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SHORT REPORT

Integrative Cardiovascular Physiology and Pathophysiology

Effect of acute intranasal insulin administration on muscle sympathetic nerve activity in healthy young adults

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Abstract

Systemic insulin increases muscle sympathetic nerve activity (MSNA) via both central actions within the brainstem and peripheral activation of the arterial baroreflex. Augmented MSNA during hyperinsulinemia likely restrains peripheral vasodilation and contributes to the maintenance of blood pressure (BP). However, in the absence of insulin action within the peripheral vasculature, whether central insulin stimulation increases MSNA and influences peripheral hemodynamics in humans remains unknown. Herein, we hypothesized intranasal insulin administration would increase MSNA and BP in healthy young adults. Participants were assigned to time control [TC, $n = 13$ (5 females/8 males), 28 ± 1 yr] or 160 IU of intranasal insulin administered over 5 min [$n = 15$ (5 females/10 males), 26 ± 2 yr]; five (1 female/4 males) participants completed both conditions. MSNA (fibular microneurography), BP (finger photoplethysmography), and leg blood flow (LBF, femoral Doppler ultrasound) were assessed at baseline, and 15 and 30 min following insulin administration. Leg vascular conductance [LVC = (LBF ÷ mean BP) × 100] was calculated. Venous insulin and glucose concentrations remained unchanged throughout ($P > 0.05$). Following intranasal insulin administration, MSNA (burst frequency; baseline = 100%; *minute 15*, 121 ± 8%; *minute 30*, 118 ± 6%; $P = 0.009$, $n = 7$) and mean BP (baseline = 100%; *minute 15*, 103 ± 1%; *minute 30*, 102 ± 1%; $P = 0.003$) increased, whereas LVC decreased (baseline = 100%; *minute 15*, 93 ± 3%; *minute 30*, 99 ± 3%; $P = 0.03$). In contrast, MSNA, mean BP, and LVC were unchanged in TC participants ($P > 0.05$). We provide the first evidence that intranasal insulin administration in healthy young adults acutely increases MSNA and BP and decreases LVC. These results enhance mechanistic understanding of the sympathetic and peripheral hemodynamic response to insulin.

NEW & NOTEWORTHY Systemic insulin increases muscle sympathetic nerve activity (MSNA) via central actions within the brainstem and peripheral activation of the arterial baroreflex. In the absence of peripheral insulin action, whether central insulin stimulation increases MSNA and influences peripheral hemodynamics in humans was unknown. We provide the first evidence that intranasal insulin administration increases MSNA and blood pressure and reduces leg vascular conductance. These results enhance mechanistic understanding of the sympathetic and hemodynamic response to insulin.

autonomic; blood pressure; insulin; sympathetic

INTRODUCTION

Increases in blood glucose stimulate insulin release from pancreatic beta cells to promote cellular glucose uptake. Insulin further augments glucose delivery to tissues through its vasodilatory effects (1). The profound vasodilatory effects of insulin require counterregulatory mechanisms to maintain blood pressure (BP) (2). As plasma insulin increases following a meal, activity of the sympathetic nervous system directed toward skeletal muscle (muscle sympathetic nerve activity, MSNA) increases (2). In the absence of a rise in MSNA after a meal, BP falls significantly (3). As such, there is a clear role for the arterial baroreflex in the increase in MSNA during hyperinsulinemia, owing to the peripheral vasodilatory effects of systemic insulin. Indeed, coinfusion of insulin and phenylephrine (to limit insulin-mediated vasodilation) prevented any rise in MSNA during hyperinsulinemia (4).

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Although data from our group support that at least a portion of the increase in MSNA during hyperinsulinemia is baroreflex mediated (4), the rise in MSNA in response to elevated systemic insulin is gradual (2). This gradual rise in insulin-mediated sympathoexcitation is accredited by others to the time required for insulin transport across the blood-brain barrier (5, 6). Supporting this idea, cerebrospinal fluid levels of insulin increase ~30 min following insulin infusion in dogs (6). Once in the brain, preclinical data support insulin's actions in the arcuate nucleus and downstream signaling to increase sympathetic activity (7). However, whether isolated central insulin administration in humans increases MSNA and influences peripheral hemodynamics remains to be fully elucidated. In addition to its physiological relevance, this question has important clinical implications, as intranasal insulin administration is being assessed as a therapeutic for chronic conditions such as Alzheimer's disease (8, 9).

