Small Decrements in Systemic Glucose Provoke Increases in Hypothalamic Blood Flow Prior to the Release of Counterregulatory Hormones

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ABSTRACT

Objective: The hypothalamus is the central brain region responsible for sensing and integrating responses to changes in circulating glucose. The aim of this study was to determine the time sequence relationship between hypothalamic activation and the initiation of the counterregulatory hormonal response to small decrements in systemic glucose.

Research Design and Methods: Nine non-diabetic volunteers underwent two hyperinsulinemic clamp sessions in which pulsed arterial spin labeling (PASL) was used to measure regional cerebral blood flow (CBF) at euglycemia (~95mg/dl) on one occasion and as glucose levels were declining to a nadir of ~50mg/dl on another occasion. Plasma glucose and counterregulatory hormones were measured during both study sessions.

Results: CBF to the hypothalamus significantly increased when glucose levels decreased to 77.2 ± 2 mg/dl when compared to the euglycemic control session when glucose levels were 95.7 ± 3 mg/dl (p=0.0009). Hypothalamic perfusion was significantly increased before there was a significant elevation in counterregulatory hormones.

Conclusions: Our data suggest that the hypothalamus is exquisitely sensitive to small decrements in systemic glucose levels in healthy, non-diabetic subjects and that hypothalamic blood flow, and presumably neuronal activity, precedes the rise in counterregulatory hormones seen during hypoglycemia.

Abbreviations:
ACC = anterior cingulate cortex
BA = Broadman’s Area
PG = plasma glucose
PASL = pulsed arterial spin labeling
CBF = cerebral blood flow
fMRI = functional magnetic resonance imaging
IFG = inferior frontal gyrus
ROI = region of interest
STG = superior temporal gyrus
The brain relies on glucose as its main energy substrate and small decrements in circulating glucose provoke an elaborate counterregulatory hormonal feedback response (1, 2). Activation of the counterregulatory response requires effective detection of a falling glucose level. While multiple glucose sensors may be involved (3,4,5,6,7) the hypothalamus has emerged as the dominant brain region responsible for sensing and integrating responses to changes in circulating glucose levels (8,9,10,11,12). While most prior studies have relied on animal models to study the neurophysiological response to changes in glucose, newer imaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) provide an in vivo method to study the effect of changes in peripheral glucose levels on human brain activity. Several fMRI studies in humans have demonstrated that a rise in systemic glucose following glucose ingestion leads to an inhibition of hypothalamic activity (13, 14, 15, 16). In addition, Musen et al (17) recently used fMRI based on the Blood Oxygenation Level Dependent (BOLD) contrast mechanism and found that insulin-induced hypoglycemia leads to hypothalamic activation. However, the fMRI-BOLD approach used in that study assesses only relative changes in oxygenated hemoglobin in specific brain regions and does not directly measure tissue perfusion. MRI pulsed arterial spin labeling (PASL) provides a method for measuring absolute blood flow responses throughout the brain to changes in circulating glucose levels. PASL magnetically tags the arterial blood prior to entering the brain and then examines the transit time for the tagged blood to reach specific tissues thereby providing a direct measure of CBF (18, 19, 20). Increased CBF has long been associated with neuronal activation dating back to the first PET studies of brain function (21).

Some studies, using positron emission tomography (22), single photon emission computed tomography (23), high field MR perfusion (24), and continuous arterial spin labeling (25) have shown region specific increases in brain CBF in response to hypoglycemia. However, none of these studies specifically demonstrated changes in blood flow to the hypothalamus during hypoglycemia. Moreover, in these studies CBF measurements were performed after hypoglycemic levels had been achieved; thus none of these studies determined the CBF response to smaller decrements in systemic glucose or the relationship between regional brain activation and the initiation of the counterregulatory hormonal response.

We employed PASL to determine the effect of small decrements in circulating glucose on hypothalamic blood flow in healthy volunteers. We performed CBF measurements as glucose levels were declining. This approach allowed us to address the following questions: 1) Does a decline in plasma glucose provoke hypothalamic activation? 2) If so, what is the plasma glucose level that correlates with hypothalamic activation? 3) How does hypothalamic activation temporally relate to the counterregulatory hormone response to hypoglycemia?

An understanding of how the hypothalamus, a key brain glucose sensing region, responds to decrements in circulating glucose levels in healthy humans provides critical information that can be used to determine how metabolic
disorders, such as diabetes, may alter this response.

