Renal Denervation reverses hepatic insulin resistance induced by high-fat diet.

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ABSTRACT

Activation of the sympathetic nervous system (SNS) constitutes a putative mechanism of obesity-induced insulin resistance. We therefore hypothesized that inhibiting SNS by using Renal Denervation (RDN) will improve insulin sensitivity (SI) in our non-hypertensive obese canine model. SI was measured using euglycemic hyperinsulinemic clamp (EGC), before (w0) and after six weeks of high-fat diet (w6-HFD) and after either RDN (HFD+RDN) or sham surgery (HFD+Sham). As expected, HFD induced insulin resistance in the liver (2.5±0.6x10^-4dl.kg^-1.min^-1.pM^-1 at w0 vs 0.7±0.6x10^-4dl.kg^-1.min^-1.pM^-1 at w6-HFD (P<0.05) in sham, 1.6±0.3 at w0 vs 0.5±0.3 at w6-HFD (P<0.001) in HFD+RDN). In sham animals, this insulin resistance persisted, yet RDN completely normalized hepatic SI in fat-fed dogs (1.8±0.3x10^-4dl.kg^-1.min^-1.pM^-1 at HFD+RDN, P<0.001 vs w6-HFD, P=ns vs w0) by reducing hepatic gluconeogenic genes including G6Pase, PEPCK and FOXO1. Our data suggests that RDN down-regulated hepatic gluconeogenesis primarily by up-regulating liver-X-receptor (LXR-α) via the natriuretic peptide pathway. In conclusion, bilateral RDN completely normalizes hepatic SI in obese canines. These preclinical data implicate a novel mechanistic role for the renal nerves in the regulation of insulin action specifically at the level of the liver and show that the renal nerves constitute a putative new therapeutic target to counteract insulin resistance.
Catheter-based renal denervation (RDN) has garnered great interest and debate as a potentially effective percutaneous intervention to treat hypertension and related major cardiac and metabolic disorders (1,2). Destruction of the renal nerves would interrupt both efferent and afferent renal nerves. Efferent renal nerves (ERN) are post-ganglionic sympathetic fibers that release norepinephrine (NE) and contribute to hypertension by causing renal vasoconstriction, renin release, and renal sodium and water retention (3). Afferent renal nerves are sensory fibers arising in the renal pelvis and cortex that signal the central nervous system of changes both in the chemical composition of the urine and in intra-renal pressure (4); when activated by uremic metabolites such as urea, ischemic metabolites such as adenosine, or increased intra-renal pressure as from excessive peri-nephric fat mass in animal models of obesity (5). Renal afferents can trigger sustained reflex increases in sympathetic nerve activity (SNA) targeted to multiple extra-renal tissues and vascular beds (6).

Initial enthusiasm for RDN to treat hypertension has waned after randomized sham-controlled Phase 3 clinical trials failed to show a significant improvement in severe drug-resistant hypertension (7). However, in the early phase trials, RDN was accompanied by reduced fasting glucose, c-peptide, and plasma insulin levels, suggesting improved SI in patients with resistant hypertension (2) and in hypertensive patients with polycystic ovary syndrome (8) and obstructive sleep apnea (9). However, this putative metabolic benefit was based on indirect evidence from small uncontrolled trials. Thus, this potentially important lead has never been pursued in a rigorous manner.

Here we thoroughly tested the hypothesis that RDN improves SI in a well-established non-hypertensive canine model of diet-induced obesity. In vivo SI was measured using both the minimal model approach and EGC with 3-3H-glucose to tease out the specific components of SI (hepatic and peripheral SI) before and after 6 weeks of HFD in dogs who then underwent either bilateral surgical RDN or sham surgery. Because a key unresolved limitation of catheter-based RDN clinical trials is the inability to ensure the completeness of renal nerve ablation (10,11), in
this initial proof-of-concept dog study we used a direct surgical approach to denervate renal nerves.

RESEARCH DESIGN AND METHODS:

Animals: Fifteen male mongrel dogs were housed in the vivarium under controlled conditions (12:12h light-dark cycle). Animals were included in this study only if judged to be in good health. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) from Cedars-Sinai Medical Center. All animals had ad libitum access to water. Weight-maintaining diet (control diet) consisted of 33% fat and hypercaloric high-fat diet (HFD) consisted of 54% fat.

Experimental Design: Baseline assessments (w0) of body weight, fasting blood and urine biochemistry and in vivo $S_i$ were performed on all the animals while on control diet. Then animals were fed HFD for 6 weeks (w6-HFD) to induce insulin resistance as previously shown (12). Dogs were then randomized into two groups: one group underwent surgical bilateral renal denervation, HFD+RDN (n=8); the other group had a sham operation, HFD+Sham (n=7). After surgery, the animals were allowed to recover for 10 days while still being maintained on HFD. Post-surgery metabolic assessment was performed in both groups (HFD+RDN and HFD+Sham) a week after the animals were completely recovered from the surgery. Tissue biopsies were collected at the end of post-surgery metabolic assessment. Fasting blood and urine biochemistry, body weight and blood pressure using a cuff on a foreleg (SurgiVet V6004 series non-invasive blood pressure monitor, Smiths Medical, Norwell, MA) were assessed every week throughout the study for all the animals.

