Insufficient fumarase contributes to generating reactive oxygen species in Dahl salt sensitive rats

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Abstract

Dahl SS rats exhibit greater levels of renal medullary oxidative stress and lower levels of fumarase activities. Fumarase insufficiencies can increase reactive oxygen species (ROS), the mechanism of which, however, is not clear. A proteomic analysis indicated fumarase knockdown in HK-2 cells resulted in changes in the expression or activity of NADPH oxidase, mitochondrial respiratory chain complex I and III, ATP synthase subunits, and α -oxoglutarate dehydrogenase, all of which are sites of ROS formation. Meantime, the activities of key antioxidant enzymes such as G6PD, 6PGD, GR, GPx and GST increased significantly too. The apparent activation of antioxidant defense appeared insufficient as glutathione precursors, glutathione and GSH/GSSG ratio were decreased. SS rats exhibited changes in redox metabolism similar to HK-2 cells with fumarase knockdown. Supplementation with fumarate and malate, the substrate and product of fumarase, increased and decreased, respectively, blood pressure and the levels of H₂O₂ and MDA in kidney tissues of SS rats. These results indicate fumarase insufficiencies cause a wide range of changes at several sites of ROS production and antioxidant mechanisms.

Key words: Dahl salt sensitive rats, Fumarase, Fumarate, Reactive oxygen species, Glutathione metabolism

1 **Introduction**

2 Salt-sensitive hypertension, a multifactorial disease affecting nearly 15% of the
3 world population, is a major risk factor for stroke, heart failure, and end-stage renal
4 disease [1-3]. Dahl salt sensitive (SS) rat, as an animal model of human salt sensitive
5 hypertension, was widely used to dissect the pathogenesis. SS.13^{BN} rats as a
6 normotensive control, which chromosome 13 of the Brown Norway (BN) rat was
7 integrated into the genetic background of the SS and result in a significant
8 reduction of blood pressure and renal injury [4], had tremendously narrowed the
9 interested gene on chromosome 13. There is only a 1.95% allelic difference over
10 the entire genome between SS and SS.13^{BN} rats. Meanwhile, SS rats renal medullary
11 exhibited obviously oxidative stress and blood pressure salt-sensitivity compared
12 with SS.13^{BN} rats [5], as H₂O₂ concentrations had also been found to be nearly twice
13 as great in the dialysate of SS rats when both rats were maintained on a 0.4% NaCl
14 diet [6]. The level was more than doubled with a 4% NaCl in diet, but it remained
15 significantly higher in SS rats [6]. Furthermore, the increased production of O₂^{·-} in
16 mTAL also could diffuses to surrounding vasa recta, contribute to decrease
17 bioavailability of NO and reduce medullary blood flow, resulting in sustained
18 hypertension in SS rats [7]. However, the mechanisms that excessive O₂^{·-} and H₂O₂
19 produced in renal medulla of SS rats, is yet not fully understood.

20 Our previous differential proteomics study found that fumarase (FH), the gene
21 of which locates on rat chromosome 13, was expressed differentially between SS
22 and SS.13^{BN} rats, and the activity of FH is significantly lower in SS rats [8, 9]. As FH

23 catalyzes the conversion between fumarate and L-malate, the insufficiency of FH
 24 activity in SS rat kidney was associated with increased levels of fumarate [8]. The
 25 intravenous infusion of a fumarate precursor diethyl-fumarate in SS.13^{BN} rats
 26 resulted in increased levels of fumarate in renal medullary and significantly
 27 exacerbated salt-induced hypertension in SS.13^{BN} rats [8]. Furthermore, excessive
 28 fumarate could also increase the level of H₂O₂ in vivo and in cultured HK-2 cells [8].
 29 Our recent research work further demonstrated that overexpressing fumarase on
 30 the background of the SS rat, SS-TgFh1 transgenic rats, could significantly attenuate
 31 hypertension and H₂O₂ production [10]. So the present study will further dissect the
 32 imbalance of redox metabolism and understand the mechanism of ROS production
 33 by a proteomic analysis in fumarase knockdown HK-2 cells.

34 **Results**

35 **Differentially expressed proteins between FH insufficient HK-2 cells and NC cells**

36 The fumarase was knocked down by siRNA in HK-2 cell line, the protein profile
 37 of the whole cells was analyzed by iTRAQ based proteomics technology, and
 38 metabolites were detected by GC/MS. Content and activity of FH were examined to
 39 ensure the efficiency of FH knockdown. The sample preparation used for proteomic
 40 analysis same as the description we reported [11] and the sample test was showed in
 41 Figure EV1.

42 A total of 3174 distinct proteins were identified and quantified reliably at the
 43 value of global FDR less than 1% and identified with more than two peptides for 95%

confidence. Details of the quantified proteins are shown in Table EV1. Compared with the NC, 267 identified proteins exhibited significant difference between FH knockdown HK-2 cells and NC cells (fold change >1.5, P<0.05) (Table EV2). Among them, 165 proteins displayed increased expression and 102 proteins displayed decreased expression compared to NC. SOD2, IDH1, IDH2 and HSP90 among the 267 differentially expressed proteins were further confirmed by Western blotting. As shown in Figure EV2, the relative protein content from western blotting was consistent with proteomic quantification.

