Deficiency in the Formation of 20-HETE Enhances Renal Ischemia-Reperfusion Injury in Dahl Salt-Sensitive Rats

SUPPLEMENTAL METHODS

Measurement of plasma 20-HETE concentration. Plasma samples (300 μl) were diluted in 0.1M sodium acetate buffer and 5% methanol after the addition of 2 ng of an internal standard, 20-HETE-d6, and loaded on a Bond Elut Prep column (Agilent Technologies, Santa Clara, CA). The samples were washed with 2 mls of 50:50% methanol/water and eluted with 2 mls of 75:25:0.1% hexane/ethyl acetate/acetic acid. The samples were dried and reconstituted in 10% solution of methanol in water and the metabolites of arachidonic acid were measured using an ABI-Sciex 4000 Q-Trap LC/MS/MS as previously described.[1-3]

Platelet preparation and aggregation assays. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described.[4] Whole blood samples were diluted in 0.11M sodium citrate solution (*9:1 v:v*). PRP was prepared by centrifugation of whole blood at 150g for 10 minutes, followed by aspiration of the upper two thirds of the plasma layer. PPP was prepared by centrifuging the lower plasma layer at 2500g for 20 minutes. Aggregation was induced by the addition of 2 μM ADP (Bio/Data Corporation, Horsham, PA) and incubation at 37^{o}C for 5 minutes. Results were quantified by measuring the rate of change of light transmission at 609 nm.[5] Baseline transmission was set to 0% with PRP prior to aggregation and 100% transmission was determined using PPP.[6]

Measurement of bleeding time. Bleeding time was measured as described previously.[7] The tail was placed in a horizontal position and was cut 3 mm from the tip. Blood was blotted onto filter paper every 30 seconds. The time until no blood appeared on the filter paper was recorded as the bleeding time.

Urine biochemical analysis. In some studies, rats were placed in metabolism cages for urine collection. Urinary sodium concentrations were measured using flame photometry (BWB Technologies USA LLC., Yorba Linda, CA) and the results were expressed as mEq excreted per day. Urine samples were also assayed by ELISA for tumor necrosis factor-α (TNFα) (Sigma-Aldrich Co., St. Louis, MO), kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL) or heme oxygenase-1 (HO-1) concentration (Enzo Life Sciences, Inc., Farmingdale, NY) and the results were expressed as ng or pg excreted per day.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Comparison of plasma 20-HETE levels and platelet function in Dahl salt-sensitive (SS) and SS.5^{BN} consomic rats. The concentration of free 20-HETE in the plasma of SS and SS.5^{BN} rats were measured by LC/MS/MS. The plasma 20-HETE levels were significantly higher in SS.5^{BN} rats compared to SS rats. There were no significant differences in platelet aggregation or bleeding time in SS and SS.5^{BN} rats. Administration of an inhibitor of the synthesis of 20-HETE, HET0016, 3 hours prior to experiments did not affect platelet function of SS.5^{BN} rats. Mean values ± SE from 6 rats per group

are presented. † indicates $P < 0.05$ from the corresponding value in SS rats.

Supplemental Figure 2. Representative images of hematoxylin and eosin (H&E)-stained section of the renal cortex of SS rats following bilateral renal IR. Examination of H&E-stained section using a fluorescent microscopy and a rhodamine filter set reveals marked staining of necrotic tubular epithelial cells and the formation of tubular casts. * indicates intact tubules; † indicates a tubular cast and ** indicates necrotic tubular epithelium.

Supplemental Figure 3. Comparison of the degree of renal tubular injury in SS and SS.5^{BN} rats following 30 minutes bilateral ischemia and 24 hours reperfusion. Panel A presents immunostaining for Atg8 positive autophagic cells (green) in the renal corticomedullary region (CMR) and outer medulla (OM) of Dahl salt-sensitive (SS), SS.5^{BN} consomic rats and SS.5^{BN} consomic rats pretreated with an inhibitor of the synthesis of 20-HETE, HET0016. Panel B presents immunostaining for ED1 positive macrophages (green) in the renal CMR and OM of SS, SS.5^{BN} rats and SS.5^{BN} rats pretreated with HET0016. These sections were counterstained with 0.001% Evans blue which exhibits red fluorescence and reduces the green autofluorescence signal in the sections. Panels C and D present a quantitative analysis of the number of stained cells on 10 random, nonoverlapping fields at 200X magnification in the renal CMR and OM. Mean values ± SE from 5 rats per group are presented. † indicates $P < 0.05$ from the corresponding value in SS rats.