**Research Methods and Design**

**Subjects.** Nine (8 male, 1 female) healthy, non-diabetic subjects participated in this study. Subject participants had a mean age (±SD) of 28±5 and a mean BMI of 23.6±2. Subjects underwent a screening history, physical examination, and laboratory testing and only individuals with no history of significant disease, including diabetes, were included in the study. Exclusion criteria also included any contraindications for MRI including pregnancy and metal implants. Before each study session, female subjects were required to have a negative urine pregnancy test. The Yale University School of Medicine Human Investigation Committee approved this study and all subjects provided informed, written consent before participation in the study.

**General Experimental Protocol.** Subjects participated in two study sessions which were separated by a minimum of 7 days. On the morning of the study, an IV catheter was inserted into a distal arm or hand vein; this arm was gently heated, allowing for sampling of arterialized venous blood. A second IV catheter was established for the administration of insulin and glucose. During the 135 minute study sessions, a primed continuous infusion of IV insulin at 2mu/kg/min was initiated, with a variable infusion of 20% glucose adjusted to achieve euglycemia (plasma glucose (PG) ~95mg/dl) on one occasion and hypoglycemia (PG ~ 50mg/dl) on the other occasion. Regional CBF measurements were performed using PASL at approximately 30 minutes after the start of the plasma glucose decline toward hypoglycemic levels and at ~90 minutes during the euglycemic session. PG levels were measured at 5 minute intervals and additional plasma samples were drawn at -20, 0, 30, 60, 120, and 135 minutes for measurement of insulin, glucagon, catecholamines, cortisol, and growth hormone. C-peptide was measured at -20, 0, 30, and 60 minute time points. The two study sessions were carried out in a single-blind fashion, in variable order across subjects.

**fMRI acquisition methods.** **Imaging Protocol.** MR Imaging was performed on a 3T Siemens Trio whole-body scanner (Siemens Medical Systems, Erlangen, Germany) with a circularly polarized head coil. Pulsed arterial spin labeling (PASL) utilizing the EPISTAR QUIPSS PASL MRI technique was used to measure CBF. The PASL acquisition parameters were: field of view = 256 × 256 mm2; matrix = 60 × 64; bandwidth = 2298 Hz/pixel; slice thickness = 6 mm; inter-slice spacing = 3 mm. Ten AC-PC aligned slices were acquired from inferior to superior in an ascending order. The whole imaging slab was positioned on the upper part of the brain, with the lowest slice passing through AC-PC to acquire the top part of the brain; the imaging slab positioned on the lower part of the brain with the 7th slice from the bottom passing through AC-PC to acquire the bottom part of the brain. Acquisition of each slice took approximately 54 ms. The repetition time was $TR = 3000$ ms; the echo time was $TE = 26$ ms. During each EPI acquisition, fat was suppressed and the phase-correction echoes were collected and applied. A bipolar gradient of encoding velocity $V_{enc} = 20$ mm/s was applied to the imaging slices for intra-vascular signal suppression.

To quantify regional CBF for both upper and lower parts of the brain, two volumes of 10 proton density weighted
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images were acquired with the same perfusion sequence, except for the following changes: \( TR = 8000 \text{ ms} \); \( TD = 0 \text{ ms} \); \( TI = 7375 \text{ ms} \) and \( TE = 26 \text{ ms} \). Mapping for the apparent longitudinal relaxation time \( T_{app} \) was performed with an ultra-fast Look-Locker echo-planar imaging T1 mapping sequence.

Two additional image acquisitions were acquired to aid in multi-subject registration. First, a high-resolution whole brain T1-weighted 3D image was acquired for each subject using MPRAGE (Magnetization Prepared Rapid Acquisition with Gradient-Echo imaging), with the following settings: 160 sagittal slices with FOV = 256 × 256 mm\(^2\); voxel size = 1 × 1 × 1 mm\(^3\); TR = 1500 ms; TI = 800 ms; TE = 2.83 ms; flip angle 15 degrees; and one average. Second, 2D T1-weighted images were acquired during each MR session using the same slice positions as the perfusion-weighted images and the following additional settings: FOV = 256 × 256 mm\(^2\); in-plane resolution 1 × 1 mm\(^2\); TR = 300 ms; TE = 3.69 ms; flip angle 60 degrees; and two averages.