GO and KEGG analysis of the differential expressed proteins

To clarify the changed metabolic pathways that involved in the response to FH knockdown, 267 differential proteins were further examined using the Omics Bean bioinformatics tool. Gene ontology (GO) enrichment analysis was conducted in biological process (BP), cellular component (CC) and molecular function (MF) categories, respectively. An overview of significantly changed proteins in BP, CC and MF categories was shown in Figure EV3 and Table EV3. KEGG pathway-based analysis showed that ribosome, carbon metabolism, glutathione metabolism, mRNA transport, pyruvate metabolism, fatty acid degradation, glycolysis /gluconeogenesis and TCA cycle were significantly enriched among 267 differential proteins (Figure EV4 and Table EV4).

The formation sites of excessive amounts of ROS and H₂O₂ in FH knockdown HK-2 cells

The activity of NOX were detected and showed significantly increased in FH

insufficient HK-2 cells compared with NC cells (Figure 1A). The differential proteomic analysis data showed that expression of NADH dehydrogenase (ubiquinone) iron-sulfur protein 5 (NDUFS5), subunit of respiratory chain complex I, reduced for 1.5 times, while the expression of succinate dehydrogenase (Complex II) has no significant difference between FH knockdown HK-2 cells and NC cells. Rieske iron-sulfur protein (RISP) and Cyt b-c1 complex subunit 6 (Complex III) reduced 1.5 fold in FH knockdown HK-2 cells. Meanwhile, the expression of OGDH and B5R (cytochrome b5 reductase) increased 1.5 and 1.7 fold. Except these defined ROS generation sites, F_1F_0 ATP synthase subunits ATP5A1, ATP5C1, ATP5I and ATP5L reduced by 1.7, 1.4, 1.5 and 2.1 times and ATP level reduced significantly (Figure 1C). Briefly, NOX, α -OGDH, B5R, ATPase and Complex I and III would be the main sites of ROS formation in FH knockdown HK-2 cells.

Glutathione metabolism and antioxidant systems in FH knockdown HK-2 cells

The redox state of the cell is finely regulated by antioxidant systems, which include antioxidant enzymes and small molecular weight antioxidants. The proteomic analysis had identified the expression of all key enzymes involved in glutathione metabolism, increased their protein expression, which included Gpx, GR, GST, GS, G6PD and 6PGD (Table EV5), meanwhile, the activities of G6PD, 6PGD, GR, GST, GPx also increased compared with NC cells (Figure1 B, D, E, F). By contrast, there was no significant difference in the expression of glutamate cysteine ligase (GCL). In addition, the expression of other antioxidant enzymes, SOD2, NNTH (nicotinamide nucleotide transhydrogenase) and IDH2 increased 1.69, 1.77, 1.43 fold, however, the CAT, SOD1

88 had no significant difference between FH knockdown HK-2 cells and NC cells. The
 89 expression of SOD2, IDH1 and IDH2 was further confirmed by western blotting
 90 (Figure EV2). Furthermore, the expression of L-lactate dehydrogenase (LDHB),
 91 NADPH-dependent alcohol dehydrogenase (AKR1A1), glutathione dependent
 92 formaldehyde dehydrogenase (ADH5) and L-glutamate gamma semialdehyde
 93 dehydrogenase (ALDH7A1) increased 1.7, 1.5, 1.5, 1.4 fold compared with NC cells.
 94 These data suggested the increased activity of dehydrogenase would supply more
 95 antioxidants such as GSH, NADH or NADPH. Unfortunately, the content of GSH
 96 reduced dramatically, especially the ratio of GSH/GSSG decreased even if the content
 97 of NADPH increased compared with NC cells. The GSH synthesis substrate, glutamate,
 98 glycine and L-cysteine reduced significantly too (Figure 2 A-F).

99 **Key enzymes involved in renal redox metabolism of Dahl SS rats**

100 The FH activity was insufficient in renal cortex and medulla of SS rats compared
 101 with SS.13^{BN}. To confirm that the renal cells exhibit the same response to the
 102 insufficient FH activity as FH knockdown HK-2 cells, the expression and activity of
 103 these key enzymes involved in redox metabolism were detected. For ROS formation,
 104 the activity of NOX had been reported increase definitely in renal medulla of SS rats
 105 [5], similar to FH knockdown HK-2 cells. The expression of OGDH increased
 106 significantly, and the expression of ATP5A1 reduced significantly in renal medulla
 107 (Figure 3). SS rats mitochondrial respiratory chain complex I and III had been
 108 reported involved in ROS production owing to the insufficient expression of EFTu
 109 (elongation factor Tu) protein in the mTAL mitochondria [12].

110 For the antioxidant response, the total GSH (GSH+GSSG), GSH reduced
111 significantly in renal medulla (Figure 4A). The GSSG increased both in renal cortex
112 and medulla (Figure 4B). The ratio of GSH/GSSG reduced significantly in renal
113 medulla ($P<0.01$), and renal cortex ($P=0.065$) (Figure 4C). The GSH synthesis substrate
114 glutamate, glycine and L-cysteine reduced significantly in renal medulla (Figure 4D).
115 Meanwhile, the activity of G6PD increased in renal cortex and medulla, but the
116 activity of 6PGD and NADP⁺-IDH only increased in renal cortex of SS rats compared
117 with SS.¹³BN (Figure 4 E, F). These data suggested SS rats renal tissue had the similar
118 redox metabolism to Fh knockdown HK2 cells.

