Glutamate receptors in the hypothalamic paraventricular nucleus contribute to insulin-induced sympathoexcitation

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Stocker SD, Gordon KW. Glutamate receptors in the hypothalamic paraventricular nucleus contribute to insulin-induced sympathoexcitation. J Neurophysiol 113: 1302–1309, 2015. First published December 4, 2014; doi:10.1152/jn.00764.2014.—The sympathoexcitatory response to insulin is mediated by neurons in the arcuate nucleus (ARC) and hypothalamic paraventricular nucleus (PVH). Previous studies have reported that stimulation of ARC neurons increases sympathetic nerve activity (SNA) and arterial blood pressure (ABP) through glutamate receptor activation in the PVH. Therefore, the purpose of the present study was to determine whether glutamatergic neurotransmission in the PVH contributes to insulin-induced sympathoexcitation. Male Sprague-Dawley rats (275–400 g) were infused with isotonic saline or insulin (3.75 mU·kg−1·min−1) plus 50% dextrose to maintain euglycemia. Intravenous infusion of insulin significantly increased lumbar SNA without a significant change in mean ABP, renal SNA, heart rate, or blood glucose. Bilateral PVH injection of the excitatory amino acid antagonist kynurenic acid (KYN) lowered lumbar SNA and ABP of animals infused with insulin. Similarly, a cocktail of the NMDA antagonist DL-2-amino-5-phosphonopentanoic acid (AP5) and non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) reduced lumbar SNA and mean ABP during infusion of insulin. In a final experiment, bilateral PVH injection of AP5 only, but not CNQX, lowered lumbar SNA and mean ABP of animals infused with insulin. The peak changes in lumbar SNA and mean ABP of insulin-treated animals were not different between KYN, AP5 plus CNQX, or AP5 alone. These drug treatments did not alter any variable in animals infused with saline. Altogether, these findings suggest that glutamatergic NMDA neurotransmission in the PVH contributes to insulin-induced sympathoexcitation.

MATERIALS AND METHODS

Animals. All of the experimental procedures conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use

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Committee at the Pennsylvania State College of Medicine. Male Sprague-Dawley rats (275–400 g, Charles River Laboratories) were housed in a temperature-controlled room (22–23°C) with a 12:12-h light-dark cycle. Rats were fed standard chow (Harlan Teklad 2018) and given access to deionized water.

General procedures. Animals were anesthetized with isoflurane (2–3% in 100% O2) and instrumented with a femoral arterial catheter for ABP measurement, a double-lumen femoral venous catheter for administration of drugs and infusions, and a brachial arterial catheter for blood sampling. The lumbar and renal sympathetic nerves were isolated through a ventral midline incision and a retroperitoneal approach for blood sampling. The lumbar and renal sympathetic nerves were given access to deionized water.

To identify the glutamate receptor subtype(s) in the PVH that contributes to insulin-induced sympathoexcitation, AP5 (5 mM, 60 nl) or CNQX (2 mM, 60 nl) alone was injected bilaterally into the PVH as described above. In these experiments, AP5 and CNQX were tested in the same animal at times separated by 45 min. The first injection was performed at 90 min, whereas the second injection was performed at 135 min after start of the insulin or saline infusion. The order of the drugs was randomized. Variables were recorded for an additional 45 min after the second injection.

Experiment 3. To establish the duration of NMDA receptor blockade achieved in the above experiments, SNA and ABP responses to PVH injection of NMDA were analyzed before and after injection of KYN or AP5. After variables stabilized, NMDA (5 mM, 20 nl) was injected unilaterally into the PVH using methods described above. At 15 min later, KYN (25 mM, 60 nl), AP5 (5 mM, 60 nl), or artificial cerebrospinal fluid (aCSF: 60 nl) was injected into the PVH using the same coordinates. The pipette was raised, rinsed with aCSF (5 times), and filled with NMDA (5 mM). SNA and ABP responses to NMDA were tested again at 5 and 25 min after injection of the antagonist or vehicle. Antagonist and NMDA injection sites were marked with 0.2% FITC or rhodamine beads added to the solutions. Only one antagonist or vehicle was tested per PVH.

