THE EFFECTS OF DIETARY WEIGHT LOSS ON INDICES OF NOREPINEPHRINE TURNOVER: MODULATORY INFLUENCE OF HYPERINSULINEMIA

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What is already known about this subject

- Chronic sympathetic nervous system overactivity is a characteristic of the metabolic syndrome that contributes to insulin resistance, target organ damage and a worse clinical prognosis.
- Weight loss, the recommended first-line treatment for the metabolic syndrome, exerts established sympathoinhibitory effects, as evidenced by reductions in muscle sympathetic nervous activity, arterial norepinephrine concentration, urinary norepinephrine excretion and whole-body norepinephrine spillover rate.

What this study adds

- We demonstrate that weight loss is associated with a down-regulation in norepinephrine turnover, but no overall alteration in norepinephrine disposition indices (neuronal uptake and plasma clearance).
- Sub-group analyses showed that hyperinsulinemic subjects derive a greater sympathoinhibitory benefit during weight loss, compared to normoinsulinemic subjects.

ABSTRACT

Objectives: This study was conducted to examine 1) the effects of dietary weight loss on indices of norepinephrine (NE) turnover and 2) whether baseline hyperinsulinemia modulates sympathetic neural adaptations.

Design and Methods: Obese individuals aged 56 ± 1 yr, BMI 32.5 ± 0.4 kg/m^2, with metabolic syndrome, underwent a 12-week hypocaloric diet (HCD, n=39) or no treatment (n=26). Neurochemical measurements comprised arterial dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylglycol (DHPG) and NE concentrations, the steady-state ratio of [^{3}H]-DHPG to [^{3}H]-NE, as an index of neuronal uptake, and calculated whole-body plasma NE clearance and spillover rates.

Results: Body weight decreased by -7.4 ± 0.5% in HCD group (P<0.001) and was accompanied by reductions in DOPA, NE and DHPG averaging -14 ± 5% (P=0.001), -23 ± 4% (P<0.001) and -5 ± 4% (P=0.03), respectively. NE spillover rate decreased by -88 ± 39 ng/min (P=0.01), whereas neuronal uptake and NE plasma clearance were unchanged. Despite similar weight loss, hyperinsulinemic subjects exhibited greater reductions in NE and NE spillover rate, compared to normoinsulinemic subjects (group by time interaction P<0.05).

Conclusions: Weight loss is associated with down regulation of sympathetic nervous activity but no overall alteration in disposition indices. Hyperinsulinemic subjects derive a greater sympathoinhibitory benefit during weight loss.

INTRODUCTION

The metabolic syndrome (MetS) is an increasingly prevalent multi-faceted condition, closely linked to the spiralling worldwide obesity epidemic (1,2), that predicts future development of cardiovascular disease, type 2 diabetes, and higher all-cause mortality (3). There is robust scientific evidence to indicate that MetS as an entity, as well as its individual components (visceral adiposity, insulin resistance, hypertension, hyperglycemia, dyslipidemia) are associated with sympathetic nervous system overactivity (4-8). Elevated efferent postganglionic muscle sympathetic nerve activity (MSNA) and urinary norepinephrine excretion, diminished heart rate variability, and baroreceptor dysfunction have been reported in MetS cases compared to controls (4,9). Chronic sympathetic activation, via both direct and indirect pathways, favours the development of obesity-related end-organ damage, including endothelial dysfunction, cardiac hypertrophy and renal impairment (10). It also determines or worsens insulin resistance, and portends an adverse clinical prognosis (11,12). Thus, sympathoinhibition is regarded as a goal in the therapeutic approach to the MetS.

Weight loss, the recommended first-line treatment for the MetS, improves both hemodynamic and metabolic components, and exerts established sympathoinhibitory effects, as evidenced by reductions in MSNA, arterial norepinephrine concentration and whole-body norepinephrine spillover rate (13,14). However, relatively little is known about the impact of weight loss on neuronal norepinephrine production and re-uptake (uptake-1). Approximately 90% of released norepinephrine is recaptured from the synaptic cleft via the norepinephrine transporter (NET), an active transport process, dependent on an inward sodium gradient (15). Our group has recently demonstrated an inverse relationship between fasting plasma insulin concentration and neuronal norepinephrine uptake in a cohort of un-medicated MetS subjects, as quantified by the steady state ratio of tritiated 3,4-dihydroxyphenylglycol ([^{3}H]-DHPG, the major intraneuronal metabolite of norepinephrine) to tritiated norepinephrine ([^{3}H]-NE), during an infusion of [^{3}H]-NE (8). This concurs with findings in rodents that acute or chronic

insulin treatment suppresses NET expression and surface availability in the brain (16,17). Reduced cardiac neuronal norepinephrine and ^{123}I-metaiodobenzylguanidine uptake have also been demonstrated in patients with essential hypertension (18) and type 2 diabetes (19), being more pronounced in those with higher visceral fat accumulation and homeostasis model assessment insulin resistance (HOMA-IR) index (19). With respect to norepinephrine synthesis, limited data in rats suggest that starvation decreases brain, but increases white adipose tissue tyrosine and tyrosine hydroxylase content (20,21).

The primary aim of the present study was to examine the impact of weight loss on indices of norepinephrine turnover in obese MetS subjects. To this end, we respectively measured arterial plasma concentrations of dihydroxyphenylalanine (DOPA), the precursor of norepinephrine synthesis within sympathetic neurones and the adrenal medulla (22), endogenous and tritiated DHPG and norepinephrine, and their respective ratios, and MSNA before and following a 12-week hypocaloric diet. A secondary aim was to elucidate whether baseline hyperinsulinemia modulates sympathetic neural adaptations. Given the reciprocal relationship between hyperinsulinemia and sympathetic activity (6), and the potential effect of insulin on NET functionality, we hypothesized that weight loss would elicit greater effects on sympathetic parameters in hyperinsulinemic subjects. Correlation and regression analyses were used to examine the drivers (metabolic, dietary, anthropometric, and cardiovascular) of change in neurochemicals during the weight loss intervention.

METHODS and PROCEDURES

Study Design

Data were pooled from three intervention trials of 12-weeks duration (13, 14 and NCT 00408850) and comprised 39 subjects who underwent a hypocaloric diet and 26 subjects who were untreated controls. Selection criteria, dietary intervention and methodologies were identical across the trials. All subjects enrolled in the weight loss or control arms of these

studies were included in order to avoid selection bias. The studies were approved by the Alfred Hospital Ethics Committee and participants gave written, witnessed, informed consent.

Participants

Eligible participants were men and postmenopausal women, aged 45-65 years with body mass indices (BMI) >27 kg/m^2. All were Caucasian, un-medicated, non-smokers, and fulfilled the harmonised MetS definition (1), using waist circumference cut-points of $\geq$102 cm (men) and $\geq$88 cm (women). All had a stable body weight ($\pm$ 1kg) in the previous 6 months. Exclusion criteria included a history of secondary hypertension, cardiovascular, cerebrovascular, renal, liver, or thyroid disease and use of drugs known to affect measured parameters. Screening investigations comprised physical examination, medical and dietary histories, 12-lead electrocardiogram, blood biochemistry and lipid analyses. Supine blood pressure was measured on three occasions one week apart as the average of 5 readings after 5 minutes rest (Dinamap, Model 1846SX, Critikon Inc, Tampa, FL, USA). The third of these measurements was defined as baseline blood pressure.

