ONLINE SUPPLEMENT

THE IgG RECEPTOR FcγRIIB PLAYS A KEY ROLE IN OBESITY-INDUCED HYPERTENSION

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Supplemental Methods

Animal Models: All experiments were performed in male mice. To study CRP-induced hypertension and FcγRIIB, CRP transgenic mice (TG-CRP) on CF1 background were crossed with FcγRIIB$^{-/-}$ B6:129S mice (Jackson Laboratory)[1] to yield FcγRIIB$^{+/+}$, FcγRIIB$^{+/+}$;TG-CRP, FcγRIIB$^{-/-}$, and FcγRIIB$^{-/-}$;TG-CRP littermates. CRP levels were measured by ELISA.[2] Mice lacking the CRP transgene had serum concentrations <1μg/mL, and serum CRP concentrations in TG-CRP mice were 10-13μg/mL and similar in FcγRIIB$^{+/+}$;TG-CRP and FcγRIIB$^{-/-}$;TG-CRP.[3] All mice were fed standard rodent chow (Teklad Global 18% Protein Rodent Diet 2018).

To study obesity-induced hypertension, C57BL/6 FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice were placed on either control diet (CON) (Research Diets, Inc. D12329, with 11 kcal% fat) or high fat diet (HFD) (Research Diets, Inc. D12331, with 58 kcal% fat) beginning at 5-6 weeks of age. Mice were maintained on their respective diet for a minimum of 12 weeks and weights were obtained every 2 weeks. This resulted in 4 experimental groups: 1) FcγRIIB$^{+/+}$ CON, 2) FcγRIIB$^{+/+}$ HFD, 3) FcγRIIB$^{-/-}$ CON, and 4) FcγRIIB$^{-/-}$HFD. All mice were maintained in animal facilities with a 12 hour light and 12 hour dark cycle. Their care and use were approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine and the University of Texas Southwestern Medical Center.

Body Composition: After 12 weeks on CON or HFD (at 17-18 weeks of age), body composition was evaluated by dual-energy x-ray absorptiometry (DEXA) in the 4 study groups interrogating HFD-induced hypertension. Mice were anesthetized using

isofluorane and body composition/densitometry was performed using a Lunar PIXImus x-ray densitometer. Measurements obtained were lean body weight and fat body weight in grams as well as percent body fat.

Tail Cuff Blood Pressure (BP) Measurement: In the first series of experiments in FcγRIIB$^{+/+}$ versus FcγRIIB$^{-/-}$ mice on CON versus HFD, mice underwent BP measurement by tail cuff after 16 weeks on their respective diet using a Visitech System BP-2000 Series II (Apex, NC) BP monitor. BP was measured 20 times over a 30 min period daily for 4 days to train the mice. The mean of the last 10 readings on the following day (day 5) was recorded as the BP value for a given mouse.[4]

Blood Pressure (BP) Measurement by Radiotelemetry: In experiments determining the role of FcγRIIB in CRP- or HFD-induced hypertension, radiotelemetry was performed as described previously.[5] In the studies of CRP, instrumentation was done at 12-16 weeks of age, and in the CON chow versus HFD experiments it was done at 17-19 weeks of age after the mice were on their respective diets for 12-13 weeks. Mice were anesthetized under isofluorane and instrumented with a left carotid artery catheter and radiotelemetry device (PA-C10, Data Sciences International, St. Paul, MN). Mice were allowed to recover for a minimum of 6 days (range 6-10 days) and until a return of circadian variation in BP and activity was observed. Then BP, heart rate, and activity were recorded every 15 min for 72 h. Inclusion criteria were pulse pressure ≥20 mmHg, standard deviation for pulse pressure ≤9 mmHg, and the presence of diurnal differences in systolic BP between the light and dark cycles.[6] BP recordings that did not meet these criteria were not used for analysis. In the CON chow versus HFD experiments, mice

were maintained on their respective study diet throughout the radiotelemetry measurements

Serum CRP, SAP and Total IgG: CRP, SAP and total IgG were measured in the serum of FcγRIIB$^{+/+}$ versus FcγRIIB$^{-/-}$ mice on CON versus HFD after 12 weeks on their respective diets. To avoid the effects of surgical stress, non-instrumented mice were employed, and samples were collected by cardiac puncture. The three parameters were quantified by ELISA (Immunology Consultants Laboratory, Inc., Portland, OR), running all samples in duplicate and employing the following dilutions: 1:200 for CRP, 1:1000 for SAP, and 1:50000 for IgG.