Histology. At the end of experiments, the animals were perfused transcardially with 4% paraformaldehyde. The brains were harvested, postfixed in 4% paraformaldehyde, and sectioned at 100 μm using a vibratome. Injection sites were identified by the addition of 0.2% fluorescent beads (rhodamine or FITC) to the drug solutions.

Statistical analysis. All data are means ± SE. Changes in rectified and integrated SNA were calculated by subtracting background noise. For all variables, 5-min segments at each time point were compared with 3 consecutive 5-min baseline periods. All data were analyzed by a two-way ANOVA with repeated measures. When significant F values were obtained, post hoc comparisons were made using independent or paired t-tests with a layered Bonferroni correction. There were two sets of specific post hoc comparisons within each group: 1) a direct comparison between baseline values and the respective time point (were values elevated with respect to baseline?) and 2) a direct comparison between 90 min (before injection) and a time after PVH injection (did PVH injection alter any variable?). Direct post hoc comparisons were also made between treatment groups (saline vs. insulin infusion or aCSF vs. KYN injection). A P value <0.05 was statistically significant.

RESULTS

Blockade of glutamate receptors in the PVH lowers lumbar SNA and ABP during infusion of insulin. The primary purpose of the present study was to determine whether excitatory amino acid or glutamate receptor activation in the PVH contributes to insulin-induced sympathoexcitation. Figure 1 illustrates examples of lumbar SNA and ABP responses before and after bilateral PVH injection of KYN or a cocktail of AP5/CNQX into animals infused intravenously with insulin or isoflontine saline. Summary data and injection sites are presented in Fig. 2. As previously reported in our laboratory (Bardgett et al. 2010; Luckett et al. 2013; Steiner et al. 2014; Ward et al. 2011), a hyperinsulinemic-euglycemic clamp significantly increased lumbar SNA without a change in renal SNA (Table 1), heart rate (Table 1), or mean ABP (Table 1). Infusion of isoflontine saline did not alter any variable.

Bilateral injection of KYN produced a fall in lumbar SNA and mean ABP of animals infused with insulin but did not alter either variable in animals infused with saline (Figs. 1 and 2). Despite the reduction in lumbar SNA of insulin treated rats, the values of lumbar SNA were significantly higher than baseline values (before insulin infusion) or the values of lumbar SNA in animals infused with saline after PVH injection of KYN (Figs.
1 and 2). Lumbar SNA returned to preinjection levels at 30 min later. Injection of KYN outside of the PVH did not significantly alter lumbar SNA (2\% H11006 7%, n/H11005 4; P/H11022 0.5) or mean ABP (1\% H11006 1 mmHg, n/H11005 4; P/H11022 0.5).

To confirm the contribution of glutamate receptor activation in the PVH to insulin-induced sympathoexcitation, an additional set of experiments was performed using a cocktail of AP5/CNQX. As illustrated in Figs. 1B and 2B, bilateral injection of AP5/CNQX produced a fall in lumbar SNA and mean BP of animals infused with insulin but did not alter either variable in animals infused with saline. Again, lumbar SNA of animals infused with insulin and injected with AP5/CNQX remained significantly elevated above baseline levels or those of animals infused with saline. Lumbar SNA and mean ABP returned to preinjection levels at 30 min later.

Blockade of NMDA, but not non-NMDA, receptors lowers lumbar SNA during infusion of insulin. Since data presented in Figs. 1 and 2 suggest glutamate receptor activation in the PVH contributes to insulin-induced sympathoexcitation, a second set of experiments was conducted to determine the contribution of NMDA vs. non-NMDA receptors. As illustrated in Fig. 3, bilateral injection of AP5 reduced lumbar SNA and mean ABP during an intravenous infusion of insulin plus dextrose. Values returned to preinjection levels within 30 min.

Fig. 1. Example of arterial blood pressure (ABP), mean ABP (grey line), integrated (\(\int\)) lumbar sympathetic nerve activity (SNA), and raw lumbar SNA of rats infused with insulin + 50% dextrose or vehicle (isotonic saline) and injected with kynurenic acid (KYN; A) or a cocktail of DL-2-amino-5-phosphono-pentanoic acid and 6-cyano-7-nitroquinoxaline-2,3-dione (AP5+CQNX; B) into the hypothalamic paraventricular nucleus (PVH) bilaterally. Insulin increased lumbar SNA without significant changes in ABP. PVH injection of KYN or AP5+CQNX lowered ABP and lumbar SNA in animals infused with insulin but did not alter any variable in animals infused with saline.