Denervation: Bilateral RDN was performed surgically and chemically. Kidneys were exposed and all the adventitial tissue was stripped. All visible nerves were surgically cut and the stripped
area was “painted” with 10% phenol in ethanol solution for 5mins. In the sham operated animals, similar surgical procedure was followed except all the visible nerves were left intact and the renal arteries were “painted” with saline for 5mins.

**Validation of Denervation:** The efficiency and specificity of successful surgical denervation was validated by the following two methods.

**Catecholamine measurement:** Tissue biopsies (~200mg) was homogenized in a buffer containing final concentrations of 1mM EDTA, 4mM sodium metabisuphite (SMS) and 0.01N HCl and the homogenate was centrifuged for 15 min at 3000 rpm (4°C). The supernatant was collected and measured using 2-CAT (A-N) ELISA (LDN, Nordhorn, Germany).

**Hypoglycemic clamp:** In a subset of animals, six denervated and six sham, hypoglycemic clamp was performed before (at w6-HFD) and after denervation. Insulin (5mU.kg⁻¹.min⁻¹) and glucose (at variable rates, 50% dextrose, 454 mg/mL; B Braun) were infused to slowly decrease glycemia to ~50mg/dL.

**Assessment of S_i:** In vivo S_i was assessed prior (w0) to and after 6 weeks of fat-feeding as well as ~3 weeks post-surgery, with both EGC and the minimal model analysis as previously described (13).

**Assays:** Plasma from blood was collected and stored for insulin, D-3-H-glucose, FFA, glycerol and peptides as previously described (14). Assays for glucose, insulin, FFAs, glycerol, c-peptide and glucagon were performed as previously described (12,14).

**Total RNA isolation and real-time PCR (RT-PCR):** RNA was extracted from tissue biopsies using the Tri-Reagent® Kit (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized using Superscript II (Invitrogen, Carlsbad, CA), from 1µg of total RNA. The RT-
PCR was performed using Light-Cycler 4.8 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol.

**Urine Collection and Analysis:** Urine sample was collected using a sterile one-way Foley catheter. Urine osmolality was estimated by a vapor pressure osmometer (Wescor Vapro 5520, ELITech Group, Princton, NJ). Specific gravity of urine, urinary pH, protein-creatinine ratio, microalbuminuria, WBC, RBC and occult blood content, urinary tract infection, urinary casts, epithelial cells, crystals, ketones and bilirubin were measured by Antech Diagnostics, Irivine, CA.

**Statistical Analyses:** Two-way repeated-measures ANOVA with Bonferroni correction was performed to compare all time course data within the groups. Student's paired t-test was used to identify the significantly different time point pairs and to compare all metabolic parameters within groups. Nonpaired t-tests were used to compare means between groups. All analyses were performed using GraphPad InStat (La Jolla, CA).

**RESULTS:**
Metabolic assessments were performed at baseline pre-fat period (w0), after six weeks of fat feeding (w6-HFD) and after three weeks of surgery (HFD+Sham or HFD+RDN) (Fig.1A). Six weeks of HFD (w6-HFD) increased body weight by approximately 6.3% (P<0.001) in the Sham and by 5.2% (P<0.001) in RDN group as compared to baseline pre-fat period (Fig.1B). However, neither sham nor RDN had any further effect on body weight during the time studied. However, no changes in caloric intake (except transient increase at w2-HFD), blood pressure and heart rate were observed with continued HFD or surgery (Fig.1C-E).
Section I] Validation of denervation: We used two methods to validate the effect of the surgical technique to denervate the kidneys and differentiate the denervation from the sham surgery.

A] Renal catecholamine content: Kidney cortex NE content is the most direct measurement of efferent renal sympathetic nerve activity (SNA). We therefore measured NE in the renal cortex in an attempt to validate the denervation. RDN animals compared to sham animals had much lower NE content in the renal cortex (Right Kidney: 546.4±89.3ng/g in sham vs 128.8±36.8ng/g in RDN, P=0.003; Left Kidney: 536.1±88.8ng/g in sham vs 196.5±51.6ng/g in RDN P=0.01; Fig.2A). We found analogous results with renal cortex epinephrine (EP) content (Right Kidney: 11.5±0.8 ng/g in sham vs 7.8±1.2ng/g in RDN, P=0.02; Left Kidney: 11.4±1.2ng/g in sham vs 7.5±1.4 ng/g in RDN P=0.09; Fig.2B). However, it is noteworthy that renal cortical EP content was only a fraction of the total catecholamine content in the kidney. We also measured the expression of adrenergic receptors, α-1A, α-2A, α-2B, β1 and β2 in the kidney. Interestingly, we found no difference in the adrenergic receptor expression in the renal cortex (Fig.S2). This indicates that removal of neural signals from the sympathetic nerves did not affect the adrenergic receptor expression in the renal cortex.