**Imaging analysis.**

a) Preprocessing of PASL images. Perfusion-weighted and the proton-density weighted images were motion-corrected using the Statistical Parametric Mapping package (SPM99), via a 6-parameter rigid-body transformation.

b) Calculation of absolute CBF. Perfusion-weighted images were obtained by pair-wise “surround” subtraction between interleaved label and control pairs (18, 19, 20) while the subject was at rest, resulting in a complete perfusion map every 2TR, i.e. 4 sec in our case. One perfusion-induced volume (\( \Delta M \)) was calculated by performing the subtraction described in Eq. 1 below between the tagged and untagged images. The mean image of the motion-corrected proton density images, \( M_0^* \), was also used for CBF mapping. The absolute CBF \( f \) (ml/100g/minute) was calculated as:

\[
\Delta M(t) = M^\text{ctrl}(t) - M^\text{label}(t) = \frac{2cf(t - \tau_a)M_0^*}{\lambda}e^{-t/T_{1a}}
\]

(1)

in which,

\( \lambda \) is the tissue-blood partition coefficient for water;

\[
c = \alpha_p \frac{1 - e^{-(t - \tau_a)1/T_{app} - 1/T_{1a}}}{(t - \tau_a)(1/T_{app} - 1/T_{1a})}
\]

(2)

is the correction factor, which accounts for exchange of labeled magnetization from intravascular to extravascular space and clearance of the labeled blood water out of the capillary bed;

\( \tau_a \) is the arterial transit time, which is the time for the labeled blood water to arrive at the capillary bed after labeling, and

\[
M_0^* = M_0 e^{-TE/2T_{2*}}
\]

(3)

\( T_{app} \) is the apparent longitudinal relaxation time, which was estimated with an ultrafast Look-Locker echo-planar imaging T1 mapping sequence (26).

Other parameters used in CBF quantification: \( T_{1a} = 1490 \text{ ms} \), \( \lambda = 0.9 \text{ ml/g} \), \( \alpha_p = 0.95 \), \( t - \tau_a = 700 \text{ ms} \), and \( TI = 1400 \text{ ms} \) for the first slice.

The activation maps were obtained by contrasting the average CBF images for two conditions. After \( M_0^*, T_{app}, \) and
ΔM have been measured on a per-voxel basis, CBF (f) can then be estimated using Equation (1).

c) **Multisubject analysis.** A standard whole brain template (MNI-1mm) was used for subject spatial normalization of the individual data. Subject integration and registration were carried out using the BioimageSuite software package (bioimagesuite.org [27]) for the PASL images under conditions of eu- and hypoglycemia. Two transformations were calculated and used in multiple subject integration: (1) an affine transformation was estimated by co-registering the 2D anatomical image to the high-resolution 3D anatomical image of each individual, and this was then used to transform the individual maps of the resting state CBF to the high-resolution 3D anatomical space of that subject; (2) a non-linear transformation was used to co-register the high-resolution 3D anatomical image of each individual to the brain template, which enabled warping of all the transformed maps of an individual subject from step (1) to a common brain space. Tri-linear interpolation was employed for image re-gridding. The mean, standard deviation, and other statistics were estimated in the common template space on the pooled-subject data. In this common reference space, voxel-wise contrasts between conditions (eu- and hypoglycemia) were estimated in the common space on the pooled-subject data using a t-statistic to test the null hypothesis. Region of interest analysis was performed on the hypothalamus.

**Laboratory analyses.** Plasma glucose was measured by an enzymatic reaction using glucose oxidase (Yellow Springs Instruments, Yellow Springs, OH). Plasma concentrations of insulin and glucagon were measured with the use of double-antibody radioimmunoassay (RIA) kits (Millipore, St. Charles, MO). Plasma epinephrine and norepinephrine were measured by high performance liquid chromatography (ESA, Chelmsford, MA). Plasma growth hormone (GH) and cortisol were measured by RIA (Irvine, CA; Diagnostic Products Corp, Los Angeles, CA), plasma C-peptide by use of double-antibody radioimmunoassay (RIA) kits (Diagnostic Products Corp, Los Angeles, CA).

**Results**

**Plasma glucose and glucoregulatory hormone concentrations.** Plasma glucose levels were not significant different at baseline prior to the euglycemic and hypoglycemic clamp sessions (Fig. 1 and Table 1). In the euglycemic hyperinsulinemic session plasma glucose levels were indistinguishable from baseline values at the time of brain perfusion acquisition. In the hypoglycemic hyperinsulinemic session plasma glucose was gradually lowered over 60 minutes and then was maintained at 52.8 ± 0.6 mg/dl for the remainder of the study (Fig. 1). During the hypoglycemic session, the brain perfusion acquisition measurements were obtained at the time of the slow glucose decline (30 min) when plasma glucose averaged 77.2 ± 2 mg/dl. During the euglycemic session brain perfusion measurements were performed at ~90 minutes when plasma glucose averaged 95.7 ± 3 mg/dl. As shown in Fig. 1, steady state plasma insulin levels during the hypoglycemic and euglycemic sessions were not significantly different (123±9 vs124±10 μU/ml, respectively). At the time that brain perfusion measurements were obtained neither plasma epinephrine, norepinephrine, cortisol nor growth hormone were altered during the hypoglycemic or euglycemic...
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clamp study but glucagon was reduced from baseline during the euglycemic session (Fig. 2 and Table 1). Subsequently, hypoglycemia provoked an increase when compared to the euglycemic session in plasma epinephrine (56±15 vs 15±3 pg/ml, p=0.02 at 60 min), glucagon (88±13 vs 49±7 pg/ml, p=0.006 at 90 min) and growth hormone (14.97±4 vs 1.91±0.22 ng/ml at 90 min, p=0.01). Small increases in plasma cortisol and norepinephrine were observed at 120 and 135 minutes, respectively (p<0.05).