119 **Redox state in kidney with SS rat oral administration of fumarate and malate**

120 The insufficiency of FH activity will result in the accumulation of fumarate and
121 slight insufficiency of malate. As FH don't directly involve in the formation of ROS ,
122 the metabolic intermediate fumarate or malate probably participate in the ROS
123 production. To further verify the contribution of fumarate and malate to ROS
124 generation, malate and fumarate were delivered by gavage in SS rats with normal salt
125 diet and high salt diet. The level of H₂O₂ and MDA were detected after malate and
126 fumarate supplementation for two weeks. The results showed the level of H₂O₂ and
127 MDA increased significantly in renal cortex and medulla of SS rats with fumarate oral
128 administration, while the blood pressure elevated significantly (Figure 5 A). On the
129 contrary, oral administration of malate didn't influence the level of H₂O₂ and MDA in
130 SS rat with normal salt diet supplied and the blood pressure reduced significantly
131 (Figure 5 B C). When SS rats fed with a high salt diet for two weeks, the level of H₂O₂

132 and MDA increased dramatically in renal medulla and the oral administration of
133 malate could attenuate significantly the increasing of H₂O₂, MDA and blood pressure
134 caused by high salt diet (Figure 5 D E). These data demonstrated that the substrate of
135 FH, fumarate, could exacerbate ROS production and hypertension in SS rats, on the
136 contrary, the product of FH, L-malate, could attenuate the hypertension induced by
137 high salt diet.

138 Discussion

139 Imbalance of redox state in FH knockdown HK-2 cells

140 GST catalyzes the conjugation of the reduced form of GSH to xenobiotic
141 substrates for the purpose of detoxification. The upregulated expression and
142 improved activity of GPx and GST suggested excessive consumption of GSH after FH
143 knockdown.

144 GR catalyzes the reduction of GSSG to the sulfhydryl form GSH via the conversion
145 of NADPH to NADP⁺, which is a critical molecule in resisting oxidative stress and
146 maintaining the reducing environment of the cell. The main source of NADPH in the
147 cell is from the pentose phosphate pathway (PPP) in which G6PD and 6PGD were
148 defined as the rate-limiting enzyme. The activity of GR, G6PD and 6PGD was
149 increased significantly as a response to FH knockdown in HK-2 cells (Figure 1). It was
150 also consistent with the increased protein expression. Except PPP, NADPH also can be
151 produced from other oxidoreductases, such as IDH2, which oxidizes a substrate by
152 reducing an electron acceptor usually as NAD⁺ or NADP⁺ and producing NADH or

153 NADPH. Indeed, the activity of NADP^+ -IDH2 increased significantly (Figure 1C).
 154 Meanwhile, the expression of other dehydrogenase such as LDHB, AKR1A1, ADH5,
 155 ALDH7A1 also increased significantly in Fh knockdown cells. These data suggested
 156 increasing the expression of dehydrogenase could provide more NADPH or NADH for
 157 the consumption of GR. In addition, oxidation of NADPH through mitochondrial
 158 electron transfer chains was decreased, owing to adrenodoxin oxidoreductase (FDXR),
 159 serving as the first electron transfer protein in all the mitochondrial P450 systems,
 160 reduced significantly. The ratio of $\text{NADPH}/\text{NADP}^+$ slightly increased (Figure 2B).

161 As all key enzymes (GR, GS, GST, GPx, G6PD and 6PGD) involved in glutathione
 162 metabolism increased their activities or protein expression, suggested that the
 163 oxidation-reduction reaction that GSH participated in was extremely active, excessive
 164 ROS and reducing molecular produced in HK-2 cells at the same time after FH
 165 knockdown.

166 The ratio of GSH/GSSG is often used as an indicator of the cellular redox state
 167 since it is the most abundant thiol-disulfide redox buffer in the cell [13]. Normally,
 168 GSH is about 30-100 fold excess over GSSG. The oxidation of only a small amount of
 169 GSH to GSSG can significantly change the ratio and the redox status of the cell.
 170 However, knockdown of FH expression resulted in a significant decreasing of
 171 GSH/GSSG in HK-2 cells (Figure 2E). The results further confirmed that the
 172 insufficient FH activity could result in the oxidative stress. Nevertheless, where did
 173 these ROS come from?

174 The activity of NADPH oxidases was detected and showed significantly increased

175 in FH knockdown HK-2 cells, which should be the contributor of ROS production
 176 (Figure 1A). Mitochondrial enzymes are so far known to generate ROS including the
 177 TCA cycle enzymes aconitase (ACO), OGDH, PDH, GPDH (glycerol-3-phosphate
 178 dehydrogenase), B5R and Complexes I, II and III [14]. Current differential proteomics
 179 analysis data showed that the expression of NDUF5, an important member of the
 180 Complex I, reduced significantly. Its content of expression modulates the efficiency of
 181 mitochondrial electron transport chain and ATP level [15]. The fully assembling of
 182 Complex I will be disturbed by insufficient expression of NDUF5, the ROS generation
 183 will be promoted [16, 17]. RISP as one of the catalytic subunits of Complex III, it had
 184 been demonstrated that deficiency of RISP influenced the stability of Complex I,
 185 Complex III and increased generation of ROS [18]. Both RISP and Cyt b-c1 complex
 186 subunit 6 reduced 1.5 fold in FH knockdown HK-2 cells. It suggested complex III
 187 contributed to the production of ROS. Meanwhile, the upregulation of both OGDH
 188 and B5R also promoted ROS production. ATP synthase had been defined as one
 189 important site of ROS generation. Its activity can modulate ROS formation and ATP
 190 level in mitochondria [19]. ATP5A1 was identified as a target for calpain-1 in diabetic
 191 hearts, leading to its proteolytic degradation, disruption of ATP5A1 may compromise
 192 mitochondrial function, resulting in excessive ROS generation in mice [20]. Current
 193 data showed that the expression of ATP synthase subunit ATP5A1, ATP5C1, ATP5I and
 194 ATP5L reduced significantly, and accordingly ATP level reduced too (Figure 1B).