B] Renin response to hypoglycemia: The rise in plasma renin activity (PRA) in response to hypoglycemia is largely mediated by the sympathetic nervous system (15). We studied the renin response to hypoglycemia before and after denervation. Interestingly, in the HFD+RDN group, PRA during steady state hypoglycemia (t=140-160mins) was modestly higher post-surgery than pre-surgery (w6-HFD), indicating higher potentiation of renin response to hypoglycemia after RDN as compared to pre-surgery (RDN: basal, 2.9±0.8ng.mL⁻¹h⁻¹ at w6-HFD vs 3.2±0.9 at HFD+RDN, P=0.1; steady state, 5.1±1.0ng.mL⁻¹h⁻¹ at w6-HFD vs 6.2±1.2 at HFD+RDN, P=0.03, Fig. 2D). No significant difference in PRA in response to hypoglycemia was observed in the sham group before and after surgery (Sham: basal, 3.3±0.8ng.mL⁻¹h⁻¹ at w6-HFD vs 3.0±0.3 at
HFD+Sham, P=0.5; steady state, 4.3±1.4ng.mL⁻¹h⁻¹ at w6-HFD vs 3.9±0.6 at HFD+Sham, P=0.4) (Fig.2C).

Catecholamine response to hypoglycemia followed a pattern similar to PRA (Fig.2K-N). Interestingly, in the HFD+RDN group there was a tendency for greater EP response to hypoglycemia as compared to pre-surgery (w6-HFD, P=0.06; Fig.2F). In addition to NE stimulation, renin response to hypoglycemia is also controlled by circulating EP secreted by adrenal medulla amongst other factors (15). It is noteworthy that renin response to insulin-induced hypoglycemia is completely abolished only when adrenal denervation is performed in addition to renal denervation (16). Our results therefore suggest that enhanced renin response with RDN may be induced by increased adrenomedullary EP secretion during a hypoglycemic challenge. These results indicate that the surgical renal denervation technique was very specific and the adrenomedullary neural circuitry was left intact.

There were no significant differences in the hypoglycemic clamp parameters, namely, baseline and steady state glucose and insulin between the RDN and Sham groups or within groups before and after surgery (Fig.S1A-D). Rate of glucose infusion (GINF) was significantly lower after RDN surgery compared to before surgery period (w6-HFD, P<0.0001, Fig.S1F), suggesting an increased resistance to hypoglycemia with RDN – a possible protective effect against hypoglycemia.

Together, the renal NE content and PRA activity during hypoglycemic challenge proved that we effectively ablated only the renal nerves in RDN but not sham animals, while leaving the adrenal nerves intact.

**Section II] Effect of nerve ablation on S_i:**

As expected, six weeks of HFD reduced S_i in both sham and RDN groups assessed by minimal model (S_i IVGTT(μU/mL⁻¹min⁻¹)), 7.6±1.3 at w0 vs 4.3±0.5 at w6-HFD (P<0.05) in sham, 5.4±0.7 at w0 vs 2.3±0.3 at w6-HFD (P<0.001) in RDN group, Fig.3A-B) as well EGC (S_i CLAMP
(x10⁴dl.kg⁻¹min⁻¹pM⁻¹) 13.7±1.5 at w0 vs 9.3±1.3 at w6-HFD (P<0.05) in sham, 9.7±0.7 at w0 vs 6.7±0.7 at w6-HFD (P<0.001) in RDN group, Fig.3G). In response to same insulin infusion rate, the rates of glucose infusion (GINF, Fig.3D-F) during the clamp were also significantly decreased with six weeks of HFD as compared to w0 (P<0.001) in both groups, indicating whole body insulin resistance. Interestingly, minimal model assessment of IVGTT showed that RDN partially restored HFD impaired $S_i$ ($S_{i, IVGTT}: 3.8±0.6\mu U/mL^{-1}min^{-1}$ at HFD+RDN, P<0.001 vs w6-HFD; Fig.3B) but sham surgery did not (4.3±0.2$\mu U/mL^{-1}min^{-1}$ at HFD+Sham, P=ns vs w6-HFD, P<0.05 vs w0; Fig.3A), although these changes were not detected by $S_{i, CLAMP}$ as GINF rates were not affected by the surgery.

In order to identify the mechanism of improved $S_i$ with RDN, we studied peripheral and hepatic $S_i$ during EGC using tritiated glucose. Interestingly, we did not find any significant changes in the rates of glucose uptake ($R_d$, Fig.4A-B) or peripheral $S_i$ ($S_{i(R_d, CLAMP)},$ Fig.4C) with HFD or surgery. Fig.3D-E shows the expected loss of suppression of endogenous glucose production (EGP) with w6-HFD. This was also reflected in the reduction of $S_i$ of EGP to insulin (hepatic insulin resistance, $S_{i(EGP, CLAMP)},$ (x10⁴dl.kg⁻¹min⁻¹pM⁻¹)) 2.5±0.6 at w0 vs 0.7±0.6 at w6-HFD (P<0.05) in sham, 1.6±0.3 at w0 vs 0.6±0.3 at w6-HFD (P<0.001 in RDN group, Fig.4F). RDN completely normalized liver $S_i$ back to baseline pre-fat w0 period despite the continuation of HFD after surgery ($S_{i(EGP, CLAMP)}$ (x10⁴dl.kg⁻¹min⁻¹pM⁻¹)) RDN: 1.8±0.3 at HFD+RDN, P<0.001 vs w6-HFD and P=ns vs w0; Sham: 1.0±0.3 at HFD+Sham, P=ns vs w6-HFD; Fig.4E-F). Renal nerve ablation totally reversed the hepatic insulin resistance induced by the HFD; sham had no such effect.

