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Eicosanoid post-mortem induction in kidney tissue is prevented by microwave irradiation

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Abstract

Previously, we, and others, have demonstrated a rapid and significant post-mortem increase in brain prostanoid (PG) levels analyzed without microwave fixation, and this is not the result of PG trapping or destruction in microwave-irradiated brain tissue. In the present study, we demonstrate a dramatic increase in kidney eicosanoid levels when analyzed without microwave fixation which was mainly accounted for by the 142-, 81-, and 62-fold increase in medullary 6-ketoPGF₁, PGE₂, and PGF₂, levels, respectively, while PGD₂ and TXB₂ levels were increased ~7-fold. Whole kidney and cortex PG were also significantly increased in nonmicrowaved tissue, but at lesser extent. Arachidonic acid and the lipoxygenase products hydroxyeicosatetraenoic acids (HETE) were also induced in whole kidney, cortex, and medulla 1.5- to 5.5- fold depending upon tissue and metabolite. Cyclooxygenase inhibition with indomethacin decreased PG mass in non-microwaved tissue to basal levels, however HETE and arachidonic acid were not decreased. These data demonstrate the critical importance of kidney tissue fixation to limiting artifacts during kidney eicosanoid analysis.

1. INTRODUCTION

Eicosanoids, including the cyclooxygenase (COX) products prostanoids (PG) and the lipoxygenase (LOX) products hydroxyeicosatetraenoic acids (HETE), are derived from polyunsaturated fatty acids and are important local regulators in kidneys under physiological, inflammatory or acute renal injury conditions. They regulate glomerular filtration rate through regulation of renal blood pressure, and microvascular hemodynamics, vascular tone, renin release, salt and water reabsorption, protein retaining, and angiotensin receptor expression [1-5]. Because of the important role for eicosanoids in normal and pathophysiological processes, their quantification is critical in understanding many biological processes where eicosanoids are involved.

Previously, we, and others, have demonstrated a rapid 30-fold increase in brain PG mass within seconds upon brain extraction from the body without microwave irradiation [6-9]. In addition, PG are synthesized during brain tissue extraction, which may also contribute to the higher values and greater variability of PG mass found in non-fixed samples [7]. Microwave

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irradiation is an effective technique to heat-denature enzymes in order to prevent post-mortem PG synthesis in brain tissue when temperature is raised to 70-80 °C [6-11]. Because lower PG levels found in microwaved tissue might be the result of PG heat-destruction or trapping in denatured proteins, this point has been addressed in several independent studies. We, and others, have demonstrated that when maximal PG formation is reached, PG levels are not different between microwaved and non-microwaved brains [7, 9], indicating that PG are not destroyed or trapped in microwaved tissue. In addition, the recovery of radiolabeled PG injected into the brain was not significantly affected by microwave irradiation [9, 10]. These data demonstrate that levels measured in microwaved brains are true basal PG levels.

Although the induction of brain PG levels during tissue analysis is well documented, to the best of our knowledge, post-mortem PG induction in kidneys which, similar to brain, have a high rate of metabolism and produce high levels of PG upon stimulation [12-14] has not been previously addressed. However, rapid tissue fixation by microwave irradiation has been applied to study renal adenylate cyclase and phosphodiesterase to rapidly heat-denature enzymes in chick kidney, and this technique was found useful for the in vivo bioassay of parathyroid hormone effect [15]

In the present study, we demonstrate a dramatic, up to 140-fold, increase in kidney eicosanoid levels when analyzed without microwave fixation. COX inhibition with indomethacin decreased PG mass in non-microwaved tissue to basal levels, however HETE and arachidonic acid (20:4n-6) were not decreased. These data demonstrate the critical importance of kidney tissue fixation to limiting artifacts during kidney eicosanoid analysis.

2. METHODS

Animals

All animal use was approved by the University of North Dakota IACUC (protocol #1111-3) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals 8th Addition. Male C57BL/6 mice (25-30g) were provided standard laboratory chow and water ad libitum. The mice were between 8 to 9 months old.