In contrast to the counterregulatory hormones, the small decline in plasma glucose at 30 minutes caused a significant fall in plasma C-peptide concentration from 0.47±0.02 to 0.34±0.02 pmol/ml (p<0.001). There was a further reduction in C-peptide to 0.19±0.02 by 60 min. In contrast, C-peptide did not significantly change during the euglycemic session.

Regional cerebral blood flow (CBF) response. Figure 3 shows the whole brain mean difference map for the hypoglycemic as compared to the euglycemic session (p<0.05, uncorrected) that was obtained when plasma glucose averaged 77.2+/−2 mg/dl. This mild decrease in plasma glucose caused a significant increase in hypothalamic blood flow. Region of interest analysis demonstrated that mean hypothalamic perfusion was 2-fold greater during the hypoglycemic session (44.523 ml/100g/min) than the euglycemic session (21.990 ml/100g/min) (P=0.0009) (Fig. 4). The right anterior cingulate cortex (ACC), left caudate, left putamen, left superior temporal gyrus (STG), left inferior frontal gyrus (IFG), and left visual association cortex (BA 18) also exhibited increased blood flow, whereas the cerebellum, right pars opercularis (BA 44), and right medial frontal gyrus (BA 46) exhibited decreased blood flow during the hypoglycemic session compared to the euglycemic session (p<0.05) (see Table 2). We used the orbitofrontal cortex (OFC) as a control region and found no difference in cerebral blood flow to either the right OFC (hypoglycemia: 16.229 ml/100g/min; euglycemia: 18.509 ml/100g/min, p=0.35) or left OFC (hypoglycemia: 21.439 ml/100g/min; euglycemia: 18.261 ml/100g/min, p=0.2) during the hypoglycemic session when compared to euglycemic session.

Discussion

We used PASL to quantify hypothalamic perfusion after small decrements in circulating glucose in healthy human volunteers. PASL is an indirect measure of neuronal activity believed to reflect changes in metabolic state since there is a clear relationship between changes in the local rate of oxygen consumption and changes in local tissue blood flow (20). Although previous studies have investigated the effects of hypoglycemia on CBF in non-diabetic subjects, the current study examined the effects of small glucose decrements within the normal range, whereas earlier studies measured CBF after moderate hypoglycemic levels (PG < 60mg/dl) were achieved (22,24,25). As a result, we were able to investigate the time sequence relationship between hypothalamic activation and the initiation of the counterregulatory hormonal response.

We focused our attention on the hypothalamic blood flow response to decrements in systemic glucose based on earlier animal studies demonstrating the importance of hypothalamic glucose sensing neurons in hypoglycemia detection and in the activation of counterregulatory hormonal responses.
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Moreover, the hypothalamus plays a central role in the regulation of appetite and energy expenditure, responses known to be stimulated by hypoglycemia. Our data are consistent with this hypothesis as well as prior studies using fMRI-BOLD demonstrating that the hypothalamus is responsive to changes in systemic glucose levels (13,14,15,16,17). On the other hand, previous studies investigating the effects of hypoglycemia on regional CBF in non-diabetic subjects have not identified an increase hypothalamic blood flow following insulin-induced hypoglycemia (22,24,25). This may be because the hypothalamus is a very small brain region, making it difficult to detect significant changes in blood flow when compared to larger brain regions. We used PASL, which may have given us greater spatial resolution than other methods for measuring CBF, such as PET. In addition, unlike previous studies, we used region of interest analysis focusing on the hypothalamus, which may also have contributed to some of the differences in regional CBF results in our study.