Fig. 2. Mean ABP, integrated lumbar SNA, and blood glucose of animals infused with saline or insulin and injected with KYN (A) or a cocktail of AP5/CQNX (B) into the PVH bilaterally. Values are means ± SE. *P < 0.05 vs. baseline values. #P < 0.05 vs. 90-min values. Schematic illustrations show PVH injection sites for KYN (C) or AP5/CQNX (D). AH, anterior hypothalamus; f, fornix.
In marked contrast, PVH injection of CNQX did not alter lumbar SNA or mean ABP in animals infused with insulin. PVH injection of AP5 or CNQX did not alter any variable in animals infused with saline.

Figure 4 summarizes the peak responses in lumbar SNA and mean ABP of animals infused with saline or insulin and injected with KYN, AP5/CNQX cocktail, AP5 only, or CNQX only into the PVN bilaterally. As noted above, injection of KYN, AP5 plus CNQX, or AP5 only reduced SNA and ABP in animals infused with insulin. The magnitude of these responses was not significantly different between drugs. The responses to CNQX were not significantly different between animals infused within insulin vs. saline.

**Time course of NMDA receptor blockade after KYN or AP5 injection.** To determine the duration of NMDA receptor blockade in the above experiments, we analyzed SNA and ABP responses to injection of NMDA before and after of KYN or AP5. As illustrated in Fig. 5, injection of NMDA into the PVH significantly increased renal SNA, lumbar SNA, mean ABP, and heart rate (data not shown). These effects were significantly attenuated at 5 min after pretreatment with KYN but restored at 25 min (Fig. 5, A and B). Similar effects were observed with AP5 pretreatment (Fig. 5B).

**DISCUSSION**

Previous studies have reported that the sympathetic and cardiovascular responses produced by ARC stimulation are mediated by glutamatergic neurotransmission in the PVH (Kawabe et al. 2012b). The purpose of the present study was to determine whether glutamatergic receptor activation in the PVH contributes to insulin-induced sympathoexcitation, a response dependent on ARC and PVH neurons (Bardgett et al. 2010; Cassaglia et al. 2011; Luckett et al. 2013; Ward et al. 2011). The present study provides several novel observations: 1) bilateral injection of KYN into the PVH attenuated the lumbar sympathoexcitatory response to insulin; 2) PVH injection of a cocktail of AP5 and CNQX also reduced lumbar SNA of animals infused with insulin; and 3) selective blockade of NMDA, but not non-NMDA, receptors in the PVH lowered lumbar SNA in insulin-treated animals. Collectively, these observations suggest that glutamatergic NMDA-dependent neurotransmission contributes to insulin-induced sympathoexcitation.

Previous studies have highlighted the importance of ARC and PVH neurons in insulin-induced sympathoexcitation and altered baroreflex function (Cassaglia et al. 2011; Luckett et al. 2013; Ward et al. 2011). Collectively, these studies suggest that insulin acts within the ARC to subsequently activate PVH neurons through melanocortin-3/4 receptors. Consistent with this notion, melanocortin receptor blockade in the PVH attenuated the sympathoexcitatory and pressor responses produced by stimulation of ARC neurons (Kawabe et al. 2012b). However, studies from the same group also reported that combined blockade of NMDA and non-NMDA receptors in the PVH...
convert the ARC-evoked sympathoexcitatory response to a decrease in splanchnic SNA and ABP (Kawabe et al. 2012a). Therefore, we investigated whether glutamatergic neurotransmission contributed to the lumbar sympathoexcitatory response during infusion of insulin. Indeed, bilateral injection of KYN or a cocktail of AP5 and CNQX attenuated the increase in lumbar SNA during a hyperinsulinemic-euglycemic clamp but had no effect on any variable on animals infused with saline. The absence of a response to PVH injection of KYN or AP5 and CNQX in control animals is consistent with previous studies (Chen et al. 2003; Li et al. 2006; Llewellyn et al. 2012). These findings suggest that glutamateric neurotransmission in the PVH contributes to the insulin-induced sympathoexcitation.

