Role of VMH ketone bodies in adjusting caloric intake to increased dietary fat content in DIO and DR rats

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Submitted 14 January 2015; accepted in final form 11 March 2015

Le Foll C, Dunn-Meynell AA, Miziorko HM, Levin BE. Role of VMH ketone bodies in adjusting caloric intake to increased dietary fat content in DIO and DR rats. Am J Physiol Regul Integr Comp Physiol 308: R872–R878, 2015. First published March 18, 2015; doi:10.1152/ajpregu.00015.2015.—The objective of this study was to determine the potential role of astrocyte-derived ketone bodies in regulating the early changes in caloric intake of diet induced-obese (DIO) versus diet-resistant (DR) rats fed a 31.5% fat high-energy (HE) diet. After 3 days on chow or HE diet, DR and DIO rats were assessed for their ventromedial hypothalamic (VMH) ketone bodies levels and neuronal ventromedial hypothalamic nucleus (VMN) sensing using microdialysis coupled to continuous food intake monitoring and calcium imaging in dissociated neurons, respectively. DIO rats ate more than DR rats over 3 days of HE diet intake. On day 3 of HE diet intake, DR rats reduced their caloric intake while DIO rats remained hyperphagic. Local VMH astrocyte ketone bodies production was similar between DR and DIO rats during the first 6 h after dark onset feeding but inhibiting VMH ketone body production in DR rats on day 3 transiently returned their intake of HE diet to the level of DIO rats consuming HE diet. In addition, dissociated VMN neurons from DIO and DR rats were equally sensitive to the largely excitatory effects of β-hydroxybutyrate. Thus while DR rats respond to increased VMH ketone levels by decreasing their intake after 3 days of HE diet, this is not the case of DIO rats. These data suggest that DIO inherent leptin resistance prevents ketone bodies inhibitory action on food intake.

Obesity and Type 2 Diabetes Mellitus are major worldwide public health issues (1, 2, 4, 12, 16, 19, 37, 38). Both obesity and Type 2 diabetes have important comorbidities that make it imperative to understand the underlying mechanisms that regulate food intake. Increased consumption of highly palatable, energy-dense foods, especially those rich in fats, represents a major cause of excess caloric intake (13). Indeed, a direct relationship exists between total fat intake and obesity (8). However, the effects and the mechanisms of chronic and excessive high-fat diet (HFD) consumption in the development of obesity are still poorly understood. Toward this end, we have used selectively bred diet-induced obese (DIO) rats as a model of human obesity (26, 27, 32) to assess the underlying factors that regulate their responses to high-energy (HE; 31.5% fat) diet intake. These rats are selectively bred to produce polygenically inherited diet-induced obesity or to remain diet resistant (DR) when fed an HE diet. DIO rats are larger but not fatter than DR rats when fed a low-fat chow diet but rapidly become hyperphagic, obese, and insulin resistant when fed an HE diet (27, 30). Most importantly, when chow-fed DIO and DR rats are fed an HE diet, both overeat for 3 days. Whereas DR rats then reduce their intake to chow-fed levels on day 3, DIO rats continue to overeat for 6–8 wk more, despite their early and persistent increase in leptin levels (29). In addition to these defects, we have previously shown that fatty acid (FA) sensing in ventromedial hypothalamic nucleus (VMN) neurons from DIO offspring were more affected by exposure of their dams to a HE diet during gestation and lactation than were those from similarly exposed DR dams (25).

Several studies have shown that food intake can be altered by ingestion of a HFD (9, 11, 20, 33, 39). Based on the knowledge that astrocytes are the major source of FA oxidation and the only source of ketone body production in the brain (6), we demonstrated that ventromedial hypothalamic (VMH) ketone body production during restricted ingestion of a very HFD (60%) inhibited caloric intake over a 6-h period (23). To further assess the importance of VMH ketone production during intake of a HFD during normal feeding, we utilized the DIO/DR rat model of early intake of HE diet to determine whether there was a differential production of or sensitivity to VMH ketone bodies that underlay the reduced intake of HE diet in DR but not DIO rats (29).