It is noteworthy that CBF increased in the hypothalamus following a very small reduction in glucose (from 93.1±3 to 77.2 ±2 mg/dl) when compared to a euglycemic control study. Our data are consistent with those of Musen and colleagues who used fMRI-BOLD and found a slightly lower glucose threshold (PG 68 ± 9 mg/dl) for hypothalamic activation in non-diabetic individuals (17). However in that study the relationship between the onset of hypothalamic activation and systemic hormone release was not examined. The current data show that hypothalamic perfusion was significantly increased before glucose levels reached a point at which there was a significant elevation of counterregulatory hormones. Since the acquisition of CBF measurements was limited to only one time point during the euglycemic and hypoglycemic clamp sessions, measurements of regional CBF at multiple time points during euglycemic and hypoglycemic clamp sessions will be required for more precise time courses of hypothalamic activation and the counterregulatory hormonal response. A potential limitation of the current study is that CBF measurements were performed at 30 minutes during the hypoglycemic session and ~90 minutes during the euglycemic session. It is noteworthy, however, that plasma insulin levels were higher at the 90 minute time point during the euglycemic session when compared to the 30 minute time point during the hypoglycemic session (124±10 vs 90+13 mU/ml). Insulin has known vasoactive effects, and a recent report by Seaquist et al shows that insulin has direct vasodilatory effects on cerebral vasculature in humans that are independent of effects on neuronal activation (28). This suggests that higher circulating insulin levels in the euglycemic study session may increase regional cerebral blood flow which would be expected to attenuate the results we observed in our study. Therefore, it is unlikely that the changes in hypothalamic CBF we observed are due to measurement time differences. As has been previously reported (2) we observed a fall in endogenous insulin secretion, as measured by C-peptide, before there was a detectable rise in counterregulatory hormones. This occurred at plasma glucose levels that were similar to those that stimulated hypothalamic blood flow. While this finding does not establish a causal relationship between hypothalamic activation and suppression of
endogenous insulin secretion, it raises the possibility that these may be coordinated events. Whether there is a neural or hormonal cue that may act to coordinate hypothalamic activation and suppression of endogenous insulin secretion as glucose levels are declining is unclear and will require further investigation.

In summary, the present analysis suggests that the hypothalamus, a key central glucose sensing region, is exquisitely sensitive to small decrements in systemic glucose levels and that hypothalamic blood flow, and presumably neuronal activity, precedes the rise in counterregulatory hormones seen during hypoglycemia. These data lay the groundwork for future studies to determine how metabolic disorders, such as diabetes, alter the hypothalamic response to changes in systemic glucose.

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Figure 1. A) Plasma glucose levels (in mg/dl) obtained during the euglycemic (diamonds) and hypoglycemic (squares) clamp sessions. B) Insulin levels were raised comparably during euglycemic and hypoglycemic clamp sessions.
Figure 2. Plasma epinephrine and glucagon response during hyperinsulinemic euglycemic (diamonds) and hypoglycemic (squares) sessions. * represents P<0.05 hypoglycemic vs euglycemic session.
**Figure 3.** Saggital (left), coronal (middle), and axial (right) images showing the mean difference (CBF euglycemia – CBF hypoglycemia) map of CBF from nine subjects. Blue represents increased and yellow/orange represents decreased blood flow during the hypoglycemic relative to euglycemic session. During hypoglycemia, flow was greater to the hypothalamus and left inferior frontal gyrus (IFG) and less to the cerebellum (other regions are noted in table 1) (P<0.05, uncorrected).
**Figure 4.** Region of interest analysis showing that blood flow to the hypothalamus was significantly higher during hypoglycemia than euglycemia (p=0.0009).
Table 1. Mean (± SE) plasma glucose and counterregulatory hormones at baseline and time of CBF acquisition for hypoglycemic and euglycemic clamp sessions and peak levels for the hypoglycemic session.

|                  | Hypoglycemic Session | Euglycemic Session |
|------------------|----------------------|--------------------|
|                  | Baseline             | Time of CBF        | Peak               | Baseline | Time of CBF |
|                  |                      | acquisition        |                    |          | acquisition |
| Glucose (mg/dl)  | 93.1 ± 3             | 77.2 ± 2 *†        | -----              | 94.3 ± 4 | 95.7 ± 3    |
| Epinephrine (pg/ml) | 16 ± 4             | 14 ± 4              | 178 ± 36*         | 13 ± 3   | 14 ± 3      |
| Norepinephrine (pg/ml) | 120 ± 13          | 124 ± 11            | 169 ± 15*         | 136 ± 15 | 123 ± 10    |
| Glucagon (pg/ml) | 67 ± 9               | 65 ± 10†            | 117 ± 20*         | 70 ± 7   | 50 ± 8*     |
| