Novel Role of Fumarate Metabolism in Dahl-Salt Sensitive Hypertension

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Abstract—In a previous proteomic study, we found dramatic differences in fumarase in the kidney between Dahl salt-sensitive rats and salt-insensitive consomic SS-13BN rats. Fumarase catalyzes the conversion between fumarate and L-malate in the tricarboxylic acid cycle. Little is known about the pathophysiological significance of fumarate metabolism in cardiovascular and renal functions, including salt-induced hypertension. The fumarase gene is located on the chromosome substituted in the SS-13BN rat. Sequencing of fumarase cDNA indicated the presence of lysine at amino acid position 481 in Dahl salt-sensitive rats and glutamic acid in Brown Norway and SS-13BN rats. Total fumarase activity was significantly lower in the kidneys of Dahl salt-sensitive rats compared with SS-13BN rats, despite an apparent compensatory increase in fumarase abundance in Dahl salt-sensitive rats. Intravenous infusion of a fumarate precursor in SS-13BN rats resulted in a fumarate excess in the renal medulla comparable to that seen in Dahl salt-sensitive rats. The infusion significantly exacerbated salt-induced hypertension in SS-13BN rats (140±3 vs 125±2 mm Hg in vehicle control at day 5 on a 4% NaCl diet; P<0.05). In addition, the fumarate infusion increased renal medullary tissue levels of H₂O₂. Treatment of cultured human renal epithelial cells with the fumarate precursor also increased cellular levels of H₂O₂. These data suggest a novel role for fumarate metabolism in salt-induced hypertension and renal medullary oxidative stress. (Hypertension. 2009;54:255-260.)

Key Words: hypertension ■ gene ■ tricarboxylic acid cycle ■ kidney ■ oxidative stress ■ rat

The Dahl salt-sensitive (SS) rat is a genetic model of human salt-sensitive forms of hypertension.¹,² The consomic SS-13BN rat has the same genomic makeup as the SS rat except for chromosome 13, which is introgressed from the Brown Norway (BN) rat and substantially attenuates salt-sensitive hypertension and renal injury.³

A tree-like network of molecular, biochemical, and physiological mechanisms is likely involved in the development of Dahl salt-sensitive hypertension and renal injury.⁴ Comparative analysis of SS and SS-13BN rats has revealed several new components of this regulatory network. Examples include increased levels of superoxide and H₂O₂,⁵,⁶ dysregulation of 11β-hydroxysteroid dehydrogenase, and alterations of glucocorticoid metabolism⁷ in the renal medulla of SS rats compared with SS-13BN rats. Additional mechanisms and particularly sequence variations of specific genes involved in the SS phenotypes remain to be discovered or validated.⁸⁻¹⁰

Fumarase was one of the proteins exhibiting dramatic differences between SS and SS-13BN rats according to a recent proteomic study.¹⁰ The analysis indicated a consistent and substantial difference in the isoelectric point of fumarase in SS and SS-13BN rats, as reflected by a significant shift of the protein spot on 2D gels. Fumarase catalyzes the reversible conversion between fumarate and L-malate in the tricarboxylic acid cycle in mitochondria. Rare loss-of-function mutations of fumarase in humans cause accumulation of fumarate and are associated with the development of hereditary leiomyomatosis, renal cell cancer, or encephalopathy.¹¹⁻¹³ Little else is known about the pathophysiological significance of fumarase and fumarate metabolism in mammals. In the present study, we discovered a sequence variation in fumarase between SS and BN alleles and demonstrated that fumarate excess exacerbated salt-induced hypertension and increased cellular levels of H₂O₂.