Indeed, nasal administration has been recently implemented to deliver insulin to the brain with limited systemic interference. Nose-to-brain delivery of insulin bypasses the blood-brain barrier to reach brain tissues in as little as 5–30 min (10, 11). Herein we applied intranasal drug delivery to examine the impact of increases in brain insulin on MSNA and peripheral hemodynamics. We hypothesized intranasal insulin would increase MSNA, promote peripheral vasoconstriction, and increase BP in healthy adults. Bursts of MSNA are generated by the synchronous firing of sympathetic action potentials (APs). Using wavelet-based approaches, sympathetic APs can be extracted from multiunit MSNA recordings to provide novel insight into neural coding and recruitment. Indeed, we recently demonstrated systemic hyperinsulinemia not only increases firing frequency of medium sized AP subpopulations, but also leads to the recruitment of previously latent, larger diameter sympathetic axons to maintain BP (12). In an exploratory analysis, we hypothesized any increase in MSNA would be achieved via both increased firing frequency of medium-sized AP subpopulations, and recruitment of previously latent, larger diameter sympathetic axons.

METHODS

Participants were young (<45 yr), and free of known acute and/or chronic diseases, including obesity (BMI < 30 kg/m^2), and taking no medications known to affect endocrine, cardiovascular, or autonomic function. Participants identified as: White/non-Hispanic (61%), White/Hispanic (9%), Asian (17%), and Black (13%). Female participants were premenopausal, had a negative pregnancy test on the morning of the study visit, and were studied in the self-reported early follicular phase of the menstrual cycle (*days 1–7*; 67% of female participants) or placebo phase of oral contraceptive use (22% of female participants). One female participant was noncycling (hormonal intrauterine device) and was studied at their convenience. Participants were asked to refrain from alcohol, caffeine, and exercise for 24 h and fast for 12 h before the study visit (13). Written informed consent was obtained from all participants and all experiments and procedures were approved by the Institutional Review Board at the University of Missouri (No. 2057288) and conformed to the Declaration of Helsinki including preregistration in a database (NCT05153395).

Participants were assigned into time control (TC, $n = 13$) or intranasal insulin ($n = 15$) groups; five (1 female/4 males) participants completed both conditions. On study days, participants were situated in a seated, semi-upright position with legs outstretched. Individuals were instrumented with a three-electrode electrocardiogram (Lead II; Bio Amp FE132, ADInstruments) and beat-to-beat BP via finger photoplethysmography (Finapres, Finapres Medical Systems) calibrated to upper arm BP (Tango M2, SunTech Medical). Superficial femoral artery diameter and blood velocity were measured using Doppler ultrasound (13). Multiunit MSNA was measured using fibular microneurography (Neuro Amp EX, ADInstruments; 14, 15).

For individuals receiving insulin, an intravenous catheter was placed in the antecubital vein for periodic blood sampling. Following instrumentation and a quiet 10-min baseline period, 160 IU of human insulin (Humulin R, U100) was administered intranasally using a commercial pump (ViaNase; Kurve Technology; Lynwood, WA; 8). Insulin was administered as four sprays over 5 min (40 IU/spray; alternating nostrils with 1 min between sprays). This dose has been shown to be safe (16, 17) and data suggest changes in autonomic function may be observed at this dose (16). Blood glucose (YSI 2300 STAT PLUS glucose analyzer) was determined at baseline, 15, and 30 min following intranasal insulin administration. Plasma was collected similarly and frozen for analysis of insulin (immunoassay; Quest Diagnostics Laboratories; Columbia, MO). Because of difficulties with catheter placement in three participants, blood glucose was monitored via fingerstick and glucometer. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated.

Analysis

Cardiovascular and MSNA measurements were obtained continuously during a 10-min baseline and for 30 min following intranasal insulin administration and TC. Data are reported as a 5-min average from 15 and 30 min following intranasal insulin administration and TC. Multiunit MSNA data are available from 16 participants [TC, $n = 9$ (4 females/5 males); insulin, $n = 7$ (1 female/6 males)] and a stable, high signal-to-noise MSNA signal required for AP analysis was acquired in 10 participants [TC, $n = 6$ (3 females/3 males); insulin, $n = 4$ (1 female/3 males)] and APs were extracted as published previously (18). Briefly, APs were extracted from the raw, bandpass-filtered MSNA neurogram at baseline and *minute 15* using a continuous wavelet transform and a matched "mother" wavelet developed for the NeuroAmp (ADInstruments) system (19). All APs were extracted, then ordered by peak-to-peak amplitude and histogram analysis, and sorted into amplitude-based clusters using Scott's rule (20). Action potential bin characteristics were normalized within each participant, and AP cluster activity was divided into 10 bins normalized to the largest AP cluster in each participant. See representative data (Fig. 1). A detailed description of data analysis can be found in the Supplemental Material (Supplement S1).