Diet Composition

A modified Dietary Approaches to Stop Hypertension (DASH) diet, with macronutrient content 22% protein, 30% fat (6% polyunsaturated, 15% monounsaturated and 9% saturated), 48% carbohydrate, was utilised at a caloric deficit of 500 to 600 kcal/day (13,14). Sodium intake was maintained in the range 100-130 mmol/day. Participants received detailed dietary instruction from the study dietician (MTG), including daily menu plans, shopping list, recipes and the keeping of dietary intake records. They attended our research centre fortnightly for body weight measurement and dietary counselling. Compliance was assessed by prospective 4-day diet records, which were analysed using Australian Food Composition Tables (FoodWorks® Professional Version 3.02, Xyris Software, Highgate Hill, Australia). Sodium intake was quantified by 24-hour urine collections. All subjects were instructed to maintain their usual level of exercise, and this was monitored by exercise records during the 12-weeks.

Control subjects consumed their usual dietary intake and were monitored 3 weekly for weight and blood pressure measurements.

Clinical Investigations

Investigations were performed at baseline and week 12, in a quiet, temperature controlled (22˚C) room, with subjects lying in a supine position. Participants attended at 8 am after a 12-hour fast, having abstained from caffeine for 18 hours, and alcohol and strenuous exercise for 36 hours. They collected a 24-hour urine specimen immediately prior to attendance and voided prior to clinical investigations. Fasting venous blood was drawn for measurement of lipids, fasting glucose and insulin, high sensitivity C-reactive protein (*hs*-CRP), non-esterified fatty acids (NEFA) and plasma renin activity (PRA). After quantification of resting sympathetic nervous system activity (norepinephrine kinetics and MSNA), subjects underwent a 75-g oral glucose tolerance test (OGTT, Glucaid, Fronine PTY, LTD, Australia), with 30 minutely blood sampling over 2 hours. HOMA-IR and the Matsuda whole-body insulin sensitivity index (SI) were used to evaluate insulin sensitivity within the cohort (13,14).

Norepinephrine Kinetics

A tracer infusion of ^{3}H-NE (levo-7-^{3}H-NE, specific activity 10 to 30 μCi/mmol Perkin-Elmer, Waltham, MA, US) in saline was administered through a forearm vein at a constant infusion rate of 0.22 μCi/min, after a priming bolus of 2.31 μCi, for the measurement of norepinephrine kinetics by isotope dilution (13,14). Brachial arterial blood samples for the measurement of DOPA, endogenous and tritiated norepinephrine and DHPG were collected at least 30 minutes after the infusion commenced, to ensure attainment of steady state. Neuronal uptake was estimated from the [^{3}H]-DHPG to [^{3}H]-NE ratio (15). Whole-body norepinephrine plasma clearance rate and spillover into the circulation were calculated as:

$$\text{Norepinephrine clearance (L/min)} = \frac{[^{3}\text{H}]\text{-NE infusion rate (dpm/min)}}{[^{3}\text{H}]\text{-NE steady state plasma concentration (dpm/ml)} \times 1000}$$

$$\text{Norepinephrine spillover (ng/min)} = \frac{\text{plasma norepinephrine (pg/ml) x clearance (ml/min)}}{1000}$$

Figure 1 illustrates the processes of norepinephrine turnover at sympathetic neuroeffector junctions and the neurochemical measurements performed herein.

Microneurography

Multiunit MSNA was recorded through the use of microneurography in the right peroneal nerve as described previously (13,14). After an acceptable nerve recording site was obtained, resting MSNA was recorded for 15 minutes and sympathetic bursts were manually counted and averaged over this period. They were expressed as burst frequency (bursts per min) and burst incidence (bursts per 100 heartbeats). Spontaneous cardiac baroreflex sensitivity was derived by the sequence method of Parati (13,14).

Laboratory Analysis

Plasma glucose and lipid profile were quantified by automated enzymatic methods (Architect C18000 analyser, Abbott Laboratories, IL, USA), *hs*-CRP by immunoturbidimetric assay, NEFA by enzymatic colorimetry (NEFA-C assay, Wako Pure Chemical industries Ltd, Osaka, Japan) and insulin and PRA by radio-immuno assay (Linco Research, Inc, Missouri, USA; REN-CT2, Cis Bio International, France). Plasma norepinephrine, DHPG and DOPA were determined by high performance liquid chromatography with electrochemical detection, following extraction by alumina adsorption. [^{3}H]-norepinephrine and [^{3}H]-DHPG in the effluent were assayed by liquid scintillation chromatography and the concentrations corrected for loss during extraction using recoveries of internal standard. Each subject's samples from baseline and week 12 were assayed together. Intra-assay CVs in our laboratory are 1.3% for norepinephrine and 2.3% for [^{3}H]-norepinephrine; inter-assay CVs are 3.8% and 4.5%, respectively. Corresponding values for DHPG and [^{3}H]-DHPG are 2.0%, 6%, 8% and 14%, and for DOPA, 9 and 15%.

Statistical Analysis

Results are presented as the mean ± SEM. Statistical analysis was carried out using SigmaStat Version 3.5 (Systat Software Inc, Point Richmond, CA, USA). Study parameters at baseline were compared between treatment groups by un-paired t-test, Mann-Whitney *U* test and chi^2 test for proportions. Two-way repeat measures ANOVA and the Holm-Sidak test for multiple post-hoc comparisons were used to quantify group effects, time effects and group x time interactions. The distribution of variables was examined by the Kolmogorov-Smirnov test and non-parametric data were log or square-root transformed ([^{3}H]-DHPG to [^{3}H]-NE ratio). Areas under the plasma concentration-time curve (AUC) during OGTT were calculated for glucose and insulin by the trapezoidal rule. For the purposes of sub-group analyses within the diet group, subjects were categorised as hyperinsulinemic if insulin $AUC_{0\text{-}120}$ was >10,000 $mU/L \cdot min^{-1}$. Baseline and longitudinal (Δ) associations between sympathetic neurochemicals and metabolic, dietary, anthropometric and cardiovascular variables were assessed using Pearson's and Spearman's rank correlations. Forward stepwise regression analyses, adjusted for age, change in body weight and urinary sodium excretion, were carried out with those univariate correlations where $P<0.05$, with change in sympathetic parameters as the dependent variables. A two-tailed P value <0.05 was regarded as statistically significant.

RESULTS

Participants

Baseline clinical characteristics of the study cohort are summarized in Table 1. The two groups were well matched for age, gender, BMI, blood pressure and indices of glucose metabolism. Habitual dietary intake averaged 2295 kcal/day, comprising 34% fat, 43% carbohydrate, 19% protein and 3% alcohol, and did not differ between control and hypocaloric diet participants (data not shown). Self-reported habitual exercise (walking and vigorous exercise combined) averaged 2.0 ± 0.4 and 2.8 ± 0.4 hours per week in the control and diet groups, respectively (P=0.32).