It is reasonable to assume that insulin suppression of liver EGP during clamps is due to suppression of gluconeogenesis. Therefore, as RDN restored liver $S_i$, it is important to see if that effect is concordant with reduction in GNG expressions. We therefore examined the expressions of key GNG in the liver and the kidney. Compared to sham, the RDN animals had significantly lower expressions of glucose 6-phosphatase (G6Pase, HFD+Sham: 0.6±0.2 vs
HFD+RDN: 0.2±0.02, P=0.05) and phosphoenolpyruvate carboxykinase (PEPCK, HFD+Sham: 0.8±0.1 vs HFD+RDN: 0.2±0.05, P=0.01, Fig.5A). We also found lower expression of transcription factors activating GNG genes, namely, forkhead box protein O1 (FOXO1, HFD+Sham: 0.6±0.2 vs HFD+RDN: 0.2±0.02, P=0.04), peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α, HFD+Sham: 1.1±0.3 vs HFD+RDN: 0.2±0.05, P=0.03) and peroxisome proliferator-activated receptor alpha (PPAR-α, HFD+Sham: 0.6±0.1 vs HFD+RDN: 0.3±0.1, P=0.03), in the RDN animals as compared to sham (Fig.5A). We did not find any significant differences in the expression of G6Pase, PEPCK and FOXO1 in the renal cortex between the two groups (Fig.S3), suggesting that the restoration of EGP sensitivity to insulin is mainly due to the liver and not the kidney. These data suggest that ablating the renal nerves reversed HFD induced resistance of EGP to insulin by reducing the expression of key GNG primarily in the liver. Liver X Receptor-α (LXR-α) controls hepatic GNG by downregulating PGC-1α, G6Pase and PEPCK (17). We found that RDN surgery greatly increased the expression of LXR-α (HFD+Sham: 0.1±0.03 vs HFD+RDN: 0.7±0.2, P=0.05, Fig.5A) in the liver as compared to sham surgery. In a recent study Cannon et al., showed that LXR-α improves glucose tolerance in cardiomyocytes by inducing the expression of A-type and B-type natriuretic peptides (18). In our model we found that RDN reduced the expression of natriuretic peptide clearance receptor (NPR-C, HFD+Sham: 1.9±0.4 vs HFD+RDN: 0.2±0.04, P=0.001, Fig.5B) but not natriuretic peptide receptor A (NPR-A) or natriuretic peptide receptor B (NPR-B). This indicates that RDN surgery increases the availability of natriuretic peptides (NPR-A/NPR-C ratio, HFD+Sham: 0.2±0.1 vs HFD+RDN: 3.6±1.4, P=0.05; NPR-B/NPR-C ratio, HFD+Sham: 0.2±0.1 vs HFD+RDN: 3.4±1.4, P=0.07; Fig.5C) in the liver by reducing their clearance, further suggesting that RDN improves hepatic SⅡ activating pathways that downregulate hepatic GNG. Natriuretic peptides have been suggested to increase thermogenesis especially in adipose tissue by interacting with the sympathetic nervous system (19). We therefore studied the expression of adrenergic receptors (ADR) in the liver. We found that RDN surgery significantly
reduced the expression of α-1A ADR (HFD+Sham: 0.5±0.2 vs HFD+RDN: 0.1±0.07, P=0.04, Fig.5D) in the liver, a G_{q/11} type G-protein coupled receptor (GPCR) that has been shown to induce GNG in the liver (20). In sharp contrast to our expectation we found significantly higher NE content in the liver of RDN group as compared to the sham group (Fig.5E), suggesting a compensatory increase in hepatic SNS activity. This increase in hepatic NE content may explain induction of gene expressions of both α-2A ADR (HFD+Sham: 0.2±0.04 vs HFD+RDN: 1.4±0.5, P=0.02, Fig.4D), a G_{i} type GPCR (inhibit adenylyl cyclase) and β-1 ADR (HFD+Sham: 1.0±0.2 vs HFD+RDN: 2.9±0.6, P=0.013, Fig.4D), a G_{s} type GPCR (stimulate adenylyl cyclase) (21) in the liver with RDN. We found no changes in the catecholamine content in any other tissue (Fig.S4). These results suggest renal denervation improves hepatic S_{i} via pathways downregulating hepatic GNG genes.

**Denervation did not alter kidney function:** Tests revealed no changes in the urine chemistry including osmolality, glucose and lactate (Fig.S5A-C) throughout the study in either the sham or RDN groups, suggesting that RDN did not impair renal function. We found reduced gene expression of NPR-A and NPR-B in the kidney of RDN animals suggesting a reduction in natriuresis to maintain normal kidney function and urine osmolality (Fig.S5D-E). No signs of urinary infection were found.