FcγRIIB Expression: To evaluate possible effects of HFD on receptor expression, following 12 week feeding of CON versus HFD in wild-type mice, primary aortic endothelial cells were obtained by collagen digestion,[7] and spleens were harvested and splenic cells dispersed by sieving. Cells then underwent fluorescence-activated cell sorting (FACS) analysis using monoclonal antibody to FcγRIIB (anti-CD16/32b, 2.4G2) and anti-CD31 (PECAM-1) or anti-CD45R(B220) antibodies (all from eBiosciences) for endothelial cell or B cell selection, respectively. The anti-CD16/32b antibody recognizes FcγRIIB and FcγRIII; however, we previously demonstrated that mouse endothelial cells express FcγRIIB and not FcγRIII,[8] and in B cells the sole Fcγ receptor expressed is FcγRIIB.[9]

NO Synthase Activity in Cultured Endothelial Cells: To evaluate possible effects of HFD on IgG action on endothelial cells, total IgG was isolated from wild-type mice following 12 weeks of CON versus HFD feeding using Melon Gel IgG Purification Resin (Pierce

Biotechnology) according to the manufacturer's instructions. Bovine aortic endothelial cells (BAEC) were incubated with either CON IgG or HFD IgG (10 μg/ml) for 15 min, and NO synthase activation by vascular endothelial growth factor (VEGF, 100 ng/ml) was then evaluated by measuring ^{14}C-L-arginine conversion to ^{14}C-L-citrulline using previously described methods.[7] Additional experiments were performed in BAEC transfected with control siRNA or siRNA targeting FcγRIIB to silence the receptor.[8]

Statistical Analysis: Statistical analysis for animal experiments was performed using 2-way ANOVA with genotype (FcγRIIB$^{+/+}$ vs. FcγRIIB$^{-/-}$) as the first factor compared and CRP (wild type vs TG-CRP) or diet (CON vs. HFD) as the second factor for comparison. Student-Newman-Keuls was used for post-hoc analysis. For endothelial cell culture experiments, 1-way ANOVA was used with Student-Newman-Keuls post-test analysis. Differences were considered significant at $p<0.05$.

References

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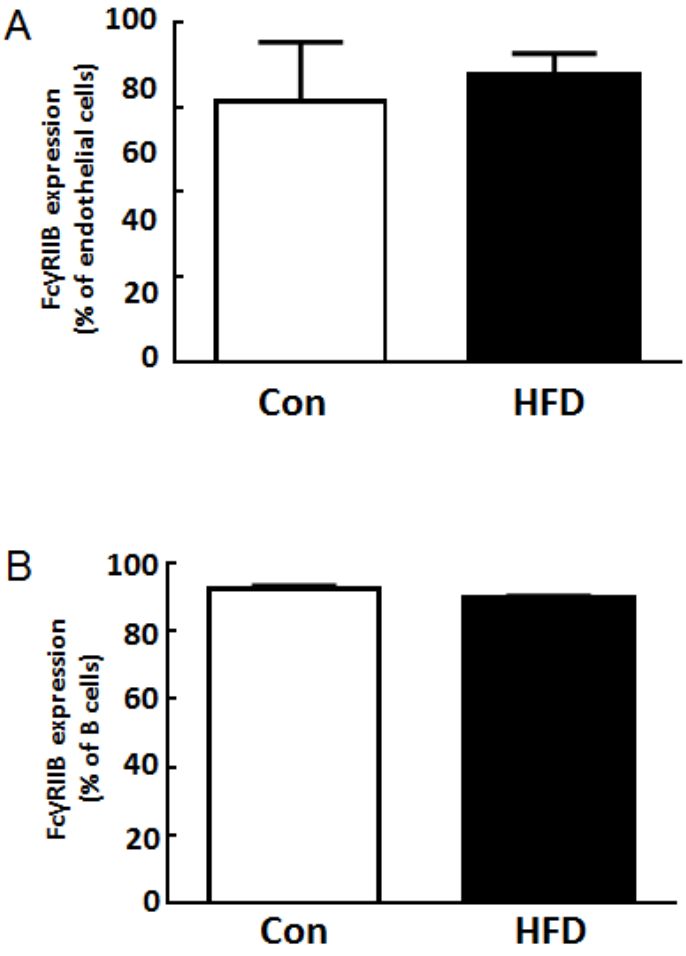
Figure S1: FcγRIIB expression is not altered in high fat diet-induced obesity.

FACS was performed on primary aortic endothelial cells (A) and spleen-derived B cells (B) from control chow (CON) or high fat diet (HFD) fed mice. Antibodies employed were 2.4G2 to detect FcγRIIB, and anti-CD31 (PECAM-1) or anti-CD45R(B220) antibodies to identify endothelial cells or B cells, respectively. Values are mean±SEM, n=4-8.

Figure S1


A
100
80
60
40
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0
FcγRIIB expression
(% of endothelial cells)
Con
HFD
B
100
80
60
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0
FcγRIIB expression
(% of B cells)
Con
HFD