Materials

9-oxo-11,15S-dihydroxyprosta-5Z,13E-dien-1-oic-17,17,18,18,19,19,20,20,20-²H₄ acid (PGE₂-d₉), arachidonic acid-d₈ (20:4n6-d₈), 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (12S-HETE-d₈), 9-Oxo-11,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid (PGE₂), 9,11,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (PGD₂), 9,11,15S-trihydroxyprosta-5Z,13E-dien-1-oic acid (PGF₂), 6-oxo-9,11,15S-trihydroxy-prosta-13E-en-1-oic acid (6-ketoPGF₁), 9,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid (TXB₂), 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5S-HETE), 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12S-HETE), and 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15S-HETE) were purchased from Cayman Chemicals (Ann Arbor, MI). Indomethacin was purchased from Sigma-Aldrich (Saint Louis, MO). All solvents of LC-MS grade or higher were purchased from Fisher Scientific (Waltham, MA).

Tissue Collection and Sample Preparation

Mice were treated with 100 µl buffer or with the COX1/2 inhibitor indomethacin (10 mg/kg dissolved in buffer containing 0.9 % NaCl, 5%NaHCO₃, 5% ethanol) via a single intraperitoneal injection. Thirty minutes after treatment, the mice were anaesthetized with 3% isoflurane and euthanized either by decapitation to collect non-fixed tissue, or by microwave irradiation at 4 kW for 2 s to denature enzymes. We used a head-focused

microwave machine (Cober Electronics, Inc., Norwalk, CT) with a rat adaptor and holder that produces a wide microwave beam to microwave an upper body of the mouse. However, we placed an animal backwards in the holder to allow for a lower body to be exposed to microwave irradiation. The final kidney temperature was 82.7 ± 2.1 °C. Microwaved and non-microwaved kidneys are well distinguished visually (Fig. 1). For both microwave and non-microwave irradiated animals, the kidneys were removed. For whole kidney analysis, the kidney was diced to approximately 1 mm size pieces and incubated for 3 min on a glass slide before being frozen in liquid nitrogen. For dissected kidney, the kidney was dissected to separate the inner medulla from the cortex before freezing in liquid nitrogen. The time for dissection was 3 min. For the time course, animals were euthanized without buffer treatment, and whole kidney was either frozen immediately (20-30 s) or placed on a glass slide at 40°C at 100% humidity for 5, 10, or 15 min before freezing in liquid nitrogen. The kidneys were pulverized to a homogenous powder under liquid nitrogen.

The tissue eicosanoids were extracted using an acetone liquid/liquid extraction protocol as previously described [7, 16, 17]. Briefly, tissue (approximately 10 mg of inner medulla or 20 mg of whole kidney or cortex) was homogenized in a Tenbroeck tissue grinder (Kontes Glass Co., Vineland, NJ) containing 3 mL of 2:1 acetone:saline with internal standards (1 ng PGE₂-d₉, 5 ng 12S-HETE-d₈, and 100 ng 20:4n6-d₈). The homogenate was transferred to a silanized (Sigmacote, Sigma Chemical Co., St. Louis, MO) screw top tube and centrifuged at 2000g for 10 min. The supernatant was transferred into a new silanized tube and extracted 3 times with 2 mL of hexane. The hexane extracts of fatty acids and HETE were combined in a new silanized glass tube. The remaining lower aqueous phase was then acidified with 30 µl of 2 M formic acid to pH 3.5 and the PG were extracted with 2 mL of chloroform. The chloroform extract was collected in a silanized tube, flushed with nitrogen, and cooled to -80°C for at least 15 min to separate any remaining aqueous phase which was removed after warming to room temperature.

The chloroform and hexane extracts were dried under nitrogen and transferred to silanized microvial inserts (Agilent, Santa Clara CA; part #5181-8872). The chloroform extracts were transferred with two 130 µl 9 : 1 chloroform : methanol washes and the hexane extracts were transferred with two 130 µl hexane washes. The samples were dried a second time under nitrogen and were re-dissolved in 22 µL of 4 : 6 acetonitrile : water.