We (Ward et al. 2011) and others (Cassaglia et al. 2011) have previously reported that inhibition of PVH neurons with microinjection of the GABA_A receptor agonist muscimol completely reverses the lumbar sympathoexcitatory response to insulin. In the present study, PVH injection of KYN, AP5 plus CNQX, or AP5 lowered lumbar SNA of animals infused with insulin. However, lumbar SNA of these animals remain significantly elevated compared with those of baseline values or control animals receiving the same PVH injection. Altogether, these findings may suggest that glutamateric receptor activation in the PVH partially contributes to the lumbar sympathoexcitatory response during hyperinsulinemia. Although identical injection volumes were used in both studies, it is difficult to determine the diffusion of the respective drugs across the entire PVH. Therefore, direct comparisons regarding the absolute magnitude of the responses to muscimol vs. glutamate receptor blockade should be interpreted with caution. Nevertheless, the time course of the responses is consistent with the ability of these drugs to block NMDA-evoked responses at 5 min but not 25 min (Fig. 5).

Chemical excitation of PVH neurons has been reported to elicit both increases and decreases in SNA and/or ABP (Chen et al. 2003; Deering and Coote 2000; Li et al. 2006; Llewellyn et al. 2012; Martin and Haywood 1993, 1992; Martin et al. 1991). However, the majority of responses are sympathoexcitatory, including studies performed in awake animals (Martin and Haywood 1993, 1992) or when PVH neurons are disinhibited (Chen et al. 2003; Kenney et al. 2001; Li et al. 2006; Martin and Haywood 1993, 1992; Martin et al. 1991). Prior studies in anesthetized animals have reported that NMDA or non-NMDA receptor activation increases SNA and ABP (Kawabe et al. 2008, 2009; Kenney et al. 2003; Ward et al. 2011).
Therefore, there was no a priori hypothesis regarding the relative contribution of NMDA vs. non-NMDA receptors in the sympathoexcitatory response to insulin. We observed that blockade of NMDA, but not non-NMDA, receptors lowered SNA in animals infused with insulin. In fact, the magnitude of the response to AP5 was not statistically different from those responses after injection of KYN or AP5 plus CNQX (Fig. 4). This suggests the sympathoexcitatory response to insulin depends, in part, on a NMDA-driven receptor activation in the PVH.

A previous report from our laboratory indicated that blockade of melanocortin-3/4 receptors in the PVH completely reversed the sympathetic response to insulin (Ward et al. 2011). Yet, the present findings also suggest glutamate receptor activation in the PVH partially contributes. How do these neurotransmitter systems work within the PVH to regulate SNA during hyperinsulinemia? Melanocortin-4 receptors are expressed both pre- and postsynaptically within the PVH (Cowley et al. 1999; Kishi et al. 2003; Liu et al. 2003). Melanocortin agonists have been reported to act presynaptically to increase either inhibitory (IPSC) or excitatory postsynaptic current (EPSC) frequency (Cowley et al. 1999; Fu and van den Pol 2008; Wan et al. 2008). Thus it is possible that insulin activates POMC neurons to subsequently modulate glutamatergic neurotransmission within the PVH. Data from direct PVH recordings are limited and controversial. Cowley et al. (1999) reported that melanocortin agonists act presynaptically to enhance electrically evoked, GABA-mediated current responses in medial parvocellular PVH neurons. On the contrary, Ye and Li (2011) reported that melanocortins act postsynaptically to depolarize and excite PVH neurons that project to the rostral ventrolateral medulla. Clearly, data are limited regarding the cellular actions of melanocortins on different neuronal populations of PVH neurons. However, the current observations together with a previous report from our laboratory suggest that both melanocortin and glutamatergic neurotransmission in the PVH interact to regulate SNA during hyperinsulinemia.