Statistical Analysis

Normality was assessed using the Shapiro–Wilk test. Glucose, insulin, and HOMA-IR were assessed via one-way

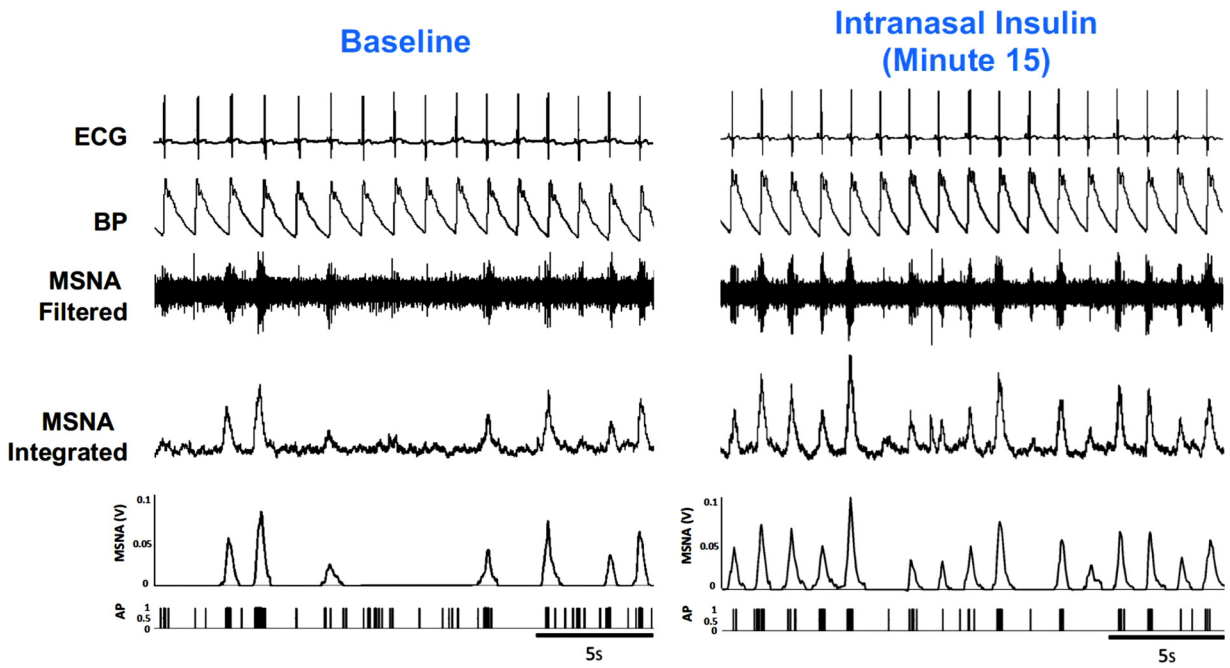

Figure 1. Representative original tracings from 1 male participant.

repeated measures analysis of variance (RMANOVA) with pairwise multiple comparisons using the Bonferroni method. Multiunit sympathetic and hemodynamic outcomes were assessed as a percentage of baseline [(*minute 15* ÷ baseline) × 100]. Outcomes were assessed using either one-way RMANOVA with Bonferroni test or the Friedman test on ranks with pairwise multiple comparisons using the Dunn's test, as appropriate. Action potential analysis focused on comparisons between baseline and *minute 15* using paired *t* tests due to the exploratory nature, limited sample size, and observed sympathetic and hemodynamic changes at this timepoint. Data are reported as means ± SE. An α of <0.05 was considered statistically significant.

RESULTS

Participants

Participants (TC, $n = 13$, 5 females/8 males; insulin, $n = 15$, 5 females/10 males) were young adults (TC, 28 ± 1 yr; insulin, 26 ± 2 yr) without obesity (TC, 25 ± 1 kg/m^2; insulin, 26 ± 1 kg/m^2). Intranasal insulin participants had normal fasting plasma insulin (7.2 ± 1.3 μIU/mL), blood glucose (76 ± 2 mg/dL), and HOMA-IR (1.3 ± 0.2 AU). Intranasal insulin administration did not alter insulin ($n = 12$; *minute 15,* 6.4 ± 0.6 μIU/mL; *minute 30,* 5.9 ± 0.7 μIU/mL; $P = 0.329$), glucose ($n = 15$; *minute 15*, 73 ± 2 mg/dL; *minute 30*, 72 ± 2 mg/dL, $P = 0.070$), nor HOMA-IR ($n = 12$; *minute 15*, 1.1 ± 0.1 AU; *minute 30*, 1.1 ± 0.2 AU; $P = 0.122$).

Hemodynamic Responses

Resting hemodynamics were within normal ranges for both groups (TC, heart rate = 63 ± 3 beats/min, mean BP = 92 ± 2 mmHg, LBF = 72 ± 7 mL/min; insulin, heart rate = 61 ± 2 beats/min, mean BP = 95 ± 2 mmHg, LBF = 92 ± 6 mL/min). Hemodynamic variables across the 30-min time course are presented as a percentage of baseline in Fig. 2. Friedman's test revealed significant differences in mean BP ($P = 0.003$) with Dunn's test showing increases at *minute 15* ($P = 0.007$) and *minute 30* ($P = 0.009$) following insulin administration (Fig. 2*A*). Although one-way RMANOVA did not reveal significant differences in LBF, a trend was observed following insulin ($P = 0.054$) (Fig. 2*B*). In the face of increased BP, one-way RMANOVA indicated significant changes in LVC ($P = 0.02$), with Bonferroni post hoc illustrating a reduction at *minute 15* following insulin ($P = 0.001$) (Fig. 2*C*).