Effects of weight loss on sympathetic neural parameters

A mean caloric deficit of 528 ± 61 kcal/day in the dietary intervention group was accompanied by reductions of -7.4 ± 0.5 % in body weight, -8.6 ± 0.6 cm in waist circumference (both $P<0.001$) and -8 ± 2 and -2 ± 1 mmHg in systolic and diastolic blood pressures ($P\leq0.01$), whilst anthropometric and cardiovascular measurements were unchanged in the control group (Table 2). Sodium intake decreased non-significantly within both the diet (by -11 ± 9 mmol/day, P=0.16) and control (by -33 ± 14 mmol/day, P=0.16) groups. Arterial concentrations of endogenous DOPA, norepinephrine and DHPG all decreased after 12 weeks hypocaloric diet, by 14 ± 5% (P=0.001), 23 ± 4% (P<0.001) and 5 ± 4% (P=0.03), respectively. There were concomitant increases in the DHPG to norepinephrine ratio and cardiac baroreflex sensitivity (by 3.1 ± 0.9 msec/mmHg), both P<0.001 (Table 2).

[^{3}H]-NE infusion rate averaged 5.1 ± 0.2 and 5.4 ± 0.3 dpm/min x 10^5 at baseline and week 12 (P=0.34), whilst infusion times were 42 ± 1 and 42 ± 2 minutes (P=0.62), respectively. The ratio of steady state [^{3}H]-DHPG to [^{3}H]-NE concentration and plasma norepinephrine clearance rate were unchanged by weight loss. However, calculated whole-body norepinephrine spillover rate decreased significantly in the weight loss group (time x group interaction, P=0.007).

Sub-group analyses of hyperinsulinemic versus normoinsulinemic subjects

Within the diet group, 19 subjects were classified as hyperinsulinemic and 20 as normoinsulinemic: insulin $AUC_{0\text{-}120}$ during OGTT averaged 14,131 ± 592 and 7035 ± 460 $mU/L \cdot min^{-1}$, respectively. Attained weight loss was similar in the two groups, averaging -7.7 ± 0.8 and -7.1 ± 0.7 % respectively (between group P=0.52). However, the decrease in insulin $AUC_{0\text{-}120}$ was greater in hyperinsulinemic subjects (-3699 ± 625 versus -919 ± 441 $mU/L \cdot min^{-1}$, P<0.001). Figure 2 summarises sympathetic neural adaptations to weight loss in these two metabolic sub-groups. Key findings were that hyperinsulinemic subjects had higher baseline arterial norepinephrine concentration and calculated norepinephrine spillover rate

(both P<0.01) and a greater magnitude of reduction following weight loss compared with normoinsulinemic subjects (norepinephrine, -111 ± 23 versus -23 ± 9 pg/ml, P<0.001; norepinephrine spillover, -185 ± 66 versus 3 ± 33 ng/min, P=0.01). Indeed, in normoinsulinemic subjects, weight loss did not alter norepinephrine spillover rate (Figure 2). Arterial DOPA level decreased in both sub-groups (hyperinsulinemic, -446 ± 182 pg/ml; normoinsulinemic, -503 ± 247 pg/ml, both P<0.05). Endogenous DHPG decreased only in the hyperinsulinemic group (by -154 ± 109 pg/ml, P=0.048). The ratio of steady state [^{3}H]-DHPG to [^{3}H]-NE concentration and plasma norepineprine clearance were not significantly altered after weight loss in either sub-group. MSNA burst frequency decreased by -9 ± 3 and -6 ± 3 bursts/min (both P<0.05) and burst incidence by -9 ± 5 (P=0.05) and -8 ± 4 (P=0.06) bursts/100 heartbeats in hyperinsulinemic and normoinsulinemic subjects, respectively. Diastolic blood pressure and heart rate decreased significantly only in hyperinsulinemic subjects (by -3 ± 1 mmHg and -5 ± 1 beats per min respectively, P both ≤ 0.01), and vis-a-vis, cardiac baroreflex sensitivity increased only in this sub-group (by 4.5 ± 1.3 msec/mmHg, P<0.001).

Correlation and regression analyses

Baseline correlation analysis (n=65) showed a positive relation between age and arterial concentrations of DOPA, norepinephrine, and DHPG (P all <0.05), and an inverse relationship between age and norepinephrine clearance (r=-0.36, P=0.004). Figure 3 displays sympathetic neural parameters in relation to plasma insulin concentrations. Insulin $AUC_{0\text{-}120}$ was positively associated with norepinephrine level (r=0.39, P=0.002) and whole-body spillover rate (r=0.42, P=0.0006), and inversely related to DHPG : norepinephrine ratio (r=-0.30, P=0.01). Fasting insulin level was inversely associated with DHPG level (r=-0.27, P=0.03). Norepinephrine plasma clearance was associated with body weight (r=0.44, P=0.0004), heart rate (r=0.28, P=0.03) and urinary sodium excretion (r=0.30, P=0.02), whilst

[^{3}H]-DHPG : [^{3}H]-NE related to plasma leptin (r=0.28, P=0.03) and DOPA (r=0.33, P=0.007, Figure 4A).

Table 3 summarises longitudinal correlations within the diet group (n=39). Key findings were that reduction in norepinephrine level was positively associated with change in plasma insulin $AUC_{0\text{-}120.}$ The increase in endogenous DHPG to NE ratio, related to the reductions in body weight, insulin and glucose $AUC_{0\text{-}120}$, and to the increase in baroreflex sensitivity (all P<0.05). Change in norepinephrine plasma clearance was inversely related to change in waist to hip ratio, fasting plasma leptin, glucose and triglyceride levels, and positively related to increase in Matsuda SI (P all <0.05). Change in neuronal norepinephrine uptake, as assessed by [^{3}H]-DHPG : [^{3}H]-NE ratio, correlated with the change in DOPA concentration (r=0.46, P=0.004, Figure 4B) and to a lesser extent with change in fasting glucose (r=-0.30, P=0.07) and norepinephrine spillover (r=0.31, P=0.06). Reduction in hyperinsulinemia (Δ insulin $AUC_{0\text{-}120}$) correlated closely with change in systolic (r=0.32, P=0.05) and diastolic (r=0.40, P=0.01) blood pressures, heart rate (r=0.41, P=0.01) and plasma triglycerides (r=0.37, P=0.02).

Stepwise regression analyses of change in neurochemicals and MSNA are presented in Table 4. Change in insulin $AUC_{0\text{-}120}$ during weight loss, independently predicted the reduction in norepinephrine level, accounting for 18% of the variance. Change in neuronal norepinephrine uptake was predicted only by change in DOPA, which explained 21% of the variance, whereas norepinephrine plasma clearance was predicted by changes in triglyceride level (inverse) and urinary sodium excretion (positive), which together explained 28% of the variance. Reduction in MSNA was independently explained by changes in PRA and leptin.