We postulated that, since DIO rats have abnormal neuronal VMH FA sensing (25) and fail to reduce their hyperphagia on HE diet on day 3 of intake (27, 29, 30), they will have defective VMH ketone production and/or neuronal ketone sensing compared with DR rats.

RESEARCH DESIGN AND METHODS

Animals. Animals were housed at 23–24°C on a reversed 12-h:12-h light-dark cycle (lights off at 0900). Male rats selectively bred to express the DR or DIO genotypes (27) were raised in our in-house colony and used for all studies. These colonies were originally derived from outbred Sprague-Dawley rats (Charles River Labs) following a breeding scheme as previously described (30). Briefly, the highest and the lowest weight gainers after 2 wk on HE diet were selected as breeding stock to produce the DIO and DR genotypes (31), respectively. These substrains have been maintained for almost 20 years in our vivarium with essentially no change in phenotype. In the current studies, litters were culled to 10 pups per dam on postnatal day 2 (P2) and weaned at P21 onto Purina Rat chow and water ad libitum. Purina Rat chow (no. 5001) contains 13.5% fat, 28.5% protein, and 58% carbohydrate as a percentage of total energy content. All work was in compliance with the Institutional Animal Care and Use Committee of the E. Orange Veterans Affairs Medical Center.

VMH β-hydroxybutyrate and feeding measurements. At 10–11 wk of age, DIO and DR rats (n = 8/group) had unilateral VMH guide cannulas (CMA 11, Harvard apparatus, Holliston, MA) and a jugular catheter implanted followed by 2 wk of recovery. Two days before microdialysis, they were fed ad libitum on HE diet containing 31.5%...
fat, 16.8% protein, and 51.4% carbohydrate as a percentage of total energy content (D12265B, Research Diet, New Brunswick, NJ). On the third day of the HE diet, at 0700, microdialysis probes [3-mm membrane length and 6-kDa pore size (CMA 11, Harvard Apparatus)] were inserted into the guide cannulas and perfused at 1.0 μl/min for 8 h with artificial cerebrospinal fluid (aCSF), and jugular catheters were connected. Microdialysis eluates and blood samples were collected every 30 min, and food intake was monitored continuously using the BioDAQ apparatus (Research Diets, New Brunswick, NJ).

A second set of DR rats (10–11 wk old, n = 8/group) were implanted with bilateral VMH guide cannulas and unilateral jugular catheters. After a 2-wk recovery period, rats were begun on the HE diet. On the third day of the HE diet, food was removed and bilateral microdialysis probes were inserted at 0700 and infused with aCSF + 0.4% DMSO vehicle or 30 μmol/l hymeglusin in aCSF + 0.4% DMSO at 1.0 μl/min for 2 h before lights off followed with aCSF for 6 h (n = 8/group). Hymeglusin is a 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) inhibitor (23, 36). The HE diet was returned at 0900 and eluates from the same microdialysis probes, and blood samples were collected at 30-min intervals for 8 h and analyzed for β-hydroxybutyric acid (β-OHB). Food intake was monitored continuously as above.

**Effects of glucose, oleic acid, and β-OHB on activity of dissociated DIO versus DR VMN neurons.** DIO and DR rats were weaned at P21 and fed either chow or HE diet for 3 days. P24 rats were perfused with an ice-cold oxygenated (95% O2-5% CO2) perfusion buffer (in mmol/l: 2.5 KCl, 1.25 NaH2PO4, 28.0 NaHCO3, 7.0 MgCl2, 0.5 CaCl2, 7.0 glucose, 1.0 ascorbate, 3.0 pyruvate, and 233 sucrose), the VMN was bilaterally punched from VMH slices, and neurons were dissociated as previously described (18, 22–24). A total of 5,000 neurons per group were removed if necessary as utilizing Systat software.

Statistics. With the use of Systat (Chicago, IL) and GraphPad Prism software (La Jolla, CA), one-way and two-way ANOVA and one-way ANOVA for repeated measures with post hoc Bonferroni corrections were carried out for the in vitro and in vivo studies. t-Tests were also performed for two-group comparisons. No more than two outliers per group were removed if necessary as utilizing Systat software.