One-way RMANOVA revealed no differences in mean BP ($P = 0.18$; Fig. 2*A*), LBF ($P = 0.62$; Fig. 2*B*), or LVC ($P = 0.74$; Fig. 2*C*) in the TC condition. One-way RMANOVA indicated a significant increase in heart rate in the TC condition ($P = 0.007$) at *minute 15* ($P = 0.004$), but not in the intranasal insulin condition ($P > 0.05$).

Sympathetic Responses

Resting MSNA values for both groups were within normal ranges (TC, 25 ± 1 bursts/min; insulin, 25 ± 1 bursts/min). Multiunit MSNA variables across the 30-min time course are presented as a percentage of baseline in Fig. 3. One-way RMANOVA revealed significant differences in burst frequency following insulin administration ($P = 0.009$), with Bonferroni post hoc denoting increases at *minute 15* ($P = 0.008$) and *minute 30* ($P = 0.025$) (Fig. 3*A*). The rise in MSNA in response to intranasal insulin occurred without changes in T50 (one-way RMANOVA, $P = 0.06$; Fig. 3*B*) or gain of the arterial baroreflex (Friedman's test, $P = 0.62$; Fig. 3*C*). No differences were observed in the TC condition for burst frequency ($P = 0.50$), arterial baroreflex gain ($P = 0.31$; Fig. 3*C*), nor T50 ($P = 0.77$; Fig. 3*B*).