DISCUSSION

Plasma norepinephrine levels reflect the dynamic interplay of synthesis, release, re-uptake (uptake 1 and 2), metabolism, and excretion. The present study was undertaken to explore the

nature of adaptations in norepinephrine turnover during a 12-week hypocaloric diet and the modulatory influence of hyperinsulinemia on these processes. There are several novel aspects to our findings, which extend previous knowledge of the effects of weight loss and negative energy balance on sympathetic neural function. First, we have demonstrated a down-regulation in norepinephrine turnover, as evidenced by reductions in arterial norepinephrine, DHPG and DOPA concentrations and calculated norepinephrine spillover rate. Second, we show that the magnitude of reduction in arterial norepinephrine concentration and spillover rate is greatest in hyperinsulinemic subjects, and independently predicted by the fall in plasma insulin levels. Third, neuronal uptake, estimated as the [^{3}H]-DHPG to [^{3}H]-NE ratio, was closely and positively associated with norepinephrine production in both cross-sectional and longitudinal analyses, but did not alter significantly with weight loss. Overall, these data suggest that the reduction in endogenous plasma norepinephrine concentration during hypocaloric diet is due to the combined effects of reduced neuronal production and release into the circulation, rather than to increased neuronal uptake or removal from plasma.

Landsberg and Young were the first to undertake a detailed evaluation of sympathetic neural adaptations under conditions of fasting and overfeeding in rodents (23). Their elegant body of work showed that norepinephrine turnover rate, estimated as the product of the rate constant of decline of [^{3}H]-norepinephrine and endogenous tissue norepinephrine content, could be reduced by ~50% during a 48 hour fast, returned to control values after refeeding, and increased above control values with overfeeding (23). These observations were consistent with the notion of adaptive thermogenesis and similar pattern of changes have been demonstrated in short-term human experiments (24,25). Although norepinephrine disposition indices did not change significantly with weight loss in the present study, correlation analyses showed that disposition was a function of both production and metabolic status. This concurs with *in vitro* and *in vivo* experimental studies demonstrating that sympathetic hyperactivity (induced by chronic depolarisation or myocardial infarction) is a major stimulus increasing

tyrosine hydroxylase and NET expression (26,27), whilst depletion of neuronal catecholamines with reserpine treatment lowers the number of NET binding sites (28). In our study, change in plasma norepinephrine clearance related positively to improvement in Matsuda SI, and inversely to change in fasting glucose and triglyceride concentration. In other words, norepinephrine clearance improved in those individuals who experienced the largest fall in blood glucose and greatest increase in insulin sensitivity, and was further modified by reductions in production which down-regulated neuronal uptake. These metabolic associations are in accord with a growing body of data showing that hyperglycemia and hyperinsulinemia/insulin resistance decrease the expression of NET (16,17,19,29,30), with resultant increases in plasma and tissue norepinephrine content and subsequent alterations in cardiac function (19,30). Importantly, these changes are observed in the absence of sympathetic denervation and may be reversed by interventions that lower hyperglycemia (29).

At baseline, fasting plasma insulin and insulin $AUC_{0\text{-}120}$ were inversely correlated with plasma DHPG level and DHPG to norepinephrine ratio in our cohort, which is consistent with an insulin-mediated inhibition of neuronal norepinephrine uptake. Albeit, it merits emphasis that DHPG is not a pure measure of uptake 1, as its levels are also influenced by leakage of norepinephrine from intraneuronal storage vesicles (15). Blockade of neuronal uptake with desipramine decreases plasma clearance of norepinephrine by 20% in humans (31). This suggests that extraneuronal uptake mainly by the liver and kidneys, predominates in the clearance of circulating norepinephrine, and factors such as affinity for organic cation transporters, changes in cardiac output or tissue perfusion may be key determinants. Although we did not directly quantify cardiac output in the present study, it is likely to have decreased with weight loss given the fall in heart rate and sympathoinhibition, which would be expected to diminish plasma norepinephrine clearance (32,33). Previous studies with similar weight loss to ours, report variable effects on cardiac output, influenced in part by concurrent changes in sodium intake (34,35). Conversely, improvements in insulin resistance and

concomitantly, in vascular endothelial function, may enhance perfusion of hepatosplanchnic tissues and thus increase extraneuronal norepinephrine clearance.

Our data emphasize the primacy of hyperinsulinemia in determining baseline sympathetic drive and the magnitude of sympathoinhibitory response to weight loss, which related to the reduction in plasma insulin. It has long been appreciated that both endogenous and exogenous hyperinsulinemia elicit sympathoexcitation (6,36,37) and insulin resistant conditions are associated with blunted baroreflex gain (5). The central neural circuitry that mediates the sympathetic effects of insulin are not fully elucidated, but likely involves activation of hypothalamic melanocortin, PI3K and MAPK signalling pathways (38,39). In tandem with greater sympathoinhibition, hyperinsulinemic subjects also derived greater benefits on diastolic blood pressure, heart rate and baroreflex sensitivity for the same magnitude of weight loss.

The main limitations of our study are that our findings likely reflect the combined effects of negative energy balance and weight loss per se, given the duration of the intervention. Furthermore, we did not quantify indices of extraneuronal uptake, cardiac output or tissue blood flow, which are mechanistically relevant and should be examined in future studies. Also, we can not exclude the possibility that change in plasma DOPA levels, were in part influenced by reduction in dietary protein intake (40), albeit reductions were small, averaging -10 $\pm$ 5 g/day in controls and -17 $\pm$ 3 g/day in the hypocaloric diet group (between group P=0.31). In summary, our study findings show that moderate weight loss is accompanied by down regulation of norepinephrine turnover, but no overall alteration in disposition indices. Hyperinsulinemic subjects derived a greater sympathoinhibitory and diastolic blood pressure benefit during weight loss and thus should be specifically targeted for lifestyle intervention programs.

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Author contributions

NES, PJN and GWL conceived the study. NES, EAL, MTG, PJN, TD, KM, CIS and MPS collected data. NE, KR and GWL performed laboratory assays. NES and EP performed data analysis. NES wrote the manuscript. All authors read and had final approval of the submitted and published versions.

DISCLOSURE SUMMARY

NES, MTG, CIS, SK, CW, NE, KLR and EAL have nothing to declare. PJN has consultative and advisory board associations with Merck Sharp & Dohme and Astra Zeneca. JBD receives competitive research grant funding from Allergan Inc. He is a consultant for Allergan Inc, Bariatric Advantage, and Scientific Intake, and is a member of the Optifast® Medical Advisory Board for Nestle Health, Australia. He is on the speakers bureaus for Eli Lilly and iNova Pharmaceuticals, has developed educational material for Novartis and iNova Pharmaceuticals and received travel assistance from GI Dynamics for an educational meeting. MPS serves on scientific advisory boards for Abbott Pharmaceuticals, Novartis Pharmaceuticals and Medtronic and has received research support and travel support, lecture fees and honoraria from Abbott, Novartis, Servier, Boehringer Ingleheim and Medtronic. GWL has acted as a consultant for Medtronic and has received honoraria from Medtronic, Pfizer and Wyeth Pharmaceuticals for presentations. These organizations played no role in the

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Table 1: Clinical characteristics of the study cohort