UPLC separation and MS Analysis

LC separation was performed on a Waters ACUITY UPLC HSS T3 column (1.8 µm, 100 Å pore diameter, 2.1×150mm, Waters, Milford, MA) with an ACUITY UPLC HSS T3 precolumn (1.8 µm, 100 Å pore diameter, 2.1×5mm, Waters) at a temperature of 55°C. The LC system consisted of a Waters ACUITY UPLC pump with a well plate autosampler at 8 °C. Ten microliters of sample was injected on column.

The separation was based on the separation described previously [18]. Solvent A consisted of water containing 0.1% formic acid and solvent B consisted of acetonitrile with 0.1% formic acid. The flow rate was 0.45 mL/min. The initial conditions of 39% B were held for 0.5 min. Solvent B was increased to 40.5% over 6.88 min, followed by an increase to 70% over 1.62 min, then increased to 75% over 3 min, and further increased to 98 over 1.5 min where it was held for 5.3 min. Finally, solvent B was returned to initial conditions over 0.2 minutes to re-equilibrate the column for 2min.

PG, 20:4n-6, and HETE were analyzed using a triple quadrupole mass spectrometer (Xevo TQ-S, Waters) with electrospray ionization operated in negative ion mode. The capillary voltage was 2.35 kV and the cone voltage was 30V. The desolvation temperature was 550 °C and the source temperature was 150 °C. The desolvation gas flow was 1000 L/hr, the

cone gas flow was 150 L/hr, and the nebulizer gas was at 7.0 Bar. MassLynx V4.1 software (Waters) was used for instrument control, acquisition, and sample analysis.

PG, 20:4n-6, and HETEs were quantified using PGE₂-d₉, 20:4n6-d₈, and 12S-HETE-d₈, respectively, as internal standards. The deuterated PGE₂ has been previously validated as an internal standard for all PG analyzed [7]. The analytes were monitored in MRM mode as previously described [16, 17, 19] with the following mass transitions: PGE₂ - 351.18/271.13; PGD₂ - 351.06/271.14; 6-ketoPGF₁ - 369.26/163.07; PGF₂ - 353.07/193.04; TXB₂ - 369.20/169.00; 20:4n6 - 303.07/259.21; 5-HETE - 319.20/115.20; 12-HETE - 319.10/267.21; 15-HETE - 319.12/219.09; PGE₂-d₉ - 360.2042/280.17; 20:4n6-d₈ - 310.93/267.21; 12S-HETE-d₈-327.12/184.04. The collision energies used were (eV): PGE₂ - 16; PGD₂ - 16; 6-ketoPGF₁ - 24; PGF₂ - 20; TXB₂ - 12; PGE₂-d₉ - 14; 20:4n6 - 12; 20:4n6-d₈ - 12; 5-HETE - 10; 12-HETE - 12; 15-HETE - 10; 12S-HETE-d₈ - 12.

Statistics

All statistical comparisons were performed using ANOVA with Tukey's *post hoc* test (GraphPad, San Diego, CA) with statistical significance defined as $p < 0.05$. All values are expressed as mean $\pm$ SD.

3. RESULTS and DISCUSSION

3.1. Prostanoid levels are significantly increased in non-microwaved kidney tissue

To validate the importance of kidney tissue fixation for measuring true basal levels of PG, we first analyzed PG levels in fixed with microwave irradiation versus non-fixed whole kidneys, cortex, and inner medulla (Fig. 2). Microwave irradiation is an effective technique to heat-denature enzymes in order to prevent post-mortem PG synthesis in tissue, and does not result in PG degradation or trapping in microwaved tissue [6-11].