PVH neurons have extensive afferent and efferent projections within the central nervous system (Sawchenko and Swanson 1982, 1983). Many of these brain regions contain glutamatergic neurons (Stornetta et al. 2002; Ziegler et al. 2002); however, the origin of all glutamatergic inputs to the PVH has not been systemically identified. Subsets of ARC POMC neurons have been reported to express mRNA for the vesicular glutamate transporter-2 (Dicken et al. 2012; Hentges et al. 2009; Meister 2007; Ziegler et al. 2002). Although insulin has been reported to hyperpolarize POMC neurons (Williams et al. 2010), recent evidence suggests that purified insulin (without Zn in the formulation) depolarizes and excites POMC neurons (Qiu et al. 2014). Therefore, insulin may activate ARC neurons to corelease α-melanocyte-stimulating hormone (α-MSH) and glutamate in the PVH. In addition, insulin has been reported to hyperpolarize agouti-related peptide/neuropeptide Y neurons (Williams et al. 2010). Agouti-related peptide acts as an endogenous antagonist of the POMC system (Schwartz et al. 2000), and neuropeptide Y receptor activation in the PVH decreases SNA and ABP (Cassaglia et al. 2014). Therefore, insulin-induced inhibition or removal of neuropeptide Y inputs to the PVH may act to disinhibit sympathetic neurons and increase SNA. Although this has not yet been directly tested, the sympathoexcitatory response to pharmacological disinhibition of the PVH via GABA_A receptor blockade is mediated, in part, by local glutamatergic receptor activation (Chen et al. 2003). Finally, a previous study reported that lesion of the anteroventral third ventricular region eliminated the lumbar sympathoexcitatory response to insulin (Muntzel et al. 1994). Although this region does not contain POMC neurons, it does contain an abundance of glutamatergic neurons (Ziegler et al. 2002). However, it is not clear whether the AV3V region contains neurons that sense changes in circulating insulin levels, provides tonic glutamatergic drive to the PVH (which is normally offset by tonic GABAergic input), or represents an integral part of the insulin neural circuit but downstream of ARC neurons. Therefore, the origin and mechanisms of glutamatergic inputs to the PVH during hyperinsulinemia need further investigation.

In the present study, an acute hyperinsulinemic-euglycemic clamp increased lumbar SNA but did not raise ABP. This observation confirms previous studies from several laboratories in which intravenous or intracerebroventricular administration of insulin does not alter ABP or produces a small pressor response (~5 mmHg) in rodents (Bardgett et al. 2010; Cassaglia et al. 2011; Luckett et al. 2013; Morgan et al. 1993; Muntzel et al. 1994; Pricher et al. 2008; Rahmouni et al. 2004; Steiner et al. 2014; Ward et al. 2011). However, it is noteworthy that chronic infusion of insulin has been reported to raise ABP in rodents (Brands et al. 1996). The lack of a robust pressor response in acute experiments may be attributed to several factors, including 1) insulin directly causes vasodilation (Manrique et al. 2014), which may negate a sympathetically mediated vasoconstriction; and 2) insulin selectively increases lumbar, but not renal, SNA. The latter effect has been repeatedly reported in our laboratory (Luckett et al. 2013; Ward et al. 2011) as well as in humans (Gudbjornsdottir et al. 1994). Acute increases in SNA to a single target tissue may not have a profound impact on ABP. In the present study, acute reversal of the lumbar sympathoexcitatory response to insulin by PVH blockade of glutamatergic receptors was associated with a fall in mean ABP. The changes in lumbar SNA paralleled the changes in mean ABP. This observation may suggest that insulin negated the lumbar sympathoexcitatory effect to increase ABP. Yet, removal of the elevated lumbar SNA left the vasodilatory actions of insulin unopposed, thereby reducing ABP.

In summary, the present findings demonstrate that glutamate receptor activation in the PVH contributes to insulin-induced sympathoexcitation and that these effects within PVH are mediated by a glutamatergic NMDA-driven pathway. Future experiments are needed to investigate how glutamate interacts with POMC neurotransmission in the PVH to regulate SNA and identify the source of glutamatergic drive.

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DISCLOSURES

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

S.D.S. and K.W.G. conception and design of research; S.D.S. and K.W.G. performed experiments; S.D.S. and K.W.G. analyzed data; S.D.S. and K.W.G. interpreted results of experiments; S.D.S. prepared figures; S.D.S. drafted manuscript; S.D.S. and K.W.G. edited and revised manuscript; S.D.S. and K.W.G. approved final version of manuscript.

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