195 Briefly, the signature of redox metabolism for FH knockdown HK-2 cells would be
 196 NOX, α -OGDH, B5R, ATPase and complex I, III worked as the main sites of ROS

197 formation, and upregulation expression of GR, GS, GST, GPx, G6PD, 6PGD, SOD2,
198 IDH2 and NNTH worked as an antagonistic response to the excessive ROS. The final
199 substantial drop in GSH/GSSG ratio was the result of FH knockdown. The redox
200 metabolism for FH knockdown HK-2 cells was summarized in Figure EV5.

201 **The oxidative stress in renal medulla and cortex of SS rats**

202 It had been demonstrated that NOX was the major source of excess superoxide
203 produced in renal medulla of SS rats [5]. The increase of NOX activity is consistent
204 with it in FH knockout HK-2 cells. The expression of OGDH increased in renal medulla,
205 on the contrary, the expression of ATP5A1 reduced significantly in renal medulla and
206 cortex of SS rats compared with SS.13^{BN}. These results suggested that OGDH and
207 ATPase may also contribute to ROS formation in renal medulla of Dahl SS rats.
208 Meanwhile, one mitochondrial proteomics analysis also suggested that Complex I
209 and Complex III were also the ROS production sites in renal medullary thick
210 ascending limb (mTAL) of the SS rats [12]. Briefly, B5R, NOX, α -OGDH, ATPase,
211 Complex I and Complex III would be the sites of ROS production in renal medulla of
212 SS rats.

213 For the antioxidant response of SS rats, the synthesis of GSH was also modulated
214 by the insufficient substrates, as the levels of glycine, glutamate and cysteine were
215 reduced in renal medulla compared with SS.13^{BN}. The levels of GSH, GSSG and total
216 GSH (GSH+GSSG) in renal medulla of SS rats were the similar to those in FH
217 knockdown HK-2 cells. Especially, the ratio of GSH/GSSG also reduced significantly.
218 GPx had been reported increase in SS medulla [21]. Other dehydrogenases such as

219 IDH2, 6GPD and G6PD also increased their expression or activity. These enzymes in
220 renal medulla of SS rats had the same response to oxidant stress as FH knockout HK-2
221 cells. The full comparison involved in redox balance between SS rats and FH knockout
222 HK-2 cells was listed in Table EV5. All the data suggested that the imbalance of redox
223 metabolism in renal medulla of SS rats was highly similar to FH knockout HK-2 cells.

224 **Fumarate and malate contribute to the imbalance of redox and hypertension in SS** 225 **rats**

226 There is no evidence to confirm that FH is the site of ROS production, but the
227 excessive fumarate formed by the insufficient FH activity could lead to oxidative
228 stress by succination of GSH [22]. This covalent adduct between fumarate and GSH
229 reduced the efficiency and level of GSH in the cell. Meanwhile, the total GSH
230 (GSH+GSSG), GSH reduced significantly in SS renal cells and HK-2 cells as inadequate
231 synthetic substrate, glycine and glutamate. Obviously, the insufficient GSH flux
232 attenuated the antioxidant ability of GSH system and promote ROS formation. Indeed,
233 the content of fumarate had been proved to increase in SS renal medulla,

234 Current data had proved extra fumarate oral administration significantly
235 increased the levels of H₂O₂ and MDA, and exacerbated the elevation of MAP (Figure
236 5). As the production of FH, excess malate oral administration could reduce the
237 generation of ROS and attenuated the high salt diet induced hypertension. The
238 mechanism of malate working is mainly through increasing synthesis of arginine and
239 NO [11].

240 **Conclusion**

241 The insufficient FH activity led to the accumulation of fumarate in renal medulla
242 of SS rats, and excessive ROS generated from NOX, OGDH, ATPase and mitochondrial
243 respiratory chain complex I, III. The increased G6PD, 6PGD, IDH and other
244 dehydrogenase activity became the main antagonistic response of cells to oxidative
245 stress induced by FH insufficiency. Glutathione metabolism, as the most important
246 antioxidant mechanism, lost its balance due to insufficient synthesis substrate and
247 resulted in the decreasing of antioxidant capacity, which made the cells under the
248 oxidative stress state. Therefore, the defect of FH gene located on chromosome 13 of
249 SS rats cause a wide range of changes at several sites of ROS production and
250 antioxidant mechanisms

251 **Materials and Methods**

252 **Cell culture and siRNA-mediated RNA interference**

253 HK-2, a human kidney epithelial cell line was obtained from and cultured as
254 suggested by Conservation Genetics CAS Kunming Cell Bank (China). RNA
255 interference was performed as described previously [9, 23]. The small interfering
256 RNA (siRNA) for human fumarase targets the CCAGGAUUAUGGUCUUGAUTT
257 sequence (Gene Phaima Technologies, China). Western blot and enzyme activity
258 analyses were performed 64 hours after transfection.