In tissue fixed with microwave irradiation, the highest PG levels were found in inner medulla (9.40 $\pm$ 3.05, 1.39 $\pm$ 0.70, 0.33 $\pm$ 0.15, 0.27 $\pm$ 0.09, and 1.25 $\pm$ 1.03 ng/gww for PGE₂, PGD₂, PGF₂, 6-ketoPGF₁, and TXB₂, respectively.), followed by whole kidney (2.38 $\pm$ 1.46, 0.82 $\pm$ 0.52, 0.26 $\pm$ 0.18, and 0.21 $\pm$ 0.1 ng/gww for PGE₂, PGD₂, PGF₂, and 6-ketoPGF₁, respectively) and cortex (1.00 $\pm$ 0.44, 0.24 $\pm$ 0.10, 0.28 $\pm$ 0.14, and 0.14 $\pm$ 0.04 ng/gww for PGE₂, PGD₂, PGF₂, and 6-ketoPGF₁, respectively). The higher PG levels in the medulla compared to the cortex is consistent with previously reported values [20, 21]. PGE₂ was found to be the most abundant PG in all kidney segments analyzed, which is in line with previous reports where the major kidney PG was also PGE₂, while PGD₂ was the lowest or below detection limits [21, 22].

When analyzed without fixation with microwave irradiation, all PG were dramatically increased in all kidney segments analyzed. The highest increase was in medullary 6-ketoPGF₁, PGE₂, and PGF₂, (142-, 81-, and 62-fold increase, respectively; Fig 2 B), followed by whole kidney and cortex PG species (38- to 4-fold increase) with the highest induction in 6-ketoPGF₁ and PGE₂ levels (Fig 2 A and C). This is in line with the previously reported 30- to 100- fold higher rate of PG synthesis in medullary versus cortical tubules [14, 23], as well as the higher levels of microsomal PGE synthase 1 (mPGES-1) and COX-2 in the medullar-associated tubular system in mouse and rat kidney [24]. Besides the role for COX-2 in PGE₂ synthesis, it is also believed to be the major source of 6-ketoPGF₁ [25], that explains both PGE₂ and 6-ketoPGF₁ uction at a similarly high rate. These data indicate the importance for kidney tissue fixation to measure true basal PG levels.

Importantly, COX inhibition with indomethacin prevented PG induction in non-fixed tissue (Fig. 2). Similar to our results, the PG levels were 100- fold decreased upon COX inhibition

in kidney [20]. These data support a COX-dependent nature of PG increase in non-fixed tissue. Notably, microwave irradiation did not change kidney basal PG levels in indomethacin treated animals (Fig. 2), indicating that PG are not destroyed or trapped in kidney tissue under microwave irradiation. Although we did not detect an effect of indomethacin on basal PG levels, this might be explained by low basal PG tissue levels when non-enzymatically produced isoPG mask the effect of the COX inhibitor.

Additionally, we assayed PG levels in fast-frozen (30 s after animal death) whole kidney. Although the time required to isolate medulla from cortex is greater than 30 s, this validation might be important when whole kidney PG analysis is performed. The PG levels in fast frozen kidney were significantly lower as compared to dissected tissue (Fig. 2 A), however they were still significantly increased (15- to 2.6- fold) except for PGF₂ as compared to microwaved kidney. These experimental conditions are considered to be a model of acute ischemia/hypoxia [6, 10]. The data indicate a dramatic PG increase in ischemic/hypoxic kidney within 20-30 sec after animal death. These data also indicate the importance of kidney tissue fixation when tissue is analyzed without dissection.