**DISCUSSION:**

The major new finding of this study is that effective surgical RDN totally reverses diet induced hepatic insulin resistance and does so primarily by decreasing hepatic-specific GNG expression.

By transecting the main renal nerves surgically under direct vision and painting the cut nerves with the neurotoxin phenol, we ensured extensive bilateral destruction of renal nerves, which was confirmed by an 80% reduction in renal tissue NE levels. That renal tissue EP levels
also was decreased after RDN provides further proof for extensive renal nerve destruction. Circulating epinephrine is taken up by postganglionic sympathetic nerve terminals—which were destroyed by RDN—and co-released with NE during sympathetic nerve stimulation (22). Here we also demonstrate that basal as well as hypoglycemia-induced plasma epinephrine response and renin activity were conserved after RDN surgery – suggesting an intact neural circuitry of the adrenals.

The seminal finding of our study is that effective RDN normalized hepatic $S_i$ as it restored the impaired ability of insulin to suppress hepatic EGP despite continued exposure to the HFD. These findings, together with the further observation that RDN partially restored whole-body $S_i$ by minimal model assessment in our canine model of diet-induced obesity, confirms and extends the uncontrolled clinical data based solely on the HOMA-IR index, which is limited because it does not accurately reflect changes in insulin resistance as previously shown (13).

Our data suggest that RDN normalizes hepatic $S_i$ by reducing the hepatic-specific expression of multiple GNG genes including G6Pase, PEPCK and transcription factors FOXO1, PGC-1α and PPAR-α. Insulin resistance previously has been characterized by increased hepatic expression of these genes (23,24). It is notable that RDN surgery was accompanied by increased expression of hepatic LXR-α, a transcription factor regulator involved in the control of GNG genes via down regulation of PGC-1α, G6Pase and PEPCK (17). LXR-α also may interact with natriuretic peptides and adrenergic receptors (18,25). RDN surgery reduced the expression of the natriuretic peptide clearance receptor, suggesting increased natriuretic peptide content in the liver (19). Further studies are necessary to elucidate whether natriuretic peptides have a direct effect on hepatic glucose output and/or mediate the observed effects of renal nerves on hepatic gluconeogenesis.
Several observations implicate hepatic adrenergic receptor mediation, as RDN was accompanied by: 1) increased hepatic NE content, suggesting increased hepatic SNA; 2) lower expression of the hepatic $\alpha$-1A ADR, which is involved in up-regulation of hepatic GNG via a $G_{q/11}$ GPCR pathway (20,26,27); 3) increased expression of both hepatic $\alpha$-2A ADR ($G_i$, adenylyl cyclase inhibitory) and hepatic $\beta$-1 ADR ($G_s$, adenylyl cyclase stimulatory). Diaz-Cruz et al. (28), have demonstrated that activation of $\alpha$-1 ADR inhibits $\beta$-ADR stimulated pathways via NOX2 activation. Together, our results suggest suppression of hepatic gluconeogenesis with RDN surgery via $\alpha$-1ADR, natriuretic peptides, and LXR-α pathway. Further investigation is necessary to develop a complete picture of the mechanistic underpinning.

It is also important to note that lower GNG expression was not reflected by lower basal EGP but by insulin-mediated suppression of EGP. This is paradoxical because the tissue biopsies were collected under fasting non-insulin stimulated conditions. Therefore one would expect lower hepatic GNG expression with RDN to be reflected in lower basal EGP during EGC. However, we only found lower EGP under insulin-stimulated condition during the clamp. Further studies investigating GNG protein expression, protein regulation and activity as well as other pathways related to liver EGP such glycogen synthesis and breakdown are required to understand this phenomenon.

It is interesting that RDN did not have an effect on peripheral $S_i$ as measured by EGC. This is likely because six weeks of HFD did not cause a significant impairment in peripheral $S_i$; therefore our study would not be designed to detect a restoration in peripheral $S_i$. This is congruent with the catecholamine content in the peripheral tissues where we did not find significant differences in NE levels in the muscle, visceral and subcutaneous fat depots between the sham and RDN groups (Fig.S4). In contrast to our findings, Rafiq et al. observed an improvement in peripheral glucose disposal with RDN surgery in diabetic rats along with lowering of blood pressure and suppression of sodium glucose co-transporter 2 (SGLT2) (29). It
is however unclear whether the insulin sensitizing effects observed by Rafiq et al., were independent or secondary to changes in cardiovascular factors.

We considered the possibility that RDN could potentially modulate hepatic EGP by decreasing plasma glucagon, the secretion of which can be decreased by central sympatholytic agents as well as adrenergic receptor antagonists (30,31). However, this is not the case because plasma glucagon levels were unchanged either with HFD alone or in combination with RDN (Fig.S6).