Variables	**Control (n=26)**	**Hypocaloric diet (n=39)**	**P-Value**
Age (yrs)	55 ± 1	57 ± 1	0.24
Gender (M/F)	15/11	24/15	0.96
Hypertension (n)	14	20	0.96
Type 2 diabetes (n)	7	6	0.41
Body mass index (kg/m^2)	33.1 ± 0.6	32.2 ± 0.6	0.30
Systolic BP (mmHg)	132 ± 4	132 ± 3	0.94
Diastolic BP (mmHg)	74 ± 2	76 ± 1	0.20
Heart rate (bpm)	63 ± 2	63 ± 2	0.99
Total cholesterol (mmol/L)	5.6 ± 0.2	5.2 ± 0.2	0.10
LDL cholesterol (mmol/L)	3.6 ± 0.1	3.3 ± 0.2	0.13
HDL cholesterol (mmol/L)	1.20 ± 0.05	1.18 ± 0.03	0.83
Triglycerides (mmol/L)	1.9 ± 0.2	1.6 ± 0.2	0.04
Fasting glucose (mmol/L)	5.9 ± 0.1	5.8 ± 0.1	0.57
Fasting insulin (mU/L)	17.8 ± 1.0	15.9 ± 0.8	0.13
HOMA-IR	4.67 ± 0.29	4.12 ± 0.23	0.13
2-h glucose (mmol/L)	10.2 ± 0.4	9.2 ± 0.4	0.09
Glucose $AUC_{0\text{-}120}$ ($mmol/L \cdot min^{-1}$)	1193 ± 33	1154 ± 29	0.39
Insulin $AUC_{0\text{-}120}$ ($mU/L \cdot min^{-1}$)	11,280 ± 1038	10,492 ± 683	0.51
High sensitivity-CRP (mg/L)	3.3 ± 0.4	3.2 ± 0.6	0.32
NEFA (mEq/L)	0.59 ± 0.04	0.51 ± 0.03	0.10
Leptin (ng/ml)	14.9 ± 1.8	17.5 ± 2.3	0.79
Urinary sodium (mmol/day)	172 ± 19	133 ± 10	0.13

Values are mean ± SEM. Hypertension defined as clinic blood pressure ≥ 130/85 mmHg (1) and type 2 diabetes as per WHO criteria. BP, blood pressure, HDL, high density lipoprotein, HOMA-IR, homeostasis model assessment insulin resistance index, LDL, low density lipoprotein, NEFA, non-esterified fatty acids.

Table 2: Sympathetic nervous system activity and selected study parameters at baseline and after 12-weeks intervention

	Control (n=26)		Hypocaloric diet (n=39)		P-values		
	Baseline	**Week 12**	**Baseline**	**Week 12**	**Time**	**Group**	**Time x Group**
Body weight (kg)	97.8 ± 2.8	98.8 ± 2.8	94.7 ± 1.9	87.7 ± 1.8***‡	<0.001	0.04	<0.001
Waist circumference (cm)	109.0 ± 1.9	108.9 ± 2.0	107.7 ± 1.5	100.9 ± 1.5***‡	<0.001	0.06	<0.001
Waist : Hip ratio	0.94 ± 0.02	0.93 ± 0.02	0.95 ± 0.01	0.93 ± 0.01***	<0.001	0.97	0.06
Systolic BP (mmHg)	132 ± 4	130 ± 4	132 ± 3	124 ± 2***	<0.001	0.45	0.01
Diastolic BP (mmHg)	74 ± 2	74 ± 2	76 ± 1	74 ± 1**	0.07	0.61	0.07
Heart rate (bpm)	63 ± 2	61 ± 1	63 ± 1	60 ± 2***	0.007	0.77	0.17
Fasting Insulin (mU/L)	17.8 ± 1.0	19.4 ± 1.7	15.9 ± 0.8	10.9 ± 0.8***‡	<0.001	<0.001	<0.001
Insulin $AUC_{0\text{-}120}$(mU/L · min^{-1})	11,280 ± 1038	11,108 ± 977	10,492 ± 683	8,219 ± 559***‡	<0.001	0.06	<0.001
Plasma renin activity (ng/ml/h)	0.75 ± 0.09	0.77 ± 0.08	0.86 ± 0.10	0.70 ± 0.06	0.69	0.94	0.52
DOPA (pg/ml)	1873 ± 94	1729 ± 119	2157 ± 160	1683 ± 101***	0.002	0.81	0.19
NE (pg/ml)	226 ± 22	230 ± 16	233 ± 17	168 ± 9***‡	0.001	0.16	<0.001
DHPG (pg/ml)	1199 ± 55	1145 ± 56	1401 ± 78	1297 ± 73*	0.03	0.15	0.57
DHPG : NE ratio	6.32 ± 0.50	5.57 ± 0.46	6.64 ± 0.43	8.17 ± 0.52***‡	0.26	0.007	<0.001
^{3}H-DHPG : ^{3}H-NE ratio	0.082 ± 0.012	0.109 ± 0.021	0.106 ± 0.016	0.103 ± 0.015	0.40	0.63	0.21
NE clearance (L/min)	2.06 ± 0.11	2.22 ± 0.15	2.03 ± 0.10	2.22 ± 0.10	0.05	0.85	0.67
Whole-body NE spillover (ng/min)	442 ± 43	491 ± 36	454 ± 36	366 ± 25**‡	0.58	0.15	0.007
MSNA (bursts/min)	37 ± 2	36 ± 2	38 ± 2	30 ± 2***†	0.005	0.32	0.02
MSNA (bursts/100hb)	62 ± 4	60 ± 3	60 ± 3	51 ± 3**	0.02	0.26	0.12
BRS (msec/mmHg)	14.0 ± 1.1	13.5 ± 1.2	10.9 ± 0.8	14.0 ± 0.9***	0.05	0.24	0.01

Values are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P\leq0.001$ versus Baseline; $^{\dagger}P\leq0.05$ and $^{\ddagger}P<0.01$ versus Control. BRS, spontaneous cardiac baroreflex sensitivity; DHPG, 3,4-dihydroxyphenylglycol; DOPA, dihydroxyphenylalanine; ^{3}H, tritiated; MSNA, muscle sympathetic nerve activity; NE, norepinephrine

Table 3: Longitudinal univariate correlation analyses with neurochemicals in the hypocaloric diet group (n=39)

	Δ log NE (pg/ml)	Δ DHPG (pg/ml)	Δ DHPG : NE	Δ Log DOPA (pg/ml)	Δ ^{3}H-DHPG : ^{3}H-NE ratio	Δ NE CL (L/min)	Δ NE spillover (ng/min)
Δ Log DOPA (pg/ml)		0.50***			0.46**		
Δ ^{3}H-DHPG : ^{3}H-NE ratio				0.46**			
Δ DHPG (pg/ml)				0.50***			0.36*
Δ Log NE (pg/ml)		0.40**					
Δ Weight (kg)			-0.33*				
Δ Waist : Hip ratio		-0.38*		-0.38*		-0.38*	-0.40*
Δ Log leptin (ng/ml)						-0.35*	
Δ Insulin $AUC_{0\text{-}120}$ (mU/L · min^{-1})	0.43**		-0.33*				
Δ Glucose $AUC_{0\text{-}120}$ (mmol/L· min^{-1})			-0.37*				
Δ Fasting glucose (mmol/L)						-0.34*	
Δ Log SI						0.37*	
Δ Log BRS (msec/mmHg)			0.38*				
Δ Heart rate (bpm)	0.39**						
Δ Plasma renin activity (ng/ml/h)				-0.39*			
Δ HDL-cholesterol (mmol/L)		0.39*					
Δ Log triglycerides (mmol/L)						-0.42**	
Δ Dietary saturated fat (g/day)				0.38*			
Δ Dietary total fat (g/day)				0.44**			

*P<0.05, **P<0.01 and ***P≤0.001. AUC, area under the curve; BRS, spontaneous cardiac baroreflex sensitivity; DHPG, 3,4-dihydroxyphenylglycol; DOPA, dihydroxyphenylalanine; ^{3}H, tritiated; HDL, high density lipoprotein; NE, norepinephrine; SI, Matsuda whole-body insulin sensitivity index.