259 **Western blotting**

260 Western blot was performed as described previously [24]. Sources and

dilutions of primary antibodies used were Fumarase, FH (CST, USA) 1:1000;
Superoxide dismutase [Mn], SOD2 (Wanleibio, China) 1:2000; NADP⁺-dependent
isocitrate dehydrogenase cytoplasmic, NADP⁺-IDH1 (Wanleibio, China) 1:2000;
NADP⁺-dependent isocitrate dehydrogenase, mitochondrial, NADP⁺-IDH2 (BOSTER,
China) 1:2000; Heat shock protein HSP 90, HSP90 (Wanleibio, China) 1:2000;
2-Oxoglutarate dehydrogenase, OGDH (CST, USA) 1:1000; ATP synthase subunit
alpha, ATP5A1 (Proteintech, USA) 1:2000; β -Actin (CW0096, China) 1:10000.
Secondary antibodies were HRP-conjugated goat anti-mouse IgG (BOSTER, China) or
HRP-conjugated goat anti-rabbit IgG antibody (Santa Cruz, USA). Signals were
developed with Thermo Super Signal West Pico Trial Kit. The intensities of the target
proteins were quantified using Image J software and β -actin was used as loading
control.

Measurement of ROS and H₂O₂

ROS level was detected using dichlorodihydrofluorescein diacetate (H₂DCF-DA)
(Sigma-Aldrich) [25]. Fluorescence was determined with a fluorescence
spectrometer (TECAN Infinite M200 PRO, Schweiz) at 485 nm (excitation) and 538
nm (emission). Cellular oxidant levels were expressed as relative DCF fluorescence
per sample protein amounts (measured using a BCA assay). H₂O₂ generation was
detected using Hydrogen Peroxide assay kit (Jiancheng Biochemical).

Proteomic analysis

(1) Protein reduce, cysteine block and digest

HK-2 cells were lysed in a buffer containing 9 mol/L Urea, 4% CHAPS, 1% DTT,

283 1% IPG buffer (GE Healthcare) at 4 °C for 1 h. The cell lysates were centrifuged at
284 15000 g for 25 min. The supernatant were collected and the protein concentration
285 was determined by Bradford method [26]. A volume corresponding to 100 µg of
286 protein were precipitated with 5 volumes of acetone at -20°C for 1h. After
287 centrifugation for 10 min at 15000 g, the deposits were collected and dried by
288 vacuum freezing dryer. Then dissolve protein pellets in 50 µL iTRAQ dissolution
289 buffer (Applied Biosystems) and add 4 µL Reducing Reagent (Applied Biosystems) at
290 60 °C for 1 h. After cooling samples to room temperature (RT), cysteine residues
291 were blocked with 2 µL 200 mmol/L methylmethanethiosulfate by incubating at RT
292 for 10 min. The protein solutions were added to 10 kDa ultrafiltration tubes and
293 cleaned by centrifugation (12000 rpm, 20 min). Then 100 µL iTRAQ dissolution
294 buffer was added in ultrafiltration tubes, centrifuged at 12000 g for 15 min and
295 repeat this step three times. Place the column in a new tube, add 50 µL
296 sequencing-grade trypsin (50 ng/µL) and incubate at 37 °C for 12 hours. Afterward,
297 centrifuge by 12000 g for 20 min and collect the peptide. Transfer the filter units to
298 new collection tube and add 50 µL iTRAQ dissolution buffers to centrifuge the tube
299 again. Combined the two filter solutions.

300 (2) Protein digestion and labeling with iTRAQ reagents

301 The labeling reactions were performed following the manufacturer's
302 recommendations. Peptide samples from negative control HK-2 cells were labeled
303 with iTRAQ reagent, 113 isobaric tag and peptide samples from FH siRNA-treated
304 HK-2 cells were labeled by adding the same amount of iTRAQ reagent, 114 isobaric

305 tag. After 2 h of incubation at RT, reactions were stopped by adding 100 μ L of water,
306 to each vial.

307 (3) Peptide fractionation by strong cation exchange chromatography

308 The mixed peptides were fractionated by strong cation exchange
309 chromatography (SCX). Samples were separated using an Agilent 1200 HPLC System
310 (Agilent), Michrom column (Poly-SEA 5 μ 300Å 2.0 x 150 mm) at a flow rate of 0.3
311 ml/min, using a nonlinear binary gradient starting with buffer A (10 mmol/L formic
312 acid, 20% acetonitrile) and transitioning to buffer B (500 mmol/L formic acid, 20%
313 acetonitrile). First, the column was washed with buffer A for 5 min and peptides
314 were eluted with a four-step gradient: first a linear gradient of 5-50% buffer B for 25
315 min, followed by a linear gradient of 50-80% buffer B for 5 min. The gradient was
316 ramped to 100% buffer B in 1 min, and held for 10 min. Collect the first segment
317 from 0-5 min, then collect each segment with 4 min interval for the 6-44 min, and
318 for the last segment from 45-50 min, with a total of 12 segments. Dry every
319 segment in a vacuum freezing dryer for LC-MSMS analysis.

320 (4) RPLC-MS/MS analysis

321 The online Nano-RPLC was employed on the Eksigent nanoLC-Ultra™ 2D
322 System (AB SCIEX). The samples were loaded on C₁₈ nano LC trap column (100 μ m×3
323 cm, C₁₈, 3 μ m, 150 Å) and washed by Nano-RPLC Buffer A (0.1% formic acid, 2%
324 acetonitrile) at 2 μ L/min for 10 min. An elution gradient of 5-35% acetonitrile (0.1%
325 formic acid) in 70 min gradient was used on an analytical Chrom XP C₁₈ column (75
326 μ m×15 cm, C₁₈, 3 μ m 120 Å) with spray tip. Data acquisition was performed with a

327 Triple TOF 5600 System (AB SCIEX, USA) fitted with a Nanospray III source (AB SCIEX,
328 USA) and a pulled quartz tip as the emitter (New Objectives, USA). Data were
329 acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of
330 5 PSI, and an interface heater temperature of 150 °C. For information-dependent
331 acquisition (IDA), survey scans were acquired in 250 ms and as many as 35 product
332 ion scans were collected if they exceeded a threshold of 150 counts per second
333 (counts/s) with a 2⁺ to 5⁺ charge-state. The total cycle time was fixed to 2.5 s. A
334 rolling collision energy setting was applied to all precursor ions for collision-induced
335 dissociation (CID). Dynamic exclusion was set for ½ of peak width (18 s), and the
336 precursor was then refreshed off the exclusion list.