Supplemental Figure 4. Comparison of the urinary excretion of renal injury biomarkers in SS and SS.5^{BN} consomic rats following 30 minutes of bilateral renal ischemia and 24 hours of reperfusion. The urinary excretion of TNFα (a), KIM-1 (b), NGAL (c) and HO-1 (d) were measured in SS and SS.5^{BN} rats 24 hrs following IR. Mean values ± SE from 6 rats per group are presented. † indicates $P < 0.05$ from the corresponding value measured in SS rats.

Supplemental Figure 5. Comparison of baseline cortical (CBF) and medullary (MBF) Laser-Doppler blood flow signals in SS and SS.5^{BN} rats. There were no significant differences in CBF or MBF in SS, SS.5^{BN} rats or SS.5^{BN} rats pretreated with the inhibitor of the synthesis of 20-HETE, HET0016 (SS.5^{BN}/HET0016). Mean values ± SE from 5 rats per group are presented.

Supplemental Figure 6. Vasocongestion in the renal cortex (CTX) and outer medulla (OM) of SS, SS.5^{BN} rats and SS.5^{BN} rats pretreated with HET0016 following 30 minutes of bilateral renal ischemia and 3 hours reperfusion. The renal medulla exhibited more vasocongestion in SS rats (b) and SS.5^{BN} rats pretreated with an inhibitor of the synthesis of 20-HETE, HET0016 (SS.5^{BN}/HET0016) (f) in comparison to the percentage of the area of red blood cells (RBC) and vascular casts seen in SS.5^{BN} rats not given HET0016 (d). Quantitative analysis was performed on 10 random, nonoverlapping fields in the renal CTX and OM at a magnification of 200X. Mean values ± SE are presented from 5 rats per group. † indicates $P < 0.05$ from the corresponding value in SS rats.

Supplemental Figure 7. Comparison of plasma creatinine concentration following 30 minutes ischemia and 24 hours reperfusion in SS.5^{BN} rats treated an inhibitor of the synthesis of 20-HETE, HET0016, 30 minutes prior to bilateral renal ischemia or 3 hours after reperfusion. Numbers in parentheses indicate the number of rats studied per group. * indicates $P < 0.05$ from the corresponding value measured in sham operated control rats. † indicates $P < 0.05$ from the corresponding value in SS.5^{BN} rats not given HET0016.

Supplemental Figure 8. Comparison of the degree of tubular injury in SS.5^{Lew} 4A^{+} (4A^{+}) and SS.5^{Lew} 4A^{-} (4A^{-}) congenic rats following 30 minutes bilateral renal ischemia and 24 hours of reperfusion. Diffuse tubular cell denudation, tubular cell necrosis, intratubular debris and tubular casts were present 24 hours after IR in the renal corticomedullary region (CMR) of 4A^{-} rats (panels A-a, g). The severity of renal tubular injury was less in 4A^{+} congenic rats with only focal areas of tubular necrosis or exfoliation of tubular cells (panels A-b, h). Pretreatment of 4A^{+} rats with the 20-HETE antagonist, 6,15-20-HEDE, increased the degree of tubular injury compared to that seen in 4A^{+} rats (panels A-c, i). Magnification=100X in panels a, b and c; 200X in panels d, e, f, g, h and i. Panel B presents quantification of the area of renal tubular injury in 4A^{+} and 4A^{-} rats and 4A^{+} rats pretreated with the 20-HETE antagonist, 6,15-20-HEDE (4^{+}/6,15-20-HEDE). Quantitative analysis was performed on 10 random, nonoverlapping fields in the renal CMR at a magnification of 200X. Mean values ± SE from 6 rats per group are presented. † indicates $P < 0.05$ from the corresponding value measured in 4A^{-} control rats.