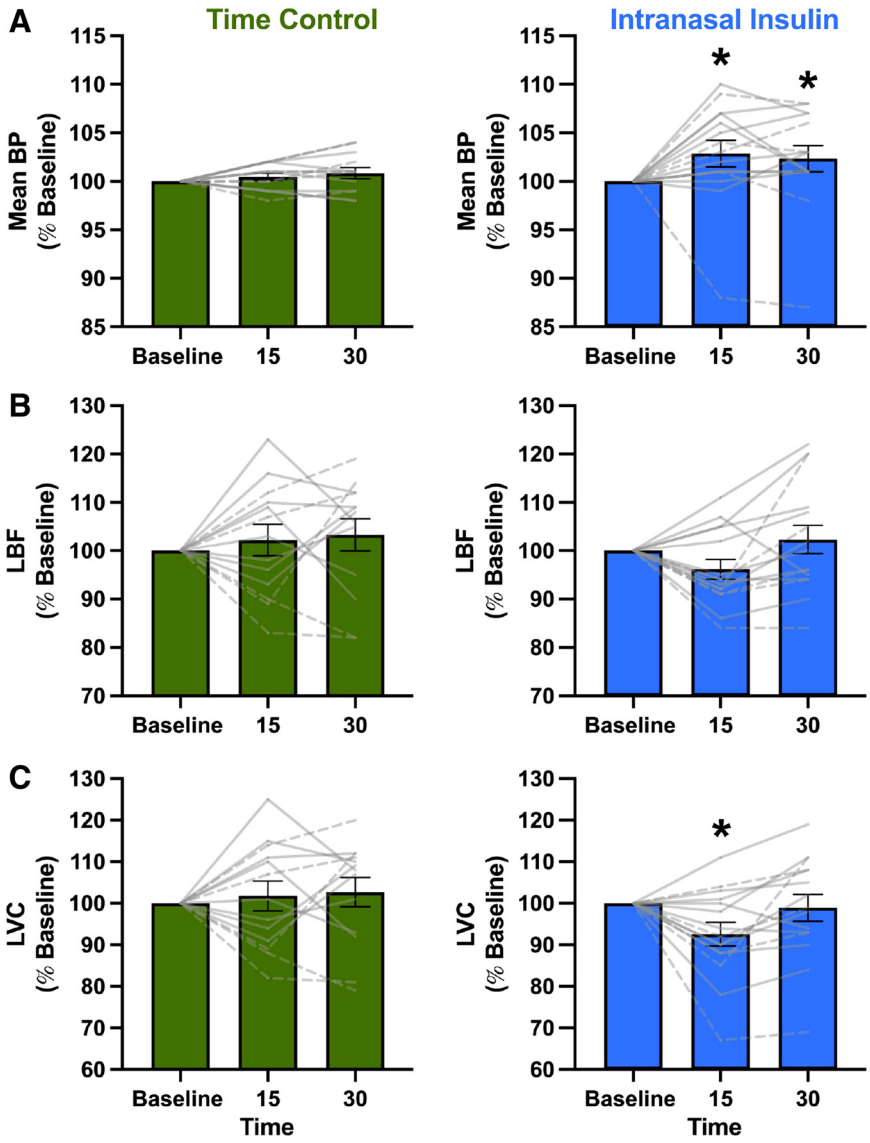

Figure 2. Intranasal insulin increases blood pressure (BP) and reduces leg vascular conductance (LVC). Individual data, as well as means ± SE, are reported for each condition [time control (TC), $n = 13$, 5 females/8 males; insulin, $n = 15$, 5 females/10 males] for mean BP (%baseline) (*A*), leg blood flow [LBF (% baseline)] (*B*), and LVC (%baseline) (*C*). Solid lines (male), dashed lines (female). One-way repeated-measures analysis of variance (RMANOVA) with Bonferroni post hoc correction or Friedman's test with Dunn's for multiple comparisons was performed, as appropriate. *$P < 0.05$ vs. baseline.

Sympathetic AP Analysis

A stable, high signal-to-noise MSNA signal required for AP analysis was acquired in a subset of 10 participants [TC, $n = 6$ (3 females/3 males); insulin, $n = 4$ (1 female/3 males)]. This analysis requires a continuous MSNA signal (e.g., no potential movement artifact, muscle tension, coughing/sneezing that could disrupt the electrode site) as well as a higher signal-to-noise ratio (18, 21). Six MSNA signals did not meet these criteria, and thus were not included within the AP subanalysis. Importantly, insulin-mediated increases in MSNA were replicated in this cohort. Paired *t* tests demonstrated an increase in burst frequency at *minute 15* following intranasal insulin (25 ± 5 to 31 ± 6 bursts/min, $P = 0.028$) with no changes in TC (22 ± 3 to 26 ± 3 bursts/min, $P = 0.27$). Analysis revealed no changes in AP frequency, mean AP per MSNA burst, mean clusters per burst, total cluster number, or AP signal-to-noise ratio ($P > 0.05$). Paired *t* test revealed that the percentage of APs firing synchronously within an MSNA burst significantly increased in the insulin condition (73 ± 7 to 86 ± 6; $P = 0.015$) (Fig. 4*A*), whereas the percentage of APs firing asynchronously decreased (28 ± 7 to 14 ± 6, $P = 0.015$) (Fig. 4*B*). Importantly, no changes were observed in the TC condition (%synchronous: $P = 0.48$, %asynchronous: $P = 0.40$) (Fig. 4, *A* and *B*). Paired *t* test showed the probability of medium-sized AP clusters firing more than once in close succession (multiple firing) increased following intranasal insulin administration (11 ± 4 to 15 ± 5, $P = 0.028$), but did not change in the TC condition ($P = 0.966$) (Fig. 4*C*). No differences were observed in multiple firing among small or large clusters in either condition ($P > 0.05$).

DISCUSSION

We provide the first evidence that intranasal insulin administration in humans acutely increases MSNA, which elicits peripheral vasoconstriction and subsequently increases BP. We further show intranasal insulin prompts an increase in MSNA, which is achieved via a higher probability of medium-sized AP clusters firing more than once in close succession. However, we did not observe recruitment of previously latent,