Consistent with previously published data in canines (14), in our study we did not find an effect of HFD on fasting glycemia (Fig.S6A). Accompanying insulin resistance was apparently compensated by fasting hyperinsulinemia (Fig.S6C). However, RDN surgery had no effect on fasting glucose or hyperinsulinemia (Fig.S6A,C) possibly due to reduced insulin clearance since fasting C-peptide levels were also not changed by surgery (Fig.S6D); indicating a liver effect. Our fasting glycemia results are similar to a recent study in hypertensive insulin resistant Norwegian subjects (32) showed only a slight effect of catheter-based RDN on hepatic $S_I$ with no measureable effect on fasting glucose. Also, in this Norwegian study the effects of RDN on hepatic $S_I$ were lost at higher insulin doses which may be due to the nature of the two step clamp; wherein the insulin dosage during the first step could potentially confound the results observed during the second step.

How does RDN suppress insulin-stimulated hepatic EGP? The direct versus indirect role of insulin in suppressing hepatic EGP has been in debate (33). We speculate that bilateral RDN suppresses the activity of both ERN and ARN, which in turn sensitizes the liver glucose output to insulin, possibly via central integration of inhibitory ARN signals or via neuro-hormonal control of renin release and the angiotensin-II and aldosterone system (Fig.6). ARNs project centrally via the dorsal root ganglia to the nucleus of the solitary tract (NTS) and evoke reflex changes in SNA to target tissues involved in blood pressure (34). Thus the ARN could potentially participate in CNS control of hepatic EGP by either reflex changes in hepatic SNA and vasculature,
changes in central insulin signaling (35,36), activation of mediobasal hypothalamic $K_{	ext{ATP}}$ channels and the efferent hepatic branch of vagal nerve (37), or ventromedial hypothalamus projections to the splanchnic sympathetic nerves that innervate the liver and control EGP (38,39). The central integration of ARN signals may also be involved in adrenergic control of adipose tissue lipolysis which in turn may suppress hepatic EGP – conforming to the ‘Single Gateway Hypothesis’ proposed by our group after experimentally demonstrating that suppression of hepatic EGP is indirectly mediated by suppression of visceral fat lipolysis by insulin (40–42). Another possible hypothesis is that RDN suppresses systemic renin-angiotensin-aldosterone system (RAAS) improving peripheral glucose metabolism; however the effects of RAAS system on glucose metabolism are thought to be mainly hemodynamic (43,44). However, we do not expect changes in angiotensin-II or aldosterone regulation to have a large effect on glucose metabolism because in clinical trials, ACE inhibitors and angiotensin receptor blockers (ARB) have had meager effects if any on impaired glucose metabolism (45,46). Further investigation is necessary to illuminate these pathways.

Our study has potential limitations. We did not measure arteriovenous difference across the kidney to quantify changes in net renal EGP or renal NE spillover with RDN. We also did not directly measure the effect of RDN on in vivo GNG using labeled substrate such as lactate and alanine and in vitro hepatic GNG. Measurement of in vivo trans-hepatic GNG substrate turnover requires catheterization of hepatic vein and portal vein (47), which was not technically feasible. Our hepatic GNG gene expression data only suggests that liver is the primary effector site of changes in glucose metabolism induced by renal nerve ablation. Further investigation is necessary to establish the molecular pathways involved, including protein levels and signaling through insulin, and other pathways that can contribute to changes in liver glucose metabolism. We do not have a direct physiological measurement of local or whole body SNA. However, there are no methods currently available that directly measures SNA (48,49). Because we did not measure hepatic SNA, we do not know whether the increased hepatic NE content was due
to increased SNA, decreased clearance, or both. We have also not established the exact neural or hormonal circuitry involved in eliciting a hepatic response to RDN; however, future studies will be designed to address this. Because the post-surgery follow-up period was rather short in our design, long term studies are needed to determine if the marked effects are sustained or permanent.

With this dog study, we were able to overcome some of the key limitations of the clinical trials including direct open surgical denervation and renal NE content to prove that we in fact ablated the nerves. Direct validation of successful RDN is technically difficult to perform in humans but essential to draw inferences from clinical trials. We cannot rule out species differences between canines and humans as a factor contributing to our results. From the perspective of potential clinical translation, it would be important in a future study to determine whether the canine results can be recapitulated in humans. It is also noteworthy that the RDN animals demonstrated resistance to insulin-induced hypoglycemia, suggesting the involvement of renal sympathetic nerves in the hypoglycemic counter-regulatory circuit, perhaps mediated through the liver – a potential clinical application of RDN in hypoglycemia management in diabetics. Our results suggest that RDN improves insulin resistance and thus, can be potentially employed to treat human diabetes and co-morbidities such as cardiovascular disease which affects 8.3% of the global population (50).