SUPPLEMENTAL REFERENCES

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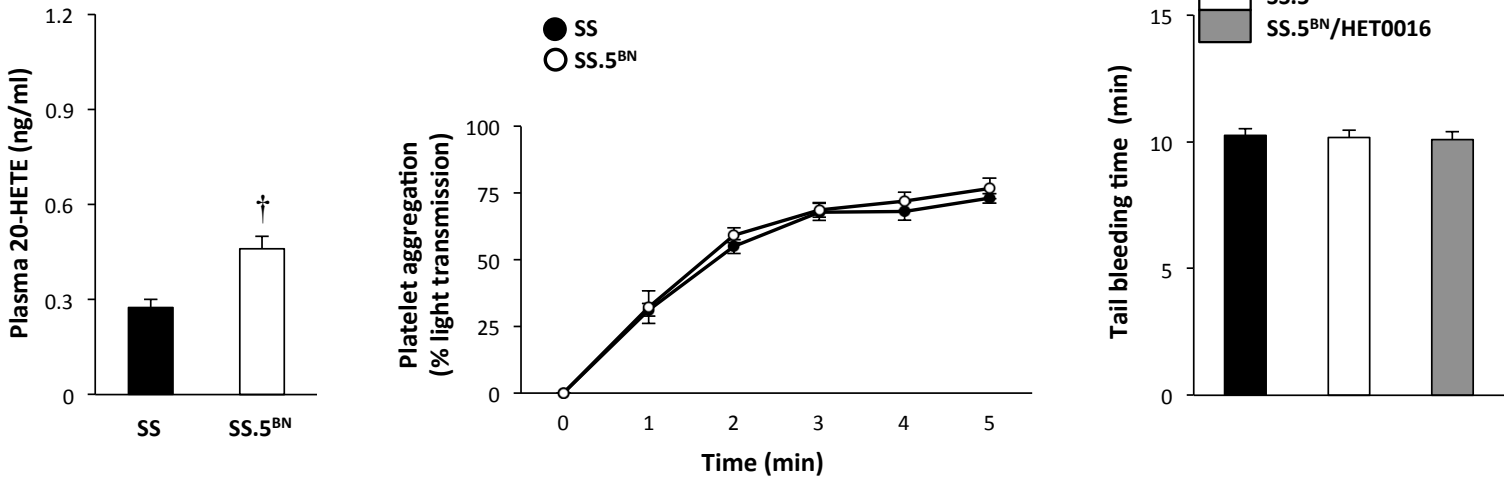
Supplemental Table 1

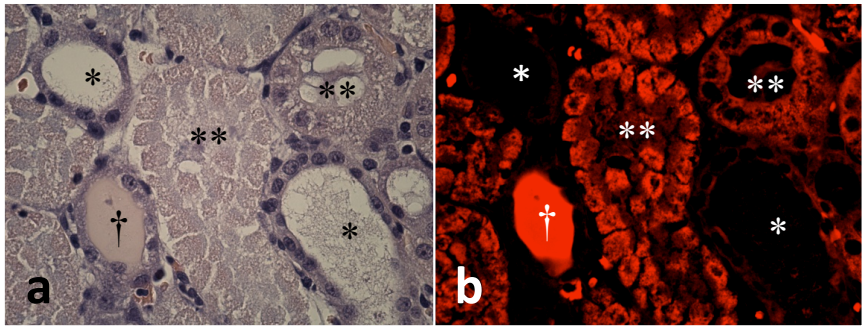
	Dahl salt-sensitive (SS)	SS.5^{BN} consomic
Body Weight (g)	291.7 ± 4.0	287.5 ± 2.1
Kidney Weight (g)	1.16 ± 0.02	1.16 ± 0.02
Systolic Blood Pressure (mmHg)	113.2 ± 1.4	111.7 ± 5.5
Urine Flow (ml/day)	11.8 ± 0.9	12.5 ± 0.5
Urinary Sodium Excretion (mEq/day)	0.26 ± 0.04	0.29 ± 0.04

Blood pressure in conscious rats was measured using a tail-cuff device (Hatteras Instruments, Cary, NC).