**RESULTS**

**Dietary effects on blood and VMH ketone levels and food intake.** We postulated that DR rats reduce their intake of the HE diet after 3 days on HE diet due to an increase in VMH ketone body production, which overrides normal FA sensing, as seen in outbred rats, whereas DIO rats have reduced ketone body production. To test this hypothesis, DIO and DR rats were fed chow diet from weaning and, beginning at 10 wk of age, were fed HE diet ad libitum for 4 days, with serum and VMH levels of β-OHB determined on day 3 (Figs. 1 and 2). DIO rats increased their caloric intake of HE diet by 31% above their previous intake of chow after 1 day and consumed more calories over all 4 days after being switched to HE diet (F1,13 = 17.126, P = 0.003; Fig. 1A) with no significant change in intake on days 3 or 4. On the other hand, DR rats significantly increased their intake of the HE diet after 1 day by 41% above their previous intake of chow and then reduced their caloric intake by 42% of day 2 intake on the third day of HE diet intake (Fig. 1A). On day 3, DIO caloric intake was significantly greater than DR rats’ intake during both 3-h intervals after feeding onset and over the entire 24-h period (Fig. 1, B and C). After the feeding onset on day 3, serum β-OHB levels peaked at 1.5 to 2.5 h and 4.5 to 5.5 h in DR rats (Fig. 1D), whereas VMH β-OHB levels were transiently higher in DIO rats at 1 h after feeding onset (Fig. 1E). This resulted in VMH-to-serum ratio (VMH/serum) spikes between 1.5 and 2 h, and 4.5 and 5.5 h in DIO rats compared with DR rats with a transiently higher ratio in DR rats at 4 h (Fig. 1F). However, overall cumulative serum, VMH, and VMH/serum β-OHB levels did not differ between DIO and DR rats over the first and second 3-h intervals after feeding onset (Fig. 2).

To test the hypothesis that the generation of ketone bodies by VMH astrocytes exposed to the 31.5% fat HE diet was responsible for the decrease in DR rats caloric intake on day 3, DR rats underwent bilateral VMH reverse dialysis of hymeglusin to inhibit local ketone body production (23) for 2 h before feeding onset on day 3 of HE diet intake. Hymeglusin decreased the production of VMH ketone bodies over the first 3-h period (F1,13 = 29.14, P = 0.041) compared with vehicle in DR rats (Fig. 3B). VMH/serum β-OHB levels were also decreased during the first 1.5 h (Fig. 3C). In parallel with the decrease in VMH/serum β-OHB levels in hymeglusin-treated DR rats, there was an increase in caloric intake by 219% over the first 3-h period and by 195% over the second 3-h period after feeding onset compared with vehicle controls (Fig. 3D). This VMH β-OHB production inhibition resulted in cumulative caloric intake over the first 3-h interval that equaled that in DIO controls (Fig. 3E). As expected with the use of a self-limited pharmacological inhibitor of ketone body production, the increased food intake of DR rats treated with VMH hymeglusin was restored to control DR levels during the second 3-h period of feeding on day 3 (Fig. 3E), as well as on the fourth day when no hymeglusin was provided (Fig. 3D). Finally, for uncertain reasons, serum β-OHB levels were transiently decreased from 2 to 3 h after feeding onset in DR rats given VMH hymeglusin (Fig. 3A).

Taken as a whole, these data suggest that local VMH astrocyte ketone body production plays an important role in the reduction of caloric intake that occurs on the third day of ad libitum HE diet intake in DR rats, an effect that does not occur in DIO rats. However, since there were no major differences in VMH ketone body production between DR and DIO rats during the first 6 h of day 3, this suggested that there might have been differences in the sensitivity to ketone bodies in VMH metabolic-sensing neurons between DR and DIO rats.

**Effect of HE diet on fatty acid and ketone sensing in DIO and DR rats.** To test the hypothesis that DR and DIO rats’ VMH metabolic-sensing neurons display differential sensitivities to ketone bodies, we assessed the effects of a range of β-OHB concentrations on the activity of dissociated DR versus DIO VMN neurons at concentrations of glucose seen in the VMH under fed (2.5 mM brain glucose level) conditions from rats fed chow or HE diet for 3 days. These assessments were
made using calcium imaging in the presence of 15 nM OA to specifically target neurons responsive to glucose, FA, and β-OHB.