337 (5) Protein identification and quantification

338 Data were processed with Protein Pilot Software v. 4.0 (AB SCIEX, USA) against
339 Homo sapiens database using the Paragon algorithm [27]. Protein identification was
340 performed with the search option: emphasis on biological modifications. The
341 database search parameters were as following: instrument was Triple TOF 5600,
342 iTRAQ 2-plex quantification, cysteine modified with iodoacetamide, biological
343 modifications were selected as the ID focus, trypsin digestion. An automatic decoy
344 database search strategy was employed to estimate the false discovery rate (FDR)
345 using the PSPEP (Proteomics System Performance Evaluation Pipeline Software,
346 integrated in the Protein Pilot Software). The FDR was calculated as the false
347 positive matches divided by the total matches. The iTRAQ 2-plex was chosen for
348 protein quantification with unique peptides during the search. A total of 3174

349 proteins were identified in HK-2 cells, with the value of global FDR from fit less than
350 1% and identified with more than two peptides for 95% confidence were
351 considered for further analysis. Proteins with a fold change of >1.50 or <0.67 were
352 considered to be significantly differentially expressed.

353 **GO and KEGG pathway enrichment analysis**

354 The multi-omics data analysis tool, Omics Bean, was used to analyze the
355 obtained proteomics data (<http://www.omicsbean.com:88>), in which distributions in
356 biological functions, subcellular locations and molecular functions were assigned to
357 each protein based on Gene Ontology (GO) categories. The Kyoto Encyclopedia of
358 Genes and Genomes (KEGG) pathway analysis was performed to enrich high-level
359 functions in the defined biological systems.

360 **Enzymes activities assays**

361 The obtained pellets were lysed by three freeze–thaw cycles to ensure that the
362 mitochondrial membrane was disrupted and enzymes were accessible. The protein
363 content was determined with BCA Protein Assay Kit (Beyotime Institute of
364 Biotechnology, China).

365 The enzyme activity of FH was measured based on a previous reported method
366 [8]. The activities of glutathione reductase (GR) were assayed using the Beyotime
367 Detection kits (Beyotime Institute of Biotechnology, China) according to the
368 corresponding protocols. The activities of glutathione S-transferase (GST),
369 glutathione peroxidase (GPx) in HK-2 cells were assayed using the Jian cheng
370 Biochemical detection kits according to the standard protocols.

371 Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate
372 dehydrogenase (6PGD) activities assay were performed as described previously [28,
373 29]. Isocitrate dehydrogenase, cytoplasmic (NADP⁺-IDH1) and isocitrate
374 dehydrogenase, mitochondrial (NADP⁺-IDH2) are NADP⁺-dependent isocitrate
375 dehydrogenase (NADP⁺-IDH). NADP⁺-IDH activity was assayed by
376 spectrophotometric monitoring of the reduction of NADP⁺ to NADPH at 340 nm
377 according to protocol by Connie S. Yarian [30]. The NADPH oxidase activity was
378 determined using the NADPH oxidase assay kit purchased from Nanjing Jiangcheng
379 Bioengineering Institute according to the manufacturer's instructions.

380 **GSH (GSSG) and NADPH (NADP⁺) measurements**

381 The determination of reduced glutathione (GSH) and oxidized glutathione
382 (GSSG) were performed using DTNB method with kits according to the
383 corresponding kit protocols (Beyotime, China). The determination of NADP⁺ and
384 NADPH were performed with kits according to the corresponding kit protocols
385 (Comin, China).

386 **GC-MS assay**

387 The extraction of metabolites in tissue and cells samples was performed
388 according to the method described in previous reports [31, 32]. Dried tissue and cell
389 residues were derivatized using a two-step procedure with a minor modification [33,
390 34]. For oximation, 80 µL of 10 mg/mL methoxyamine hydrochloride (Sigma-Aldrich)
391 dissolved in pyridine was mixed with a lyophilized sample, and kept at 30°C for 90
392 min. Then, 80 µL of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% TMCS

(Sigma-Aldrich) was added for derivatization, and heated to 70°C for 60 minutes.

GC-MS analysis was performed according to our previous report [33], using a 7890A GC/5975C Inert MSD (Agilent Technologies, Wilmington, DE), coupled with a DB-5 column (30 m × 0.25 mm I.D.; film thickness: 0.25 µm; Agilent J&W Scientific, USA). Helium was used as carrier gas at a constant flow rate of 1 ml/min. The GC temperature programming was set to 2 min isothermal heating at 80°C, followed by 10°C/min oven temperature ramps to 120°C, 5°C/min to 260°C, and then increased at a rate of 10 °C /min to 300°C, where it was held for 2 min. Fumarate, L-malate, glutamate, glycine, L-cysteine standards were analyzed under identical experimental conditions for further identification. One µL of the final derivatized aliquots was injected into the GC-MS.