In conclusion, bilateral RDN improved $S_i$ in diet-induced insulin resistant mainly by improving hepatic insulin sensitivity, supporting a novel crosstalk between the renal nerves and the liver. The translational potential of this work to the clinical setting will be fascinating to investigate.
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FIGURE LEGENDS:

Fig.1: Experimental Design (A), Body weight (B), Food Intake (C), blood pressure (D) and heart rate (E). Vertical line represents the week of surgery. Panel B-C and E: Open circles=HFD+Sham and closed circles=HFD+RDN. Panels D-E: Open circle=systolic blood pressure in HFD+Sham, open square=diastolic blood pressure in HFD+Sham, closed circle=systolic blood pressure in HFD+RDN, closed square=diastolic blood pressure in HFD+RDN. N=7 in sham and N=8 in RDN group. Data are means±SEM. ## P<0.001 vs week -1 or week 0 (pre-fat period), #P<0.01 vs week 0 as measured by Two Way ANOVA.

Fig.2: Validation of Denervation: Renal norepinephrine (A), and epinephrine (B) content, and plasma renin activity (E) and plasma epinephrine (F) during hypoglycemic clamp: Kidney cortex norepinephrine (A) and epinephrine (B) content are lower in RDN group as compared to Sham indicating successful denervation. (C-D) plasma renin activity (PRA) and (E-F) plasma epinephrine assessed during the hypoglycemic clamp show that RDN induces higher renin and epinephrine response to hypoglycemia as compared to sham. Panels C and E represent sham; Panels D and F represent RDN group. Panels A-B: White bars=HFD+Sham, black bars=HFD+RDN; Panels C-F: Hatched bars=w6AHFD, grey bars=HFD+Sham, and black bars=HFD+RDN. N=6 in sham and N=6 in RDN group. *P<0.05 as measured by Mann-Whitney U non-parametric t-test.

Fig.3: Whole Body Insulin Sensitivity Measured by Minimal Model (A-B) and Euglycemic Clamp (C-G): S_{IVGTT} (A-B) as assessed by minimal model shows that RDN surgery reverses HFD induced insulin resistance. Time course data of (C, E) plasma insulin concentration, (D, F) and glucose infusion rate; (G) S_{CLAMP}, whole-body S_l assessed during the EGC show that RDN reverses insulin resistance to EGP. Panels A, C and D represent sham; Panels B, E and F represent RDN group. Panels C-G: Open circles and white bars=w0, closed circles and hatched
bars=w6-HFD, inverted triangles and grey bars=HFD+Sham, upright triangles and black bars=HFD+RDN. N=7 in sham and N=8 in RDN group. *P<0.05 vs w0; #P<0.05 vs w6-HFD as measured by Two Way ANOVA.

Fig.4: Peripheral (A-C) and Hepatic Insulin Sensitivity (D-F): Time course data of (A-B) rate of peripheral glucose disposal (R_d); (C) S_i(Rd CLAMP), peripheral S_i (D-E); time course data of (D-E) EGC; (F) S_i(EGP CLAMP), hepatic S_i assessed during the EGC show that RDN reverses insulin resistance to EGP. Panels A and D represent sham group; Panels B and E represent RDN group. Open circles and white bars=w0, closed circles and hatched bars=w6-HFD, inverted triangles and grey bars=HFD+Sham, upright triangles and black bars=HFD+RDN. N=7 in group and N=8 in RDN group. *P<0.05 vs w0; #P<0.05 vs w6-HFD as measured by Two Way ANOVA.

Fig.5: Expression of gluconeogenic genes (A), natriuretic peptide receptors (B-C), adrenergic receptors (D), norepinephrine content (E) and epinephrine content (F) in the liver. RDN reduced the expression of G6Pase, PEPCK, FOXO1, PGC-1α, PPAR-α and LXR-α in the liver. RDN also reduced the expression of NPR-C and α-1A adrenergic receptor while it increased the expression of α-2A adrenergic receptor and β-1 adrenergic receptor. White bars represent sham group and black bars represent RDN group. White bars=HFD+Sham and black bars=HFD+RDN group. N=7 in sham and N=8 in RDN group. Data are means±SEM. *P<0.05, aP=0.07 as measured by Mann Whitney non-parametric t-test.

Fig.6: Working hypotheses of indirect control of hepatic glucose production. Liver glucose output can be regulated indirectly by renal afferent and efferent nerves by either central integration of renal afferent nerve signals, central insulin signaling, adrenergic control of adipose tissue lipolysis or neurogenic control of renin-angiotensin-aldosterone system (RAAS).
Fig. 1: Experimental Design (A), Body weight (B), Food Intake (C), blood pressure (D) and heart rate (E). Vertical line represents the week of surgery. Panel B-C and E: Open circles=HFD+Sham and closed circles=HFD+RDN. Panels D-E: Open circle=systolic blood pressure in HFD+Sham, open square=diastolic blood pressure in HFD+Sham, closed circle=systolic blood pressure in HFD+RDN, closed square=diastolic blood pressure in HFD+RDN. N=7 in sham and N=8 in RDN group. Data are means±SEM. ** P<0.001 vs week -1 or week 0 (pre-fat period), *P<0.01 vs week 0 as measured by Two Way ANOVA.
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434x194mm (96 x 96 DPI)
Fig. 4: Peripheral (A-C) and Hepatic Insulin Sensitivity (D-F): Time course data of (A-B) rate of peripheral glucose disposal ($R_d$); (C) $S_I(R_d \text{ CLAMP})$, peripheral $S_I$ (D-E); time course data of (D-E) EGC; (F) $S_I(\text{EGP CLAMP})$, hepatic $S_I$ assessed during the EGC show that RDN reverses insulin resistance to EGP. Panels A and D represent sham group; Panels B and E represent RDN group. Open circles and white bars=w0, closed circles and hatched bars=w6-HFD, inverted triangles and grey bars=HFD+Sham, upright triangles and black bars=HFD+RDN. N=7 in group and N=8 in RDN group. *P<0.05 vs w0; †P<0.05 vs w6-HFD as measured by Two Way ANOVA.