First, 3 days of HE diet intake led to 31% fewer GE neurons and 57% more GI neurons in DR rats, whereas it did not affect the number of DIO glucosensing neurons (Table 1). In the equivalent of the fed state (2.5 mM glucose, 15 nM OA), the major effects of prior intake of HE diet were seen in both the neuronal responses to FA and β-OHB primarily in DIO rats. While there were equivalent percentages of VMN neurons that were excited and inhibited by OA in chow-fed DR and DIO rats, after 3 days on HE diet, only DIO rats had a 59% increase in the percentage of GI neurons that were excited by OA (Table 1).

VMN neurons were next assessed for their responses to a range of β-OHB concentrations as a function of their FA-sensing properties (Table 2). Overall, β-OHB had a predominantly excitatory effect with VMN neurons being two to three times more excited than inhibited by β-OHB (Table 2, Fig. 4). When taking in account their FA-sensing properties, chow-fed DIO rats had significantly fewer OAE neurons that were excited by β-OHB than all other groups, and this deficit was essentially corrected by 3-day intake of HE diet. On the other hand, 3 days of HE diet intake in DIO rats reduced the...
percentage of OAI neurons excited by β-OHB compared with the other groups (Table 2). Next the effect of increasing concentrations of β-OHB (0.1 nM to 1 μM) on VMN neuronal FA sensing was assessed at 2.5 mM glucose and 15 nM OA. Most importantly, neither OAE nor OAI neurons demonstrated a concentration-response to β-OHB (Fig. 4). However, some individual effects were observed. At 0.1 nM β-OHB, neurons excited by OA from DIO rats fed the HE diet were 10-fold more excited by β-OHB than those from chow-fed DIO rats and two- to threefold more excited than those from DR rats fed either chow or HE diet (Fig. 4A). The same effect was also observed in OAI neurons that were inhibited by 1 nM β-OHB.

Table 1. Effects of 2.5 mM glucose and 15 nM oleic acid on dissociated VMN neurons from P24 male DR and DIO rats fed chow or HE diet for 3 days

<table>
<thead>
<tr>
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<th>DR Chow</th>
<th>DR HE</th>
<th>DIO Chow</th>
<th>DIO HE</th>
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<tbody>
<tr>
<td>GE 1 mmol/l Hymeglusin</td>
<td>13 ± 1*</td>
<td>9 ± 1**</td>
<td>14 ± 2**</td>
<td>10 ± 2**</td>
</tr>
<tr>
<td>GI 1 mmol/l Hymeglusin</td>
<td>7 ± 1*</td>
<td>11 ± 1**</td>
<td>10 ± 1**</td>
<td>9 ± 1**</td>
</tr>
<tr>
<td>Total</td>
<td>100 (632)</td>
<td>100 (546)</td>
<td>100 (678)</td>
<td>100 (692)</td>
</tr>
</tbody>
</table>

Data are means ± SE percentage of total neurons tested in each category; n = 8–10 rats/group. Dissociated ventromedial hypothalamic nuclei (VMN) neurons from diet-resistant (DR) and diet-induced obese (DIO) rats were assessed by fura-2 calcium imaging at P24. Neurons were first classified by glucosensing categories as glucose was changed from 2.5 to 0.5 to 2.5 mmol/l and then for fatty acid (FA) sensing to 15 mmol/l oleic acid (OA) at 2.5 mmol/l glucose. Neurons were classified as OA excited (OAE) or inhibited (OAI). GE, glucose excited; GI, glucose inhibited; HE, high energy Total, total percentage of each category of neurons at each glucose concentration, irrespective of their glucosensing properties with the number of neurons tested in each group divided by the total number tested in parentheses. *P < 0.05 one-way ANOVA. **P < 0.05 two-way ANOVA.
Table 2. Effect of 2.5 glucose and 15 nM of oleic acid and a range of β-hydroxybutyrate concentration on dissociated VMN neurons from P24 DR and DIO rats fed chow or HE diet for 3 days