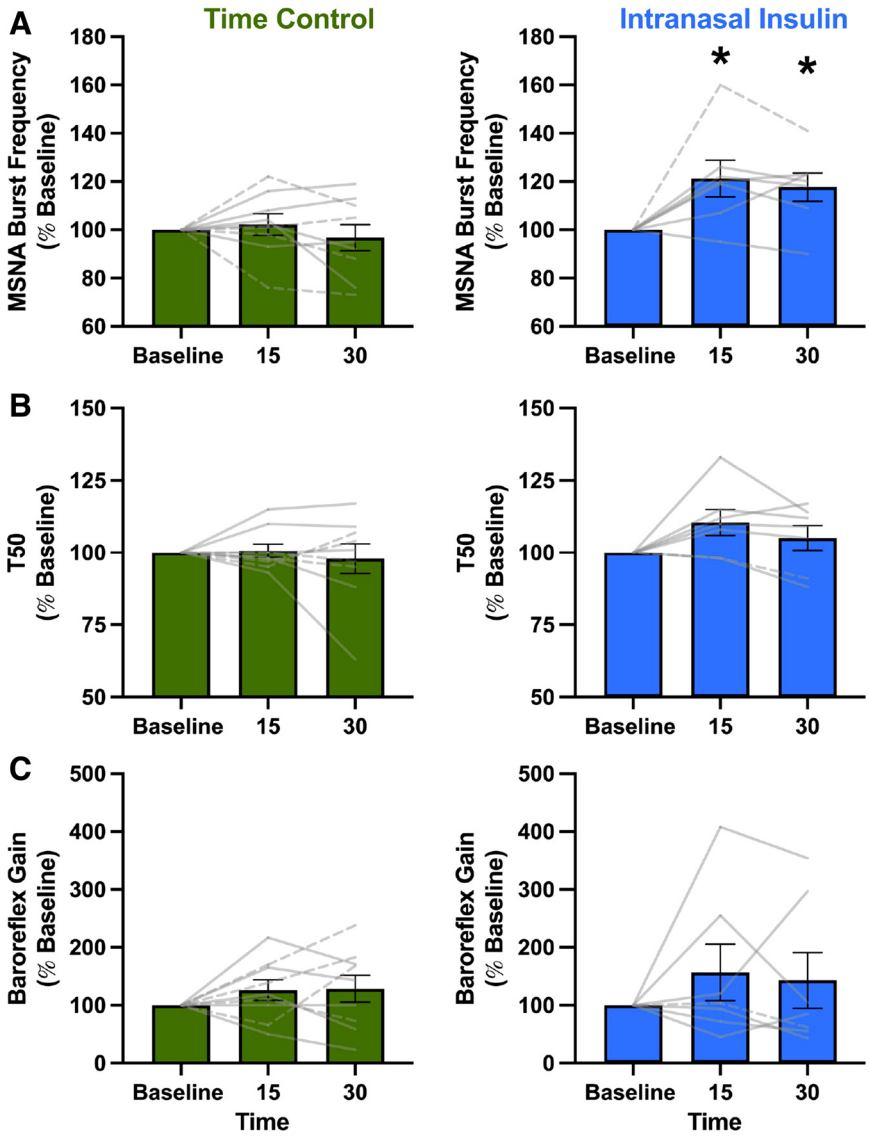

Figure 3. Intranasal insulin increases muscle sympathetic nerve activity (MSNA). Individual data, as well as means ± SE, are reported for each condition [time control (TC), $n = 9$, 4 females/5 males; insulin, $n = 7$, 1 female/6 males] for MSNA (%baseline) (*A*), T50 (% baseline) (*B*), and Baroreflex Gain (%baseline) (*C*). Solid lines (male), dashed lines (female). One-way repeated-measures analysis of variance (RMANOVA) with Bonferroni post hoc correction or Friedman's test with Dunn's for multiple comparisons was performed, as appropriate. $^*P < 0.05$ vs. baseline.

larger-diameter sympathetic axons when healthy young adults were exposed to insulin intranasally. These novel data highlight potential differences in the sympathetic response to central versus peripheral insulin exposure in healthy young adults.

Preclinical data suggest insulin-mediated increases in MSNA are due to insulin's effects within the central nervous system, particularly via action in the arcuate nucleus (7). Unfortunately, the mechanistic understanding of insulin's central effects on MSNA in humans was previously limited because of administration of insulin systemically through intravenous methods. Intravenous insulin has profound vasodilatory effects within the peripheral vasculature (1, 22), thereby making it challenging to separate the direct central versus peripheral effects of insulin on MSNA. Indeed, high-dose insulin given intravenously increases MSNA within 15 min, before any central effects, presumably to maintain total peripheral resistance and preserve BP in healthy young adults (4).

Insulin receptors are distributed widely, among many major cell types in the human brain (9). Peripheral insulin crosses the blood-brain barrier through a gradual, saturable transport system (5, 23, 24), whereas insulin administered intranasally allows for prompt entry into brain regions (i.e., olfactory bulb, hippocampus, hypothalamus, cerebellum). Entry through the olfactory and trigeminal nerve pathways provides direct connections between the nasal lamina propria and central nervous system (10, 11, 25). Importantly, following intranasal insulin administration in rats, insulin receptor phosphorylation (tyrosine 1185) increased in brain homogenates, supporting delivery of bioactive insulin (11). Human clinical trials confirm intranasal insulin administration delivers a bioactive form, as changes in functional connectivity between brain regions, alterations in tissue perfusion, and improvements in memory-related tasks have been documented (16, 17, 26). Important to this, and in agreement with our data, intranasal insulin elicits little-to-no changes to plasma insulin and glucose levels (16, 26, 27). As such, intranasal administration is a novel strategy to assess the isolated central effects of insulin on MSNA and downstream hemodynamics.