3.2. LOX products and 20:4n-6 levels are significantly increased in non-microwaved kidney tissue

In addition to COX products, we validated the effect of microwave irradiation on levels of 20:4n-6 and LOX products (Fig. 3). Kidneys express 5-, 12- and 15-lipoxygenases that produce 5-, 12-, and 15- HETE, respectively, as one of their major products [5, 26]. In contrast to PG basal levels, HETE and 20:4n-6 were not different between whole kidney, cortex, and inner medulla except for 15-HETE that had 2.4 fold lower levels in whole kidney compared to cortex or inner medulla. This might be explained by a higher tissue purity achieved when cortex and medulla are separated from surrounding tissue and fluids, thus limiting dilution with compartments containing low HETE levels. Among LOX products, 12-HETE had the highest concentration in all kidney tissues analyzed (~ 15 ng/gww), and 5-HETE had the lowest concentration (~ 3 ng/gww). However, consistent with PG data, 12-HETE was increased 1.7-, 2.8-, and 5.4- fold in non-microwaved whole kidney, cortex, and inner medulla, respectively, while 15-HETE was increased 5.5-fold in inner medulla only, and 5-HETE was unchanged in all kidney segments (Fig. 3). Additionally, consistent with PLA₂ activation under injury/ischemia/hypoxia conditions, 20:4n-6 levels were also increased 1.5-, 1.8-, and 2.5- fold in non-microwaved whole kidney, cortex, and inner medulla, respectively. These results are consistent with 20:4n-6 increase in ischemic brain [8, 9, 27-29], but opposite to non-microwaved heart tissue [30] where fatty acids were decreased under ischemic non-microwaved conditions. Brain 20:4n-6 increase is associated with activation of phospholipases and diacylglycerol lipases [31-33], while the heart fatty acid decrease is explained by use of fatty acids as an energy source [30]. Fatty acid increase in ischemic kidney is associated with decreased β -oxidation [34] and phospholipase A₂ activation [35]. However, the specificity and mechanism for fatty acid, including 20:4n-6, release in renal tissue under acute ischemia has not been extensively studied. Further studies are required to address this question, and microwave technique might be a useful tool to assay true basal fatty acid levels in ischemia models. Importantly, similar to PG levels, the most dramatic alterations in HETE and 20:4n-6 levels were found in medulla. This indicates the importance of kidney tissue fixation with microwave irradiation to prevent post-mortem LOX and lipase activation. However, in contrast to PG data, LOX inhibition with indomethacin did not affect kidney 20:4n-6 and HETE mass, supporting the enzyme-dependent HETE and 20:4n-6 induction rather than metabolite destruction/trapping during microwave irradiation.

3.3. Time course for eicosanoid induction during tissue homogenate incubation

To further validate the kinetics for post-mortem induction of kidney eicosanoids, we measured whole kidney COX and LOX products, and 20:4n-6 after 0, 5, 10, and 15 min of kidney homogenate incubation (Fig. 4). Most PG were continuously synthesized during incubation (Fig. 4 A), which is consistent with the high level of PG synthesis during kidney homogenate incubation [12, 21, 36]. Similar to PG increase in non-fixed tissue (Fig. 2 A), all PG assayed were significantly increased at 15 min of kidney homogenate incubation. PGE₂ and 6-ketoPGF₁ were increased to the highest extent. Interestingly, PGE₂ was increased from 6.32 ng/gww to 203.27 ng/gww at 15 min in a linear manner, while 6-ketoPGF₁ was induced mainly at 15 min of incubation and reached 17.72 ng/gww. Other PG were increased in a manner similar to PGE₂, but at a slower rate. However, initially induced 20:4n-6 levels were unchanged at 10 and 15 min of incubation, while 12-HETE mass was further increased at 15 min after initial induction at 5 min (Fig. 4 B). These data indicate different dynamics of post-mortem activation of different enzymes in kidneys. Together, these data further support the importance for kidney tissue fixation during sample handling and analysis to assay true basal levels for rapidly changing pools of metabolites.

4. CONCLUSIONS

In the present study, we report a dramatic increase in non-fixed kidney eicosanoid levels during analysis. This indicates the importance for kidney tissue fixation with microwave irradiation to prevent post-mortem COX, LOX, and lipase activation. COX inhibition with indomethacin decreased PG mass in non-microwaved tissue to basal levels. However, HETE and 20:4n-6 were not decreased, supporting the enzyme-dependent HETE and 20:4n-6 induction rather than metabolite destruction/trapping during microwave irradiation. These data demonstrate the critical importance of kidney tissue fixation to limiting artifacts during kidney eicosanoid and 20:4n-6 analysis.