<table>
<thead>
<tr>
<th></th>
<th>DR Chow</th>
<th>DR HE</th>
<th>DIO Chow</th>
<th>DIO HE</th>
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<tr>
<td>% of Total</td>
<td>β-OHBE</td>
<td>β-OHBI</td>
<td>β-OHBE</td>
<td>β-OHBI</td>
</tr>
<tr>
<td>OAE</td>
<td>28 ± 5</td>
<td>39 ± 4b</td>
<td>32 ± 5</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>OAI</td>
<td>15 ± 2</td>
<td>73 ± 7b</td>
<td>11 ± 6</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>Total</td>
<td>100 (632)</td>
<td>49 ± 4</td>
<td>18 ± 3</td>
<td>17 ± 5</td>
</tr>
</tbody>
</table>

Data are in percentage of total neurons ± SE tested in each category; n = 8–10 rats/group. At 2.5 mmol/l glucose, VMN neurons were classified as OAE or OAI by alterations in intracellular Ca²⁺ concentration ([Ca²⁺]) oscillations produced by exposure to 15 mmol/l OA. They were then exposed to a range of concentration β-hydroxybutyrate (β-OHB) from 0.1 mmol/l to 1 μmol/l in the presence of 15 mmol/l OA and were then classified as β-OHBE excited (β-OHBE) or inhibited (β-OHBI). Total, total percentage of each category of neurons for each β-OHB category, irrespective of their OA-sensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. *Data with differing superscripts within the same category differ from each other in at the P < 0.05 level after two-way ANOVA, followed by Bonferroni test.

This suggests that neuronal uptake of ketone bodies via MCT2 transporters (3, 10, 34, 35) is not a key regulatory step and that production of ATP and/or ROS from these ketone bodies in astrocytes utilizes FA to produce ketone bodies that reduce caloric intake back to control, chow-fed levels, whereas DIO rats continued their increased intake of HE diet. Contrary to our initial hypothesis, local VMH astrocyte ketone body production was similar between DIO and DR rats during the first 6 h after dark onset feeding. In addition, dissociated VMN neurons from DIO and DR rats were equally sensitive to the largely excitatory effects of β-OHB. Nevertheless, while DIO rats had continued hyperphagia, the increase in VMH β-OHB levels seen in DR rats was sufficient to reduce HE diet intake in DR rats on day 3. This was supported by their increased intake when VMH β-OHB production was inhibited with hymegulcin. Thus, whereas DR rats do seem to respond to increased VMH local ketone body production by decreasing their intake after 3 days of HE diet, this is not the case of DIO rats, which are equally as responsive to both the individual neuronal effects of ketone bodies and VMH astrocyte ketone body production on HE diet. This suggests that something else overrides what should be an inhibitory effect of VMH ketone bodies on HE diet intake in DIO rats.

Our previous studies suggest that the inherent leptin resistance of DIO rats (15, 26, 27, 29) might override the otherwise powerful inhibitory effect of VMH ketone production on HE diet intake seen in DR rats. We previously showed that despite a major increase in leptin after 3 days on the HE diet, DIO rats do not respond to this by decreasing their food intake (29). However, the overriding effect of leptin on ketone bodies sensing in DIO rats is hypothetical and needs to be further assessed. Regardless of the reason for the resistance of DIO rats to the inhibitory effect of VMH ketone bodies on HE diet intake, it is important to recognize the impressive role that they do play in reducing intake in the DR rats. At the single neuron level, β-OHB mostly overrode the actions of glucose and OA with a predominantly excitatory over inhibitory effect in both DIO and DR rats. Importantly, and contrary to our previous findings with both glucose (17) and FA (24), β-OHB produced its effects without a concentration-dependent responsiveness in dissociated neurons assessed in the absence of surrounding glial cells. Thus even very small concentrations (100 pmol/l) produced an almost maximal effect in many cases. This suggests that neuronal uptake of ketone bodies via MCT2 transporters (3, 10, 34, 35) is not a key regulatory step and that production of ATP and/or ROS from these ketone bodies in neurons is likely to be the main factor overriding both glucose and FA sensing (5, 24). On the other hand, 3 days of prior HE diet intake significantly altered VMN neuronal glucose sensing in GI neurons of DIO rats, while it had less to no impact in DR rats. HE diet intake also altered responsiveness to FA and β-OHB in DIO rats but not in DR rats. Yet, despite this increased responsiveness, DIO rats still failed to reduce their intake of HE diet supporting a role for other factors besides VMN neuronal metabolic sensing as regulators of the short-term intake of HE diet in DIO rats.