Measurement of ATP

The ATP assay was performed with a kit from Promega (G7570) according to manufacturer's instruction. The luminescence was recorded in a TECAN Infinite M200 PRO (Schweiz) with an integration time of 5s per well.

Animals and tissues

Male SS rats (SS/JrHsdMcwi) and consomic, salt-insensitive SS.13^{BN} rats [35] were bred in a pathogen-free animal house and maintained on a purified AIN-76A rodent diet (Dyets) containing 0.4% NaCl with free access to water. For tissue collection, kidneys were flushed in situ with cold saline and the renal cortex and medulla were quickly removed and snap-frozen in liquid nitrogen and stored at -80°C. Frozen tissues were used for preparation of tissue homogenates to perform activity

assays, Western blots and metabolite analysis. The experiments were approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University.

Fumarate and malate supplementation

Male SS rats were fed a 0.4% NaCl diet since weaning. After three days of stable baseline blood pressure at approximately 7 weeks of age, rats were switched to an 8% NaCl diet (HS). Fumarate (50 mg/kg/d) [36], malate (600 mg/kg/d) [37], or an equal volume of distilled water was delivered by gavage. Serum and kidneys were harvested at the end of blood pressure measurement on day 17 of the HS diet.

Measurement of blood pressure

Blood pressure of conscious rats was measured using tail-cuff plethysmography with a CODA-4 computerized system (Kent Scientific Corporation, Torrington, Connecticut, USA). Mean arterial pressure (MAP) was measured between 14:00 and 16:00 by a single experienced operator. Rats were trained for the procedure for five consecutive days. After calibrating the tail-cuff apparatus, rats were placed on the platform and allowed to rest in a glass restrainer. A black conical plastic piece with a nose opening was placed over the head region of the rat to cover the eyes and allow the rat to rest. The platform was heated to 30–32 °C to increase the detection of the oscillation waveforms generated by the blood flow in the tail. Each session was composed of 15 successive measurements of the systolic and diastolic pressure, which were averaged. Heart rate was recorded at the same time.

Measurement of H₂O₂ and MDA in the kidney of SS rats

H₂O₂ and malondialdehyde (MDA) generation were detected using Hydrogen

437 Peroxide assay kit (Jiancheng Biochemical).

438 **Statistical analysis**

439 All values are presented as mean $\pm$ SEM. All data were analyzed using the
440 Student's t-test or ANOVA analysis. Probabilities of < 0.05 were considered to be
441 statistically significant. All of the statistical tests were performed with the Graph Pad
442 Prism software, version 6.0 (Graph Pad Software Inc., San Diego, CA).

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445 **Conflict of interest**

446 The authors declare that they have no conflict of interest.

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562 mice. *Physiological research* **56**: 213

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564

565 **Figure legends**

566 **Figure 1 - NADPH oxidase and key enzymes involved in glutathione metabolism** 567 **activities in fumarase insufficient HK-2 cells.**

568 A NADPH oxidase activity enhanced in fumarase insufficiency HK-2 cells.

569 B Fumarase insufficiency attenuated ATP production.

570 C Glucose-6-phosphate 1-dehydrogenase (G6PD), 6-Phosphogluconate
571 dehydrogenase (6PGD) and NADP⁺-IDH activity.

572 D Glutathione reductase (GR) activity.

573 E Glutathione S-transferase (GST) activity.

574 F Glutathione peroxidase (GPx) activity. Mock siRNA targeting fumarase that had no
575 sequence homology with any mammalian genes as a negative control.

576 Data are presented as mean values $\pm$ SEM, n = 4, *p < 0.05, **p < 0.01 compared to
577 negative control.

578

579 **Figure 2 - Fumarase insufficiency is associated with increased NADPH, NADP⁺ and** 580 **reduced total GSH, GSH/GSSG ratio.**

581 A NADPH and NADP⁺ levels were detected in negative control and fumarase
582 insufficient HK-2 cells.

583 B NADPH/NADP⁺ ratio.

584 C Total GSH (GSH+GSSH) and GSH levels.

585 D GSSG levels.

586 E GSH/GSSG ratio.

587 F Fold changes of the glutamate, glycine and L-cysteine levels in fumarase insufficient
588 HK-2 cells was calculated as a ratio relative to negative control. Mock siRNA targeting
589 fumarase that had no sequence homology with any mammalian genes as a negative
590 control. Data are presented as mean values $\pm$ SEM, n = 6, *p < 0.05, **p < 0.01
591 compared to negative control.

592

593 **Figure 3 - Western blot analysis of protein abundance of OGDH and ATP5A1 in the**
594 **kidney of SS and SS.13^{BN} rats.**

595 A Abundance of α -oxoglutarate dehydrogenase (OGDH) and ATP synthase subunit
596 alpha (ATP5A1) in the renal cortex.

597 B Abundance of OGDH and ATP5A1 in the renal medulla. β -actin is used as the
598 internal loading control. Data are presented as mean values $\pm$ SEM, n = 4, *p < 0.05
599 compared to negative control.

600

601 Figure 4 - Total GSH levels and GSH/GSSG ratio were reduced in the kidney of SS rats.

602 A Total GSH (GSH+GSSH) and GSH levels reduced in the renal cortex and medulla.

603 B GSSG levels.

604 C GSH/GSSG ratio.

605 D Fold changes of the glutamate, glycine and L-cysteine in the renal medulla.

606 E G6PD, 6PGD activities in the renal cortex and medulla.

607 F NADP⁺-IDH activity in the renal cortex and medulla.

608 Data are presented as mean values $\pm$ SEM, n = 5, *p < 0.05 compared to negative

609 control.

610 **Figure 5 - Levels of H₂O₂ and MAD in the kidney of SS rats with fumarate or malate**
 611 **supplementation.**

612 A Mean arterial blood pressure (MAP) of SS rats on 0.4% salt (normal salt, NS) diet
 613 with or without malate (fumarate) or 8% salt (high salt, HS) diet with or without
 614 malate supplementation by gavage.

615 B Levels of H₂O₂ in the renal cortex and medulla of SS rats on 0.4% salt with or
 616 without malate (fumarate) supplementation by gavage.

617 C Levels of MDA in the renal cortex and medulla of SS rats on 0.4% salt with or
 618 without malate (fumarate) supplementation by gavage.

619 D Levels of H₂O₂ in the renal cortex and medulla of SS rats on 0.4% salt or 8% salt
 620 diet with or without malate supplementation by gavage.

621 E Levels of MDA in the renal cortex and medulla of SS rats on 0.4% salt or 8% salt
 622 diet with or without malate supplementation by gavage. Data are presented as
 623 mean values $\pm$ SEM. n = 5-6, *p<0.05, **p < 0.01.

624

625 **Expanded View Figure legends**

626 **Figure EV1 - Down-regulation of fumarase expression and activity is associated with** 627 **the accumulation of fumarate, ROS and H₂O₂ in the HK-2 cells.**

628 A Western blotting densitometric analysis of fumarase of HK-2 cells on both negative control
629 (NC) and FH-siRNA treated (FH-SiRNA) with the associated gel shown.

630 B Fumarase (FH) activity.

631 C Fold changes of fumarate and L-malate level in fumarase insufficient HK-2 cells were
632 calculated as a ratio relative to negative control.

633 D ROS levels.

634 E, H₂O₂ levels. Mock siRNA targeting fumarase that had no sequence homology with any
635 mammalian genes as a negative control.

636 Data are presented as mean values $\pm$ SEM, n = 4-6, *p < 0.05, **p < 0.01 compared to
637 negative control.

638

639 **Figure EV2 - Expressional changes of SOD2, IDH1, IDH2 and HSP90 in the HK-2 cells** 640 **induced by fumarase insufficiency.**

641 Left: a representative result of western blotting shows the expressions of SOD2, IDH1, IDH2,
642 and HSP90 in fumarase insufficient HK-2 cells compared to negative control.

643 Right: histogram shows the expression levels of the four proteins in these HK-2 cells as
644 determined by densitometric analysis.

645 β -actin is used as the internal loading control. Mock siRNA targeting fumarase that had no
646 sequence homology with any mammalian genes as a negative control.

647 Data are presented as mean values $\pm$ SEM. n=4, *p < 0.05, **p < 0.01 significant difference

648 from negative control.

649

650 **Figure EV3 - GO annotation of identified significant changes proteins in three**
651 **categories: biological process (BP), cellular component (CC) and molecular function**
652 **(MF).**

653 All of biological processes were ranked in term of the enrichment of the differentially
654 expressed proteins, and the top 10 are presented.

655

656 **Figure EV4 - Distribution of enriched KEGG Pathway. Top 10 enriched pathway are**
657 **shown here.**

658 P value =0.01 (red) and P value =0.05 (blue) as two selected cutoff are highlighted on the
659 figure, as an indicator to show how significant the results are based on KEGG enrichment.

660

661 **Figure EV5. Role of fumarase insufficiency in reactive oxygen species metabolism.**

662 The proteins or compounds involved in ROS generation (red stars) and antioxidant defence
663 (blue stars). The red arrows indicate proteins or compounds identified as significantly
664 increased in our study. The green arrows indicate proteins or compounds identified as
665 significantly decreased in our study. FH, Fumarase; OGDH, 2-oxoglutarate dehydrogenase; C I,
666 C II and C III, the electron-transport chain (ETC) complexes I, II and III; B5R, cytochrome b5
667 reductase; NNTH, Nicotinamide nucleotide transhydrogenase; G6PD, Glucose-6-phosphate
668 1-dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; IDH2, Isocitrate
669 dehydrogenase [NADP+], mitochondrial; PRDX5, Peroxiredoxin-5, mitochondrial; FDXR,
670 NADPH:adrenodoxin oxidoreductase, mitochondrial; SOD2, Superoxide dismutase [Mn]; GS,
671 Glutathione synthetase; GR, Glutathione reductase; GPx, Glutathione peroxidase; GSH,
672 reduced glutathione; GSSG, oxidized glutathione. PM, Plasma membrane; OMM, Outer
673 mitochondrial membrane; IMM, Inner mitochondrial membrane.

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676 **Expanded View Table legends**

677 **Table EV1.** Summary of the total proteins were identified and quantified reliably by
678 iTRAQ.

679

680 **Table EV2.** Differentially expressed proteins in FH-siRNA treated HK-2 cell line
681 identified from iTRAQ analysis.

682

683 **Table EV3.** GO enrichment of the total significant changes proteins.

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685 **Table EV4.** KEGG enrichment of the total significant changes proteins.

686

687 **Table EV5.** The enzymes and metabolites involved in ROS generation and
688 antioxidant defence in fumarase insufficient HK-2 cell and SS rats.

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Insufficient fumarase contributes to generating reactive oxygen species in Dahl salt sensitive rats

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5