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Fig. 1. Images of non microwaved and microwaved kidneys

Mice were anaesthetized with 3% isoflurane and euthanized either by decapitation to collect non-fixed tissue, or by microwave irradiation at 4 kW for 2 s to denature enzymes. A head-focused microwave machine (Coher Electronics, Inc., Norwalk, CT) with a rat adaptor and holder was used to produce a wide microwave beam to microwave an upper body of the mouse. However, an animal was placed backwards in the holder to allow for a lower body to be exposed to microwave irradiation. The final kidney temperature was 82.7 ± 2.1 °C.

A

B

C

Fig. 2. Effect of microwave tissue fixation and COX inhibition on kidney PG basal levels

Mice were treated with indomethacin (Indo) to inhibit COX or buffer only, anesthetized with isoflurane (1-3%), and euthanized by either high energy microwave irradiation to heat-denature proteins (MW) or decapitation (Non MW). Kidneys were removed immediately (20-30 s) after animal death and either frozen in liquid nitrogen (Frozen Whole Kidney) or dissected to prepare whole kidney homogenate (**A**, particle size 1 mm or less) or to separate inner medulla (**B**) and cortex (**C**) and frozen in liquid nitrogen 3 min after animal death. 10-20 mg of tissue samples were extracted with the acetone liquid/liquid method, and the chloroform fractions were analyzed using the LC-MS/MS approach as described in the Method section. Values are expressed as mean $\pm$ SD (n=3 for all conditions except for Not MW Frozen Whole Kidney with n=4). * - $p < 0.05$ compared to other conditions; ** - $p < 0.05$ compared to other conditions except for not microwaved (Not MW Whole Kidney) tissue.

A

B

C

Fig. 3. Effect of microwave tissue fixation and COX inhibition on kidney basal levels of arachidonic acid and LOX products

Mice were treated with indomethacin (Indo) to inhibit COX or buffer only, anesthetized with isoflurane (1-3%), and euthanized by either high energy microwave irradiation to heat-denature proteins (MW) or decapitation (Non MW). Kidneys were removed immediately (20-30 s) after animal death and either frozen in liquid nitrogen (Frozen Whole Kidney) or dissected to prepare whole kidney homogenate (**A**, particle size 1 mm or less) or to separate inner medulla (**B**) and cortex (**C**) and frozen in liquid nitrogen 3 min after animal death. 10-20 mg of tissue samples were extracted with the acetone liquid/liquid method, and the hexane fractions were analyzed using the LC-MS/MS approach as described in the Method section. Values are expressed as mean $\pm$ SD (n=3 for all conditions except for Not MW Frozen Whole Kidney with n=4). * - $p < 0.05$ compared to microwaved tissue.

A

B

Fig. 4. Time course for eicosanoid production in non-fixed whole kidney tissue

Mice were anesthetized with isoflurane (1-3%), and euthanized by decapitation. Kidneys were removed immediately (20-30 s) after animal death and either frozen in liquid nitrogen (0 min) or dissected to prepare whole kidney homogenate (particle size 1 mm or less) and incubated at 40 °C and 100% humidity for 5, 10, and 15 min. At the end of incubation, whole kidney homogenates were frozen in liquid nitrogen and 22.0 ± 1.2 mg of tissue samples were extracted with the acetone liquid/liquid method, and the chloroform and hexane fractions were analyzed for prostaglandin (PG, **A**) or hydroxyeicosatetraenoic (HETE) and arachidonic (20:4n6) acid (**B**) levels, respectively, using the LC-MS/MS

approach as described in the Method section. Values are expressed as mean $\pm$ SD (n=4). a - p < 0.05 compared to 0 min; b - p < 0.05 compared to 5 min; c - p < 0.05 compared to 10 min; d - p < 0.05 compared to 15 min of incubation.