Supplemental Figure 1



Supplemental Figure 2



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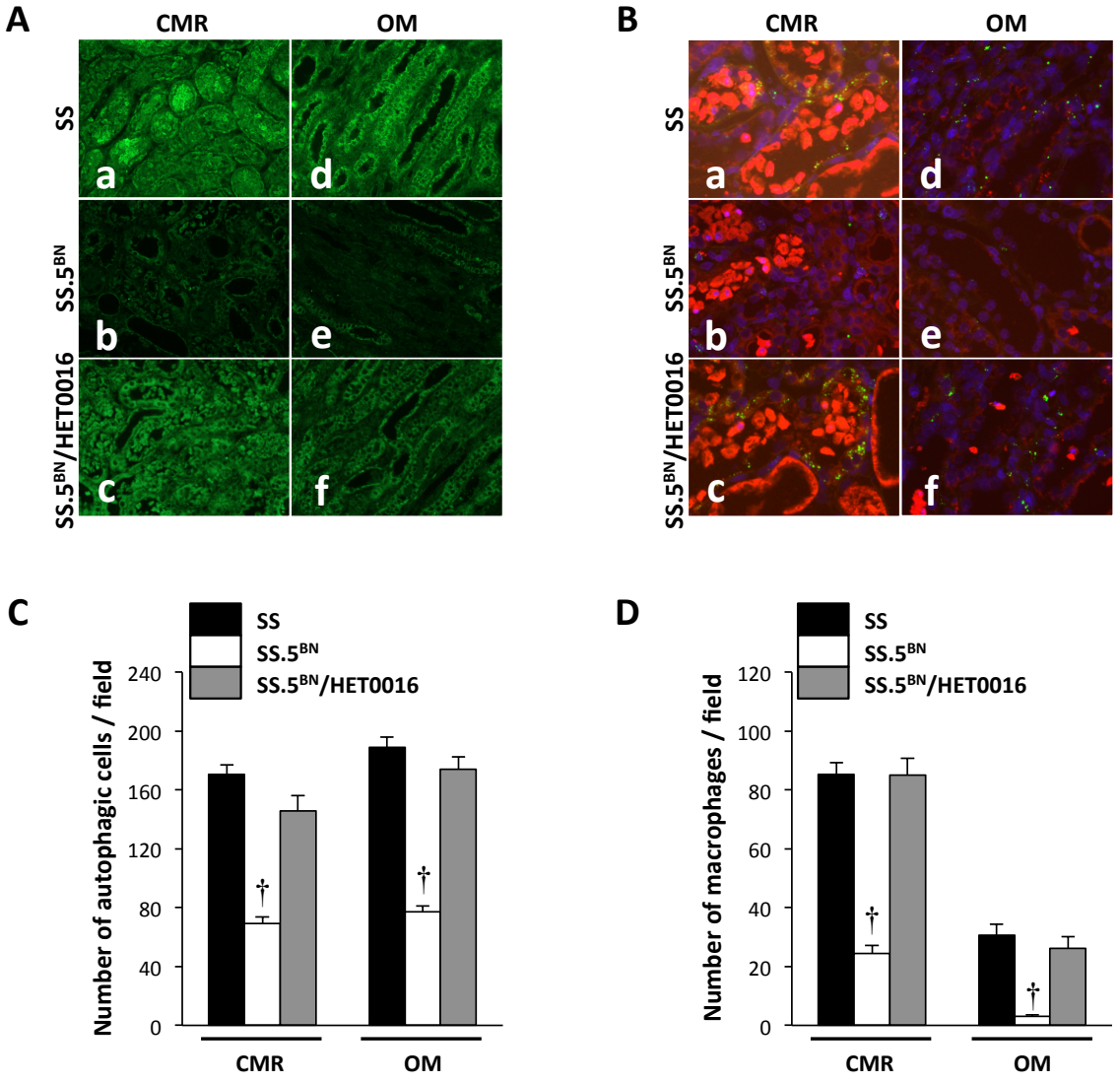

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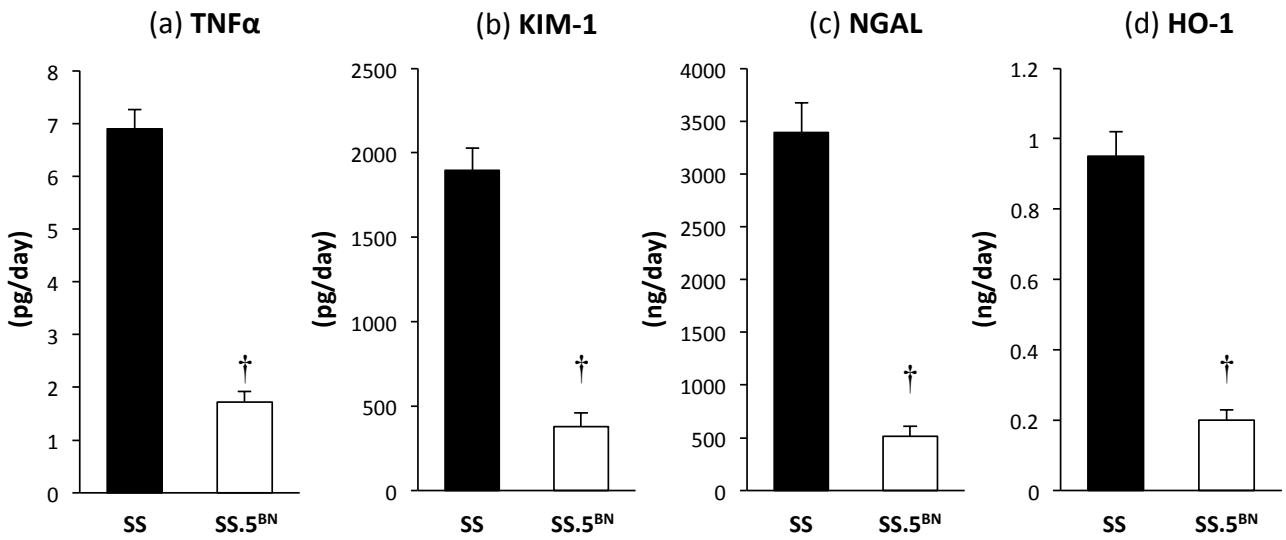