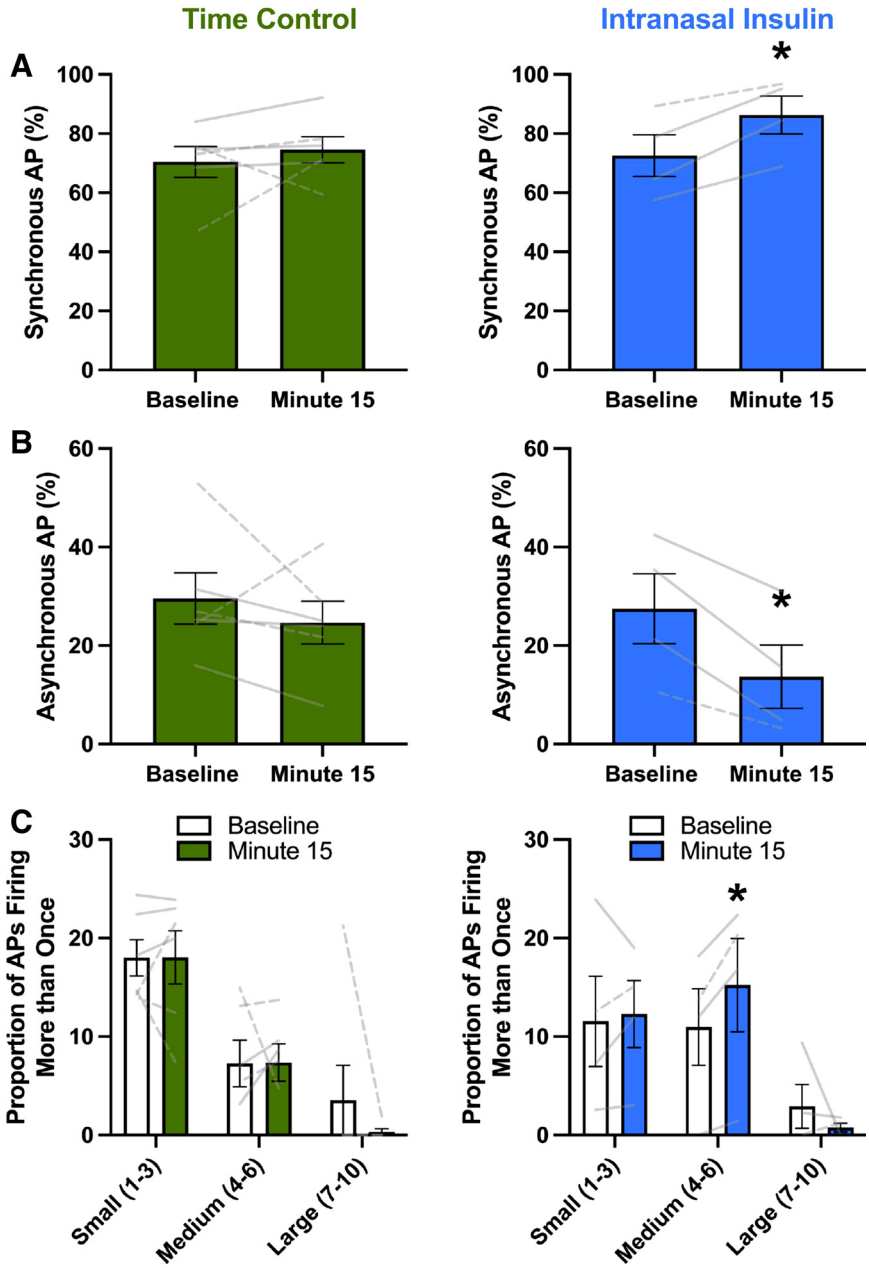

Figure 4. Sympathetic action potential (AP) response to intranasal insulin. Individual data, as well as means ± SE, are reported for each condition [time control (TC), $n = 6$, 3 females/3 males; insulin, $n = 4$, 1 female/3 males] for synchronous APs (*A*), asynchronous APs (*B*), and proportion of APs firing more than once (*C*). Solid lines (male), dashed lines (female). Paired *t* test was used. *$P < 0.05$ vs. baseline.

To date, only one study attempted to assess the influence of intranasal insulin on efferent MSNA in humans. Prior work in eight young males (24.8 yr; BMI, 22.6 kg/m^2) suggests that acute intranasal insulin (Insulin Actrapid HM, Novo Nordisk) raises BP ~14%; however, these effects were not observed until 90–120 min after administration (27). In contrast, the authors found heart rate and MSNA to be numerically, but not statistically, increased; an observation which is inconsistent with preclinical data (28). The authors conclude that increasing brain insulin levels contributes to increased BP via central mechanisms which downregulate the sensitivity of the arterial and cardiovagal baroreflex—although baroreflex sensitivity was not assessed. It should be noted that immediately before insulin exposure, participants exhibited resting BPs in the hypertensive range (152/92 mmHg) (27), and hypertension can alter the sympathetic and BP-raising effects of insulin (29). In addition, MSNA recordings were averaged across 25 min, limiting the ability to assess dynamic changes characteristic of the baroreflex (27). Finally, it is possible that the lack of effect was due to the repeated, low-dose application (20 IU of insulin administered every 10 min), which is inconsistent with the bolus given in a majority of clinical trials (8, 16, 17).

