs that diffuse to adjacent arteries and cause relaxation. Adrenic acid-derived DH-EETs may represent another possible vasorelaxing factor from ZG cells.

ZG cells metabolize adrenic acid by COX to the DH-PG, by LO to DH-HETE and DH-dihydroxyeicosatetraenoic acid, and by CYP450 to DH-EET and dihydroxyeicosatrienoic acid (Figure 6). Identification of these metabolites was based on reverse-phase HPLC comigration with known DH-PG and DH-EET standards and analysis of tandem mass spectrometry analysis spectra. Thus, adrenic acid is metabolized by ZG cells to a myriad of docosanoids by COX, LO, and CYP450 enzymatic pathways. Bovine coronary vessels produce a
similar array of COX, LO, and CYP450 metabolites of adrenic acid. Moreover, adrenic acid is metabolized to DH-thromboxane by COX and DH-14-EET by 12-LO in human platelets, DH-PG and DH-thromboxane by the renal medulla, and DH-PGI2 by COX in human endothelial cells.

Because both the endothelium-mediated and ZG cell-mediated relaxations to adrenic acid involve CYP450 metabolism and require K+ channel activation, a DH-EET is a likely metabolite contributing to these relaxations. DH-16,17-EET caused concentration-dependent relaxations of preconstricted adrenal cortical arteries. These relaxations were attenuated by EET antagonism and abolished by high K+.

Moreover, DH-16,17-EET activated iberiotoxin-sensitive outward K+ currents in isolated bovine adrenal cortical smooth muscle cells. These results are similar to results concerning DH-16,17-EET in bovine coronary arteries from a previous study. Thus, the mechanism of relaxation induced by DH-16,17-EET is similar to EET, which diffuses to the vascular smooth muscle and activate membrane K+ channels, resulting in hyperpolarization and vascular relaxation.

**Perspectives**

Endothelium-derived and ZG cell-derived adrenic acid metabolites may play a role in the regulation of adrenal vascular tone. Adrenic acid relaxations of adrenal cortical arteries are mediated by endothelial and ZG cell CYP450 metabolites. Moreover, the closely associated ZG cells produce COX, LO, and CYP450 metabolites with potential vasoactivity, including DH-PG, DH-HETE, DH-dihydroxyeicosatetraenoic acid, and DH-EET. The CYP450-derived DH-16,17-EET induces vasorelaxation and represents a potential endogenous adrenic acid metabolite that functions as an endothelium-derived and ZG cell-derived hyperpolarizing factor in the adrenal vasculature. Whereas the role of these metabolites in the regulation and maintenance of vascular tone is unknown, adrenic acid and its metabolites should be considered in future studies examining the regulation of vascular tone, especially in tissues with known abundance of adrenic acid.

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**Disclosures**

None.

**References**


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**Figure 6.** Schematic of adrenic acid metabolism by bovine ZG cells. Adrenic acid is metabolized by the COX, LO, and CYP450 pathways.


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