Methods and Materials

Animals

Male SS and consomic SS-13BN rats were obtained and maintained as described previously.¹³,¹⁴,¹⁰ Rats were fed a purified AIN-76A rodent diet (Dyets) containing 0.4% NaCl and had free access to water. The diet was switched to one containing 4% NaCl (Dyets), as required by specific protocols. Rats were 6 to 8 weeks old when entering the present study. The animal protocols were approved by...
the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Molecular Cloning and Sequencing of Fumarase
Molecular cloning and sequencing were performed essentially as we described previously. Briefly, for cDNA sequencing, total RNA was extracted from rat liver, reverse transcribed using an oligo-dT primer, and PCR amplified using the following primers: forward, 5′-ctttccatccacgtacagcagcacc-3′ and reverse, 5′-taactgcttgacgacgcatgt-3′. The PCR product of ~1.5 kb was recovered and inserted into the T-easy vector (Promega). The plasmid was propagated in competent Escherichia coli, extracted, and sequenced with 3 reads to obtain the fumarase cDNA sequence containing the complete protein-coding region. For the analysis of genomic DNA, the primers used to amplify a 658-bp segment of the fumarase gene were as follows: forward, 5-tggtcaaatctccctctctctc-3, and reverse, 5-tctaccctcataactagcctca-3. The forward primer binds to an intron of the gene, ensuring amplification from genomic DNA. A second set of primers was used for the sequencing reaction: forward, 5-aaaaatttttaggtgctgccttaga-3, and reverse, 5-ttcaccctcactcatcaaactgc-3.

Fumarase Activity Assay
Fumarase activity is conventionally measured in the direction of l-malate to fumaric acid because of the ease of the assay. Tissue specimens were homogenized and sonicated extensively in a cold HEPES solution (20 mmol/L [pH 7.5], volume:weight: 3:1). The homogenate was centrifuged at 500g, 4°C, for 5 minutes and then at 18 000g, 4°C, for 10 minutes. Protein concentrations of the extracts were measured using the Bio-Rad DC protein assay and adjusted to be the same across all of the samples. The reaction recipe was 20 μL of Tris-acetate (pH 7.5), 178 μL of l-malate acid (50 mmol/L prepared in Tris-acetate [pH 7.5]), and 2 μL of the tissue homogenate. Absorbance at 240 nm was monitored at 30-second intervals using the kinetic mode of a microplate reader.

Fumarate, l-Malate, and Succinate Assay
Fumarate and l-malate levels were measured essentially as described previously. Tissue specimens were crushed in liquid nitrogen and homogenized in 3.5 volumes (volume:weight) of 8% perchloric acid in 40% ethanol. The homogenate was centrifuged at 40 000g, 4°C, for 10 minutes. The pellet was extracted with 2.5 volumes of 6% perchloric acid and the supernatants combined. The extract was neutralized with 0.75 volumes of 3.0 mol/L of K2CO3 with 0.5 mol/L of triethanolamine and centrifuged. The supernatant was heated at 18 000g, 100°C as the tissue extract. The reaction recipe consisted of 200 μL of the reaction buffer (400 mmol/L of hydrazine hydrate, 5 mmol/L of EDTA, 10 mmol/L of MgSO4, and 100 mmol/L of Tris-acetate [pH 8.5]), 2 μL of nicotinamide-adenine dinucleotide (80 μg/μL), 2 μL of tissue extract, 15 U of nicotinamide-adenine dinucleotide-malate dehydrogenase, and 3 U of fumarase. The chemicals and enzymes were from Sigma. Each tissue extract sample was assayed with 3 sets of duplicate reactions. The first set did not contain any enzymes. The second and the third sets contained malate dehydrogenase. The conversion of nicotinamide-adenine dinucleotide to reduced nicotinamide-adenine dinucleotide was monitored using a fluorescent microplate reader with excitation and emission wave-lengths of 360 nm and 465 nm, respectively, and operating on the kinetic mode. The reactions were incubated at room temperature for 60 minutes until the fluorescent intensity reached a plateau, indicating that the endogenous l-malate had been completely metabolized. Fumarase was then added to the third set of reactions, and the measurement of fluorescent signals in all of the reactions continued.

Western Blot
Western blotting was performed as described previously, using Fumarase antibody from Santa Cruz Biotechnology.