Table 4: Stepwise regression analyses of change in sympathetic neural parameters with weight loss (n=39)

Dependent Variables	Step	Independent Predictor Variables	R^2	Association	P
Δ Log DOPA (pg) ml)	1	Δ DHPG (pg/ml)	0.24	positive	0.003
	2	Δ Plasma renin activity (ng/ml/h)	0.37	inverse	0.004
	3	Δ [^{3}H]-DHPG : [^{3}H]-NE ratio	0.50	positive	0.007
Δ Log arterial NE (pg/mL)	1	Δ Insulin $AUC_{0\text{-}120}$ (mU/L · min^{-1})	0.18	positive	0.007
Δ DHPG (pg/ml)	1	Δ Log DOPA (pg/ml)	0.25	positive	0.004
	2	Δ HDL cholesterol (mmol/L)	0.34	positive	0.038
Δ DHPG : NE ratio	1	Δ Log baroreflex sensitivity (msec/mmHg)	0.14	positive	0.017
	2	Δ Glucose $AUC_{0\text{-}120}$ (mmol/L · min^{-1})	0.27	inverse	0.018
Δ Norepinephrine clearance (L/min)	1	Δ Log triglycerides (mmol/L)	0.17	inverse	0.003
	2	Δ 24-h urinary sodium (mmol/day)	0.28	positive	0.028
Δ [^{3}H]-DHPG : [^{3}H]-NE ratio	1	Δ Log DOPA (pg) ml)	0.21	positive	0.004
Δ NE spillover rate (ng/min)	1	Δ Waist : hip ratio	0.16	inverse	0.015
Δ MSNA burst frequency (bursts/min)	1	Age (yrs)	0.22	inverse	0.005
	2	Δ Plasma renin activity (ng/ml/h)	0.33	positive	0.034
Δ MSNA burst incidence (bursts/100hb)	1	Δ Log leptin (ng/ml)	0.17	positive	0.034
	2	Δ Plasma renin activity (ng/ml/h)	0.31	positive	0.018
	3	Age (yrs)	0.41	inverse	0.045

DHPG, 3,4-dihydroxyphenylglycol; DOPA, dihydroxyphenylalanine; MSNA, muscle sympathetic nerve activity; NE, norepinephrine

Figure 1: Norepinephrine turnover at sympathetic neuroeffector junctions, showing neurochemicals measured in arterial blood. Abbreviations: COMT, catechol-*O*-methyltransferase; DA, dopamine; DHPG, 3,4-dihydroxyphenylglycol; DOPA, dihydroxyphenylalanine; [^{3}H]-DHPG, tritiated 3,4-dihydroxyphenylglycol; [^{3}H]-NE, tritiated norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; MAO, monoamine oxidase; NE, norepinephrine; NET, norepinephrine transporter (black box); NMN, normetanephrine; OCT3, organic cation transporter 3 (black triangle); TYR, tyrosine. Adapted from Eisenhofer *et al* 1989 (15).

Figure 2: Bar diagrams displaying endogenous and steady state tritiated neurochemical concentrations in arterial blood at baseline (black) and after 12-weeks weight loss (white) in hyperinsulinemic (hyper, n=19) and normoinsulinemic (normo, n=20) subjects. Mean weight changes were -7.7 $\pm$ 0.8% and -7.1 $\pm$ 0.7 %, respectively. **A.** Dihydroxyphenylalanine (DOPA), time effect P=0.003. **B.** Norepinephrine, time effect P<0.001; group effect P=0.006; group x time interaction P=0.002. **C.** Dihydroxyphenylglycol (DHPG), time effect P=0.055. **D.** DHPG : NE ratio, time effect P<0.001; group effect, p=0.012; group x time interaction, P=0.047. **E.** Neuronal norepinephrine uptake, estimated as the ratio of [^{3}H]-DHPG : [^{3}H]-NE, no time, group or interaction effects. **F.** Calculated whole-body norepinephrine (NE) spillover rate into plasma, time effect P=0.017; group effect P=0.04, group x time interaction, P=0.031. *P<0.05, **P<0.01 and ***P<0.001 versus baseline; $^{\#}$P<0.01 versus normoinsulinemic group.

Figure 3: Relation of sympathetic neural parameters to plasma insulin concentrations at baseline (n=65). **A.** Arterial 3,4-dihydroxyphenylglycol (DHPG) versus fasting plasma insulin concentration (r=-0.27, P=0.03). **B.** Endogenous dihydroxyphenylglycol to norepinephrine ratio (DHPG : NE) versus insulin area under the curve during oral glucose tolerance test (r=-

0.30, P=0.01). **C.** Whole-body norepinephrine (NE) spillover rate versus insulin area under the curve during oral glucose tolerance test (r=0.42, P=0.0006).

Figure 4: Cross-sectional and longitudinal inter-relationships between norepinephrine production and neuronal uptake. **A**. Plasma DOPA concentration versus [^{3}H]-DHPG to [^{3}H]-NE ratio at baseline (r=0.33, P=0.007, n=65). **B.** Change in plasma DOPA concentration versus change in [^{3}H]-DHPG to [^{3}H]-NE ratio after 12-weeks weight loss (r=0.46, P=0.004, n=39). Change represents baseline minus week 12 values.