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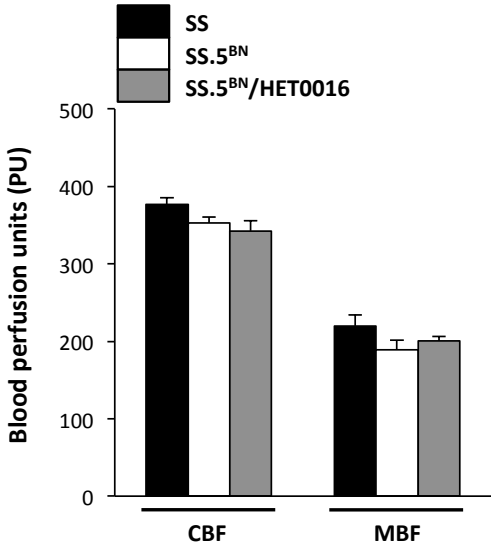

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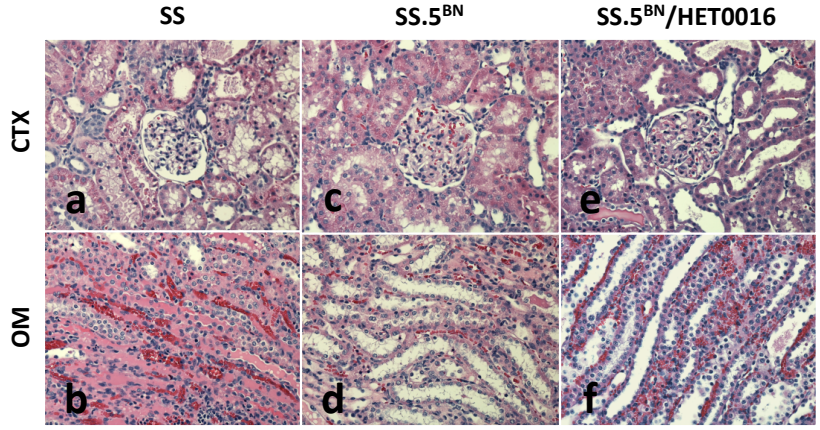
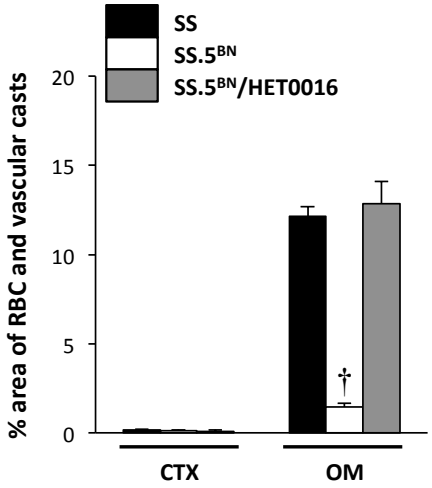
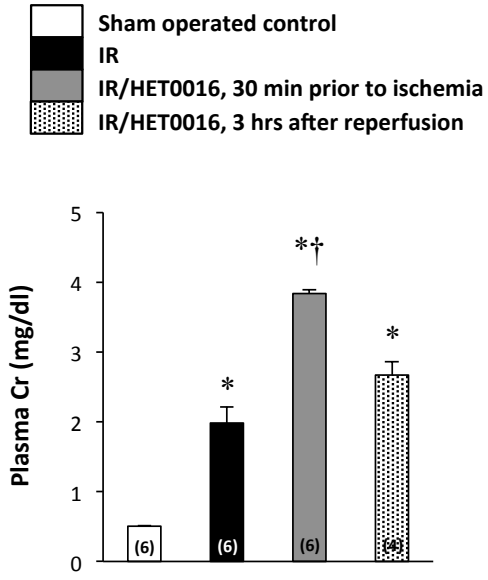




Supplemental Figure 7



Supplemental Figure 8