Chronic Intravenous Infusion and Blood Pressure Measurement in Conscious Rats
Chronic instrumentation in conscious rats was performed as described previously. Briefly, an arterial catheter and an infusion catheter were implanted into the left femoral artery and vein, respectively. The venous line was continuously infused with normal saline at 8 μL/min. Rats were allowed 1 week to recover before the initiation of daily blood pressure recording through the arterial catheter. After a baseline period, normal saline was replaced by diethyl-fumarate or vehicle control at the concentrations described in the Results section.

H2O2 Assay
Acid extraction of tissue or cell samples was performed as described above for the fumarate and l-malate assay. H2O2 levels in the neutralized acid extract were measured as catalase-inhibitable signals using an Amplex Red assay kit (Invitrogen), similar to that described previously.

Cell Culture
HK2 and HRE cells were obtained from and cultured as suggested by American Type Culture Collection and Cambrex, respectively. HK2 cells are immortalized human kidney epithelial cells with characteristics of proximal tubular and medullary cells. HRE cells are primary cultures of human renal epithelial cells.

Statistics
Data were analyzed using Student t test or multiple-group ANOVA. Data are shown as mean±SEM.

Results
K481E Variation in Fumarase in SS and SS-13BN Rats
Fh1, the rat gene encoding fumarase is located on rat chromosome 13q25 at ~91 520 000- to 91 545 000-bp positions (http://rgd.mcw.edu). Chromosome 13 is the only chromosome in SS-13BN rats that is of the BN origin.

The entire protein-coding region of fumarase cDNA was cloned from SS, SS-13BN, and BN rats and sequenced. The nucleotide at position 1441, relative to the start codon, was A in SS-13BN rats than in SS rats by 22% (n = 4; P < 0.05; Figure above for the fumarate and l-malate assay. The entire fumarase coding region was of the BN origin.

The entire fumarase coding region was of the BN origin. The same nucleotide difference was found in genomic DNA. The nucleotide difference at 1441 would be translated into lysine (K) in SS fumarase but glutamic acid (E) in SS-13BN and BN (Figure 1). The difference in amino acid sequence at the amino acid position 481 (Figure 1).

Fumarate Excess in SS Rats
Fumarase catalytic activity was measured in the renal medulla of 6-week-old SS and SS-13BN rats on the 0.4% NaCl diet. The renal medulla is a kidney region that has been shown to play an important role in long-term blood pressure regulation and the development of Dahl salt-sensitive hypertension.

Fumarase activity was consistently and significantly higher in SS-13BN rats than in SS rats by 22% (n = 4; P < 0.05; Figure above for the fumarate and l-malate assay. The entire fumarase coding region was of the BN origin. The same nucleotide difference was found in genomic DNA. The nucleotide difference at 1441 would be translated into lysine (K) in SS fumarase but glutamic acid (E) in SS-13BN and BN (Figure 1). The difference in amino acid sequence at the amino acid position 481 (Figure 1).
SS  ...AACCTAAAGAAACGGCTATT...  
SS-13BN  ...AACCTAAAGAAACGGCTATT...  
BN  ...AACCTAAAGAAACGGCTATT...

| SS | ...AHKNGSTLETAIELGYLTAEQ... |
| SS-13BN | ...AHKNGSTLETAIELGYLTAEQ... |
| BN | ...AHKNGSTLETAIELGYLTAEQ... |

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Figure 1. An amino acid difference between SS and BN fumarase. Sequencing of the entire protein-coding region of fumarase cDNA indicated a single nucleotide difference between SS rats and SS-13BN and BN rats. The codon AAA in SS fumarase encodes lysine (K), whereas the codon GAA in SS-13BN and BN fumarase encodes glutamic acid (E).

2A). The protein abundance of fumarase, estimated by Western blotting, was 2.3-fold higher in SS rats (Figure 2B). The specific activity of fumarase, calculated as total activity normalized by fumarase protein abundance, was 2.9-fold higher in SS-13BN rats than in SS rats by 28% (n=5; P<0.05). C, Specific activity of fumarase was much lower in SS rats than in SS-13BN rats. Fumarase activity shown in A was normal-

ized by fumarase abundance shown in B for each individual rat. n=4; *P<0.05.