We do know that neuronal FA sensing in the arcuate plus VMN (VMH) is important during chronic intake of a HFD in DIO but not DR rats. Inhibiting FA sensing by depletion of the FA sensor CD36 causes DIO rats to become hyperphagic and obese on 45% fat diet, whereas VMH CD36 depletion has no effect on long-term intake of HFD in DR rats, even though they still become obese on such diets. Once again, these data support the contention that factors other than VMH neuronal metabolic sensing or responses to ketone bodies determine the short-term intake of HE diet in DIO rats, whereas local VMH ketone body production plays a major role in the early intake of HE diet in DR rats. On the other hand, VMH FA sensing, as mediated by CD36, appears to be an important regulator of the long-term intake of HFD in DIO but not DR rats. This may possibly be due to the fact that high-fat intake selectively alters the responsiveness of VMH neurons to FA in DIO but not DR rats.
In conclusion, this study show that, in DR rats, local ketone body VMH production associated to normal ketone body sensing after 3 days on HE diet is sufficient to decrease HE diet intake to the levels of Chow diet intake. However, in DIO rats, even though their ketone bodies VMH levels and sensing are similar to DR rats, it is not sufficient to override their inherent leptin resistance that could prevent them from decreasing their HE diet intake.

Perspectives and Significance

The increased consumption of palatable, HFD contributes to the excess caloric intake that leads to the development of obesity. Thus it is important to understand the mechanism underlying the relationship between HFD consumption and the regulation of feeding. We have shown that specialized hypothalamic metabolic-sensing neurons respond to changes in ambient brain levels of substrates such as glucose, FA, and ketone bodies as signaling molecules to alter their activity (17, 22, 23). Using a restricted feeding schedule (3 h/day) of a 60% fat diet in outbred rats, we previously demonstrated that there was a delayed inhibition of intake and that this inhibition was reversed by transiently inhibiting local VMH astrocyte production of ketone bodies (23). The current studies were initiated to examine the potential role of VMH ketone body production in modulating ad libitum intake of a diet of much lower (31.5%) fat content. We chose the selectively bred DIO/DR model because of our previous finding that DR, but not DIO rats, reduce their intake of this diet to low-fat control levels after only 3 days (29). Our hypothesis was that DR rats might either have increased levels of VMH ketone body production and/or that their VMH metabolic-sensing neurons were more sensitive to the largely excitatory effects of ketone bodies than were those in DIO rats. In fact, neither of these postulates was correct, even though inhibiting VMH ketone production clearly did prevent DR rats from reducing their intake of 31.5% fat HE diet to control levels on day 3. Since we know that DIO rats are inherently leptin resistant (7, 14, 26, 28) and fail to reduce their intake for up to 6–8 wk after onset of HE diet intake, despite very high leptin levels (29), our findings here strongly suggest that VMH ketone bodies and fatty acid-sensing neurons clearly are important regulators of feeding, energy, and glucose homeostasis in rats with normal leptin sensitivity (22, 23). However, with or without obesity the presence of underlying leptin resistance appears to override normal VMH fatty acid and ketone body sensing in the regulation of feeding in the early response to increase in dietary fat content. Thus there appears to be a hierarchy of control mechanisms regulating feeding in which normal metabolic sensing is dependent on normal leptin sensitivity.

ACKNOWLEDGMENTS

The authors thank Sunny Lee, Antoinette Moralisvili, and Charlie Salter (all VA Medical Center) for technical assistance. Hymeglusin was kindly supplied by H. Miziorko.

GRANTS

This work was supported by the Research Service of the Department of Veterans Affairs (to B. E. Levin and A. Dunn-Meynell) and by the National Institute of Diabetes and Digestive and Kidney Diseases (DR-53181 to B. E. Levin).
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