Fig. 4
331x179mm (96 x 96 DPI)
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Hypotheses: Indirect Regulation of Liver Glucose Production
ONLINE APPENDIX

Renal Denervation reverses hepatic insulin resistance induced by high-fat diet.

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SUPPLEMENTAL FIGURE LEGENDS:

**Fig.S1: Hypoglycemic Clamp:** Time course data of (A-B) plasma insulin concentration, (C-D) plasma glucose; (E-F) glucose infusion rate (GINF); (G-H) plasma norepinephrine and (I-J) plasma glucagon assessed during the hypoglycemic clamp. Panels A, C, E, G and I represent sham group; Panels B, D, F, H and J represent RDN group. RDN significantly lowers the GINF as compared to sham during insulin induced hypoglycemic clamp. Closed circles and hatched bars=w6-HFD, inverted triangles and grey bars=HFD+Sham, upright triangles and black bars=HFD+RDN. N=6 in each group. #P<0.05 vs w6-HFD as measured by two way ANOVA; *P<0.05 as measured by Mann-Whitney U non-parametric t-test.

**Fig.S2: Adrenergic receptor gene expression in the kidney.** White bars are HFD+Sham group and black bars are HFD+RDN group. ADR=adrenergic receptor. Data from right and left kidneys are combined for presentation. N=7 in sham group and N=8 in RDN group. Data are Mean±SEM. P=ns as measured by Mann Whitney non-parametric t-test.

**Fig.S3: Gluconeogenic gene expressions in the kidney cortex.** White bars are HFD+Sham group and black bars are HFD+RDN group. Data are from right and left kidneys are combined for presentation. N=7 in sham group and N=8 in RDN group. Data are Mean±SEM. P=ns as measured by Mann Whitney non-parametric t-test.

**Fig.S4: Tissue Catecholamine Content.** Panels A-D are tissue norepinephrine levels and panels E-H are tissue epinephrine levels. White bars=HFD+Sham group and black bars=HFD+RDN group. N=7 in sham group and N=8 in RDN group. Data are Mean±SEM. *P<0.05 as measured by Mann Whitney non-parametric t-test.

**Fig.S5: Urine osmolality (A), urinary glucose (B), urinary lactate (C) and natriuretic peptide receptor expressions (D-E) in the kidney.** Panel A: white bar=w0, hatched bar=w6-HFD, grey bar=HFD+Sham and black bar=HFD+RDN. Panels B-C: Renal denervation does not induce significant changes in urinary glucose and lactate. The vertical line represents the week
of surgery. Open circles=HFD+Sham and closed circles= HFD+RDN. Panels D-E: Renal
denervation reduces renal natriuresis by lower the expression of NPR-A and NPR-B as
compared to sham. NPR = natriuretic peptide receptor. White bars=HFD+Sham group and
black bars=HFD+RDN group. Data from right and left kidneys are combined for presentation.
N=7 in sham group and N=8 in RDN group. Data are Mean±SEM. *P<0.05 as measured by
Mann Whitney non-parametric t-test.

**Fig.S6: Fasting parameters.** RDN has no effect on fasting plasma glucose (A), lactate (B), insulin (C), C-peptide (D), glucagon (E), FFA (F), glycerol (G), triglycerides (H), NE (I) and EP (J) as compared to w6-HFD. White bar=w0, hatched bar=w6-
HFD, grey bar=HFD+Sham and black bar=HFD+RDN. N=7 in sham group and N=8 in RDN
group in all panels except D where N=6 in sham group and N=6 in RDN group. Data are
means±SEM. FFA = free-fatty acids. #P<0.01 vs w0 as measured by Two Way ANOVA.
SUPPLEMENTAL FIGURES:

**Fig. S1:** Hypoglycemic Clamp: Time course data of (A-B) plasma insulin concentration, (C-D) plasma glucose; (E-F) glucose infusion rate (GINF); (G-H) plasma norepinephrine and (I-J) plasma glucagon assessed during the hypoglycemic clamp. Panels A, C, E, G and I represent sham group; Panels B, D, F, H and J represent RDN group. RDN significantly lowers the GINF as compared to sham during insulin induced hypoglycemic clamp. Closed circles and hatched bars=w6-HFD, inverted triangles and grey bars=HFD+Sham, upright triangles and black bars=HFD+RDN. N=6 in each group. *P<0.05 vs w6-HFD as measured by two way ANOVA; *P<0.05 as measured by Mann-Whitney U non-parametric t-test.
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