D, Fumarate levels were higher in SS rats than in SS-13BN rats by 1.7±0.2-fold (n=4; P<0.05). The specific fumarase activity in the cortex, normalized by Western blot density, was 2.5±0.4-fold higher in SS-13BN rats. Fumarate levels in the cortex, however, were not significantly different between SS (100±11%) and SS-13BN rats (111±15%; n=5; P value not significant).

Infusion of Fumarate Exacerbated Salt-Induced Hypertension in SS-13BN Rats

SS-13BN rats were prepared for chronic intravenous infusion and blood pressure measurement. Three groups of rats were studied. After 2 days of stable baseline recording, 1 group of rats received a vehicle infusion (0.5% ethanol in normal saline; n=6), and another group received an intravenous infusion of diethyl-fumarate at the dose of 0.2 mmol/kg of body weight per day (n=7), whereas the third group received diethyl-fumarate at the dose of 1 mmol/kg per day (n=5). Diethyl-fumarate is cell membrane permeable and can be converted to fumarate in the cell. Seven days later, the dietary salt content was increased from 0.4% to 4.0%, and the intravenous infusion and blood pressure recording were continued for another 5 days.

As shown in Figure 3A, intravenous infusion of diethyl-fumarate at 1 mmol/kg per day significantly exacerbated high salt–induced hypertension in SS-13BN rats. Mean arterial pressure was increased by ∼10 mm Hg after 5 days on the 4% NaCl diet in control rats and rats receiving diethyl-fumarate at 0.2 mmol/kg per day. The increase in the mean arterial pressure was ∼20 mm Hg in rats receiving 1 mmol/kg per day of diethyl-fumarate.

Concomitantly, tissue fumarate levels in the renal medulla were 74% higher in rats receiving diethyl-fumarate at 1 mmol/kg per day (n=5 to 7; P<0.05; Figure 3B). The increase in renal medullary levels of fumarate resulting from the infusion of diethyl-fumarate resembled the higher level of fumarate observed in the renal medulla of SS rats compared with SS-13BN rats (see Figure 2D). Diethyl-fumarate at the dose of 0.2 mmol/kg per day did not significantly affect renal medullary levels of fumarate, probably because fumarase in SS-13BN rats was sufficient for metabolizing fumarate infused at the lower dose. Fumarase protein abundance in the renal medullary homogenate in 6-week–old male SS and SS-13BN rats maintained on the 0.4% NaCl diet, n=4; *P<0.05. B, Fumarase abundance was higher in SS rats than in SS-13BN rats. Fumarase abundance was measured by Western blotting in the same rats used for the activity assay. n=4; *P<0.05. C, Specific activity of fumarase was much lower in SS rats than in SS-13BN rats. Fumarase activity shown in A was normalized by fumarase abundance shown in B for each individual rat. n=4; *P<0.05. D, Fumarate levels were higher in SS rats than in SS-13BN rats. Fumarate levels were measured in acid extracts of renal medulla tissues from rats similar to those described above. n=5; *P<0.05.
The present study discovered an amino acid sequence difference in fumarase between SS and BN alleles, demonstrated a novel functional role of fumarase metabolism in salt-induced hypertension, and provided insights into possible mechanisms.

The K481E amino acid difference was the first amino acid variation identified between SS and SS-13BN rats. The variation likely contributes to the much higher specific activity of fumarase in SS-13BN rats. The differences in fumarase activity and abundance were observed in both the renal medulla and the renal cortex, which is consistent with a genetically determined mechanism. It is possible, however, that the amino acid difference leads to additional differences in protein modification that collectively cause the change in catalytic activity. The differential in gel electrophoresis analysis indicated the presence of ≥5 fumarase spots, with spots in SS having more basic isoelectric points than those in SS-13BN rats. Bioinformatic analysis indicated that the single amino acid difference was consistent with, but might not be sufficient to explain, the shift of the isoelectric point. In addition, fumarase levels were higher in SS rats only in the renal medulla, and fumarase infusion did not induce a compensatory increase in fumarase abundance in the medulla of SS-13BN rats. These data suggest that fumarase metabolism might be regulated by multiple mechanisms. The precise effect of the K481E variation on fumarase structure and fumarase metabolism warrants further analysis. Moreover, gene transfer study would be required to determine the definitive role of the K481E variation in the SS phenotypes.

Abnormalities in the tricarboxylic acid cycle and related metabolic pathways might represent an important mechanism of blood pressure regulation that is not yet fully appreciated. It has been shown that excess succinate, which is upstream of fumarase in the tricarboxylic acid cycle, can cause hypertension in mice. We have shown in the present study that excess fumarate could dose-dependently exacerbate salt-induced hypertension in rats. The importance of maintaining normal fumarate metabolism was supported by the apparent compensatory increase in fumarase abundance in SS rats. The increase in abundance, however, was not sufficient to bring total fumarase activity up to the level seen in SS-13BN rats. It would be interesting to determine whether fumarate excess affects the levels of other intermediates of the tricarboxylic acid cycle, although succinate levels in the renal medulla were not significantly different between SS and SS-13BN rats. In humans, fumarase mutations cause fibroid syndromes, and hypertension is associated with an increased risk of fibroids. Alterations of other metabolic pathways may also be involved in the development of Dahl salt-sensitive hypertension. For example, the renal proteomic differences between SS and SS-13BN rats suggested an upregulation of fatty acid metabolism in SS rats.

Metabolic abnormalities might contribute to SS phenotypes through changes in bioenergetics or mechanisms other than bioenergetics. Production of reactive oxygen species might be one such mechanism. It has been shown that the renal medulla of SS rats had higher levels of reactive oxygen species, including superoxide anions and H$_2$O$_2$. Pharmacological inhibition of oxidative stress, including the reduction

**Fumarate Increased H$_2$O$_2$ In Vivo and in Cultured Human Cells**

The renal medulla of SS rats has been shown to contain higher levels of H$_2$O$_2$ than SS-13BN rats, which contributes to salt-induced hypertension. Interestingly, renal medullary levels of H$_2$O$_2$ were significantly elevated in SS-13BN rats receiving diethyl-fumarate at 1 mmol/kg per day (Figure 4A).

The effect of diethyl-fumarate on H$_2$O$_2$ levels was confirmed in cultured human renal epithelial cells. Cellular levels of H$_2$O$_2$ were increased significantly in HK2 cells treated with 2 μmol/L of diethyl-fumarate and in HRE cells treated with 10 μmol/L of diethyl-fumarate (Figure 4B and 4C).
of superoxide and H₂O₂ specifically in the renal medulla, attenuates salt-induced hypertension in SS rats.³⁶,³⁷,³⁸ The present study provided in vivo and in vitro evidence suggesting that excess fumarate might contribute to the elevation of reactive oxygen species. An increase of reactive oxygen species has been proposed as a possible link between fumarase mutations and tumorigenesis.²⁷ Fumarase deficiencies have been shown to block succinate dehydrogenase, presumably through fumarate accumulation.¹¹ Succinate dehydrogenase deficiencies could cause an increase in the superoxide anion.²⁷ In addition, fumarase C in E. coli is part of the soxRS regulon of antioxidant defense.²⁸,²⁹

Fumarate excess has also been shown to inhibit prolyl hydroxylase and stabilize hypoxia-inducible factor 1α (HIF1α).¹⁸,³⁰ It has been suggested that elevation of HIF1α might contribute to the development of tumors in patients with fumarase mutations. It remains to be determined whether HIF1α stabilization contributes to the role of fumarate excess in salt-induced hypertension and renal injury. The concentrations of diethyl-fumarate that increased H₂O₂ in cultured cells shown in the present study were lower than or at the low end of the concentrations reported previously to increase H₂O₂. These differences on HIF1α are unknown.

Perspectives
Abnormalities in fumarate metabolism appear to be novel mechanisms contributing to oxidative stress and salt-induced hypertension. The findings suggest several new directions for future research that could further delineate the regulatory tree underlying the molecular pathophysiology of hypertension.

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Disclosures
None.

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