To address limitations in prior work, we measured MSNA, LBF, and BP before and for 30 min immediately following intranasal insulin administration. Our comprehensive assessment of the sympathetic and hemodynamic response to intranasal insulin resulted in two major findings. First, we observed a significant increase in MSNA burst frequency and mean BP within 15 min of intranasal insulin administration, which was accompanied by a reduction in LVC. Second, any changes in MSNA and BP following

intranasal insulin administration were independent of a change in arterial baroreflex gain. The lack of an effect of centrally administrated insulin on baroreflex sensitivity disagrees with experiments conducted in rats, which show intracerebroventricular insulin enhances the gain of the baroreflex and reduces BP (7, 28). Discrepancies between results may be due to several factors, including fundamental differences in route of application (intranasal vs. intracerebroventricular) and concentration of bioavailable insulin. Although it is difficult to directly compare preclinical work and present data, these first-in-human data clearly show that intranasal insulin increases MSNA and BP without obvious changes to the gain of the baroreflex.

To provide insight into insulin-mediated changes in MSNA, we conducted an exploratory analysis examining sympathetic neural firing patterns. Sympathetic APs modulate size and frequency of integrated MSNA bursts through increased firing of previously active AP populations (rate coding), recruitment of previously latent AP populations (population coding) (30), or by modifying the synchronicity of AP firing (31). In response to a euglycemic-hyperinsulinemic infusion, the probability of medium-sized AP clusters and the firing of previously latent, larger-diameter axons increased (12). Interestingly, despite an increase in multiunit MSNA burst frequency, intranasal insulin administration did not reveal any changes in total APs nor AP frequency, nor did we observe recruitment of previously latent, larger diameter axons (i.e., increase in maximum cluster number). The differential response in AP firing patterns suggests that rate and population coding strategies to modulate MSNA may be more pertinent in the setting of systemic insulin infusion. In contrast, intranasal insulin administration resulted in significant changes in the proportion of APs firing synchronously (with concomitant reductions in APs firing asynchronously). Synchronous AP firing has relevance for peripheral vasomotor control (12, 21, 31, 32). Indeed, recent human studies (12, 21, 32), in addition to preclinical evidence (33), show that augmented synchronous AP discharge produces a vasoconstrictive effect in the periphery. In the setting of central insulin administration, our data suggest that the combination of a greater number of APs firing synchronously, in conjunction with a higher proportion of medium-sized APs firing more than once, may be sufficient to promote greater neurotransmitter release from presynaptic nerve terminals, ultimately promoting peripheral vasoconstriction and increases in BP.

Experimental Considerations

Although this study is the first of its kind to assess the central effects of insulin on MSNA, baroreflex sensitivity, and peripheral hemodynamics in healthy young adults, there are some important limitations. First, due to challenges surrounding intranasal drug administration, a TC, rather than a placebo or sham control, was applied. Second, conclusions are limited to the acute dose (160 IU) administered and cannot be extrapolated to other doses. Third, bioavailability of insulin, and the dose of insulin that reaches the brain, is not completely known and may be specific to the device used. Fourth, we acknowledge sex-related differences exist in basal MSNA (34), the impact of MSNA on BP (35), as well as the central sympathetic response to insulin (36). Although we were not powered to examine results by sex, this will be a focus of future assessments. Of note, one female participant who received intranasal insulin had a robust increase in MSNA burst frequency (Fig. 3*A*). Importantly, the observed effect ($P = 0.016$) was retained even after removing this individual from the analysis, with Bonferroni indicating significant increases in MSNA burst frequency (%baseline) at *minute 15* ($P = 0.019$) and *minute 30* ($P = 0.037$). Lastly, sample sizes for MSNA and AP analysis were low due to challenges involved with obtaining a consistent, high quality MSNA recording and thus results are exploratory in nature. Inclusion of more participants, particularly females, would enhance the ability to assess sex differences.

Conclusions

We provide the first evidence that intranasal insulin administration in healthy young adults acutely increases MSNA and BP. These observations may be due to enhanced synchronicity of APs and increased proportion of medium-sized APs firing multiple times promoting peripheral vasoconstriction following insulin exposure. The neural coding patterns observed offer insight into how changes in MSNA may be acutely induce peripheral vascular resistance in response to central insulin. These findings enhance our understanding of cardiovascular control mechanisms during hyperinsulinemia.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental S1:

ACKNOWLEDGMENTS

We appreciate the time and effort put in by all research participants. A licensed version of BioRender.com was used to create the graphical abstract.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.M.-A., J.P., and J.K.L. conceived and designed research; N.J.M., D.W.J., B.S., J.L.H., and J.K.L. performed experiments; N.J.M. analyzed data; N.J.M., G.E.F., J.P., and J.K.L. interpreted results of experiments; N.J.M. prepared figures; N.J.M., J.P., and J.K.L. drafted

manuscript; N.J.M., D.W.J., B.S., J.L.H., G.E.F., C.M.-A., J.P., and J.K.L. edited and revised manuscript; N.J.M., D.W.J., B.S., J.L.H., G.E.F., C.M.-A., J.P., and J.K.L. approved final version of manuscript.

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