Figure 1

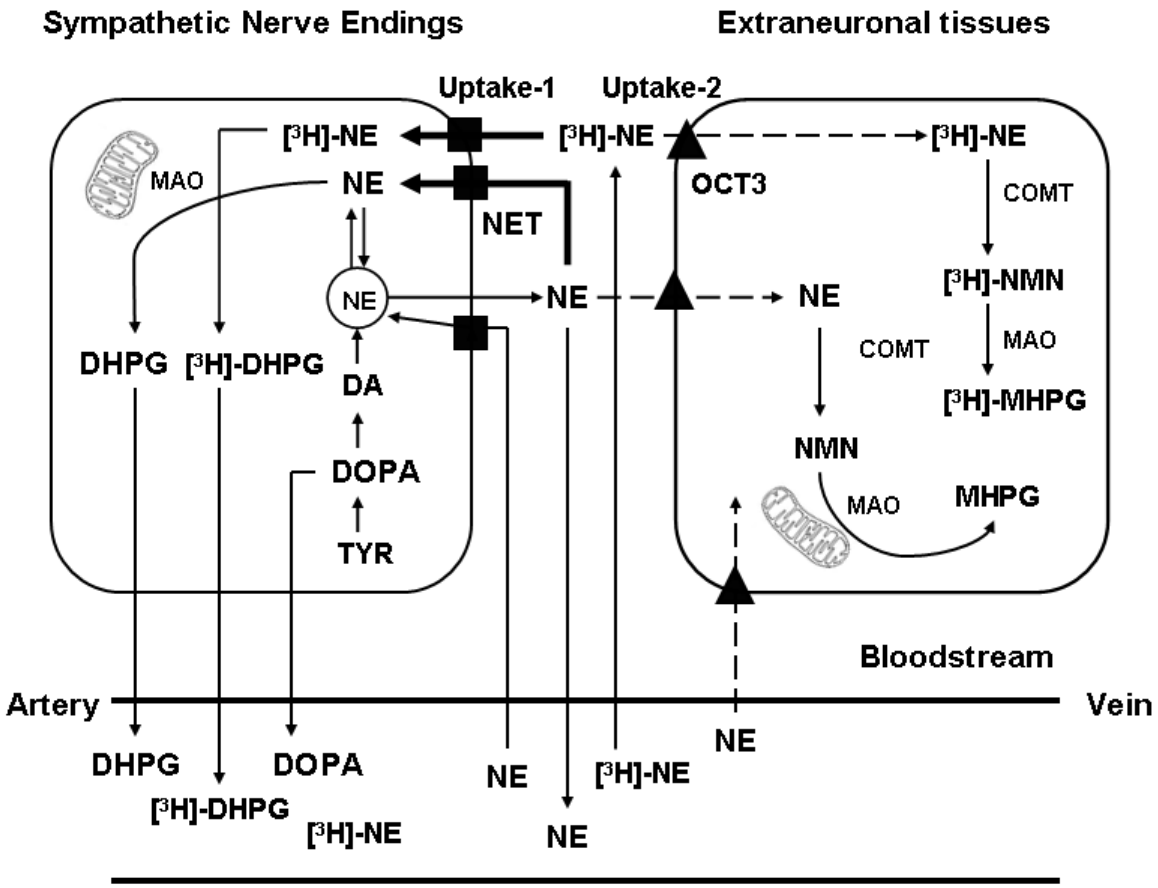

Sympathetic Nerve Endings
Extraneuronal tissues
Uptake-1
Uptake-2
[3H]-NE
[3H]-NE
[3H]-NE
MAO
NE
OCT3
COMT
NET
[3H]-NMN
NE
NE
NE
COMT
MAO
DHPG
[3H]-DHPG
DA
[3H]-MHPG
NMN
DOPA
MAO
MHPG
TYR
Bloodstream
Artery
Vein
NE
DHPG
DOPA
NE
[3H]-NE
[3H]-DHPG
[3H]-NE
NE

Figure 2

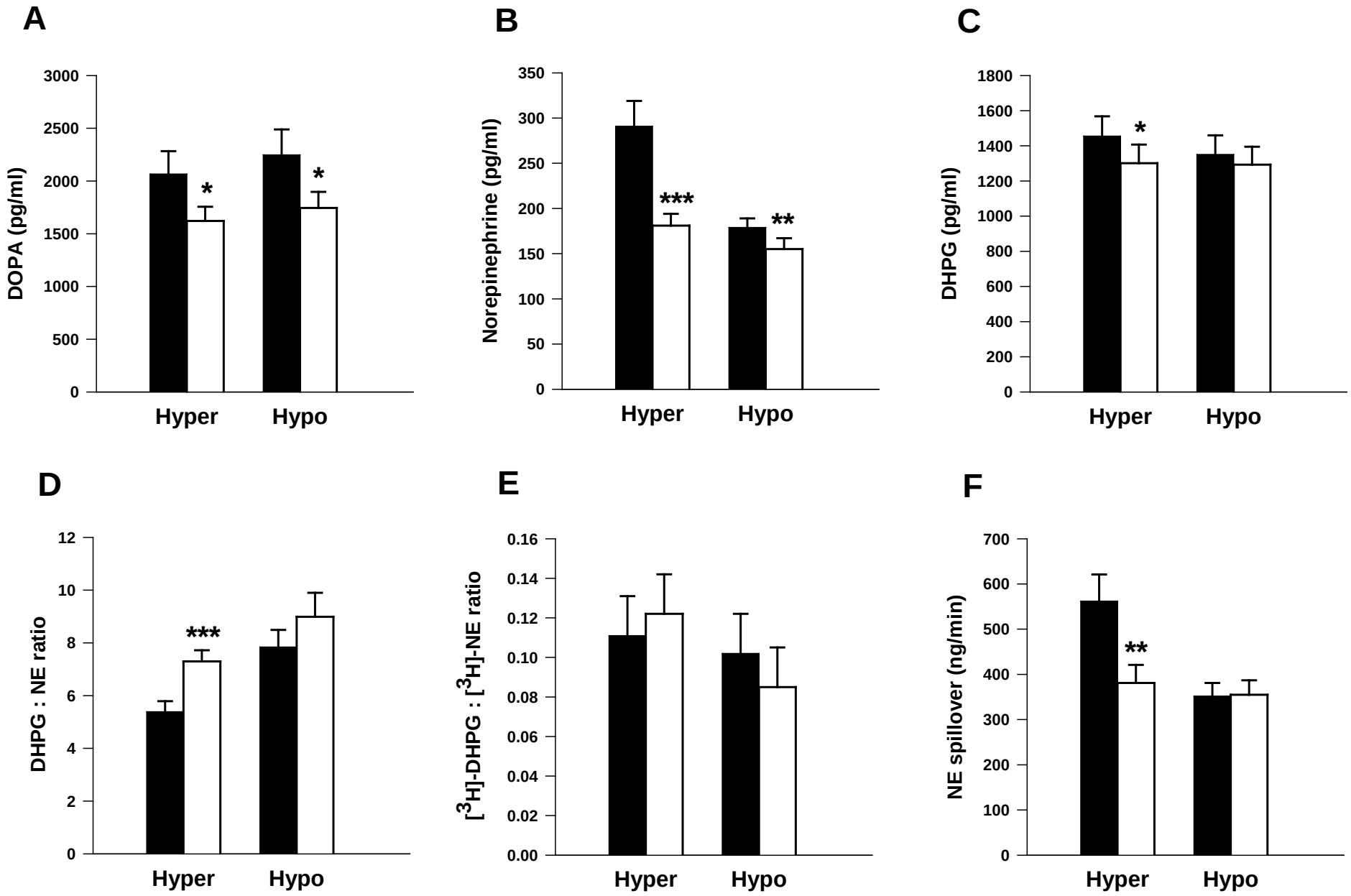

A
DOPA (pg/ml)
3000
2500
2000
1500
1000
500
0
*
*
Hyper
Hypo
B
Norepinephrine (pg/ml)
350
300
250
200
150
100
50
0
***
**
Hyper
Hypo
C
DHPG (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
*
Hyper
Hypo
D
DHPG : NE ratio
12
10
8
6
4
2
0
***
Hyper
Hypo
E
[3H]-DHPG : [3H]-NE ratio
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Hyper
Hypo
F
NE spillover (ng/min)
700
600
500
400
300
200
100
0
**
Hyper
Hypo

Figure 3

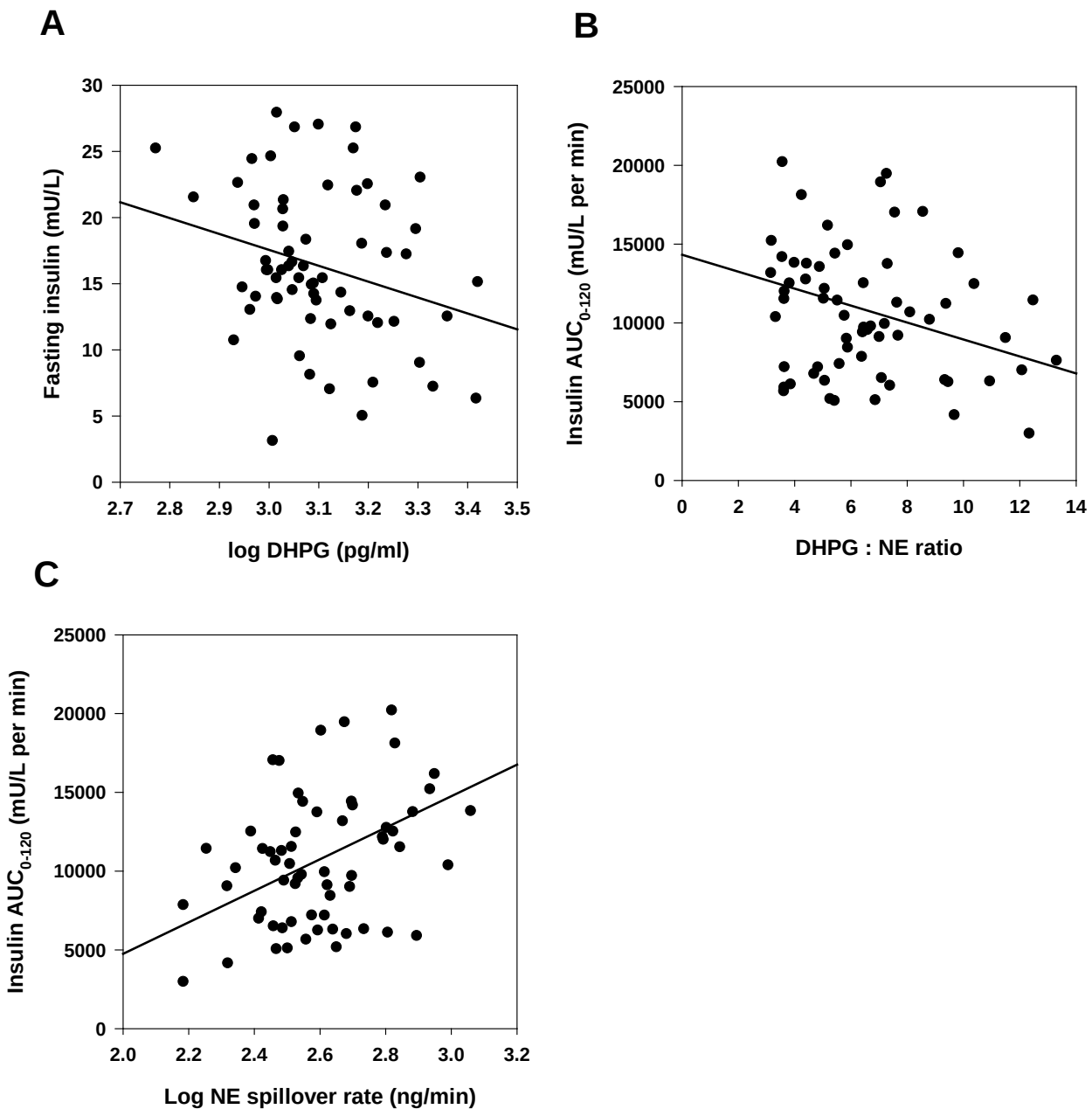
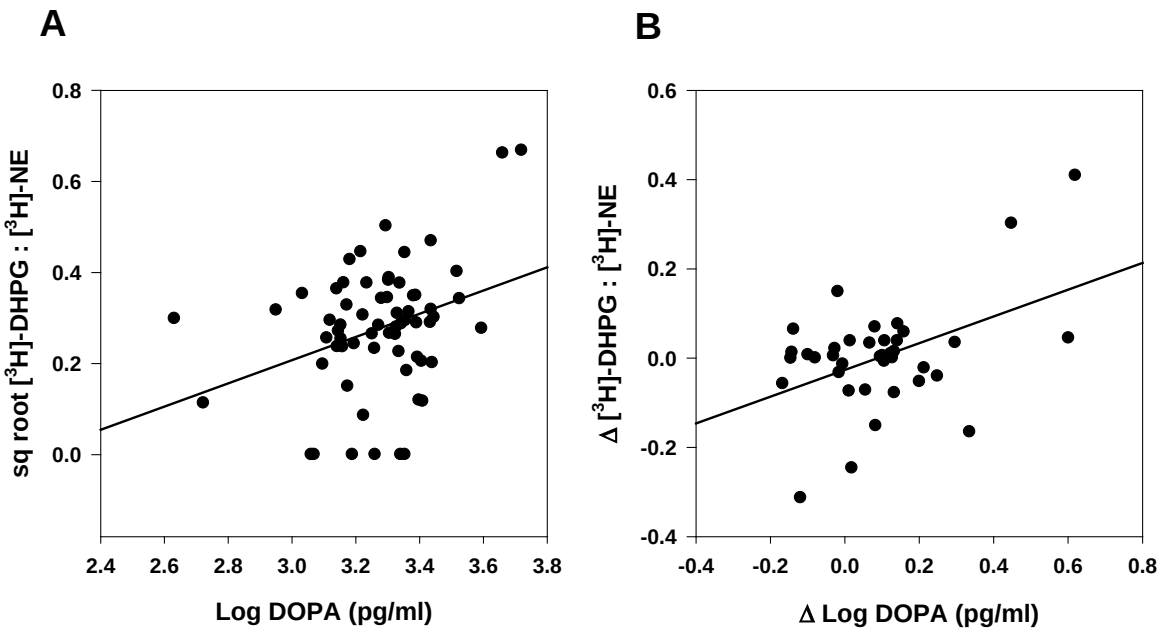

A
Fasting insulin (mU/L)
log DHPG (pg/ml)
B
Insulin AUC0-120 (mU/L per min)
DHPG : NE ratio
C
Insulin AUC0-120 (mU/L per min)
Log NE spillover rate (ng/min)

Figure 4


A
B
sq root [3H]-DHPG : [3H]-NE
Log DOPA (pg/ml)
Δ [3H]-DHPG : [3H]-NE
Δ Log DOPA (pg/ml)
0.8
0.6
0.4
0.2
0.0
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8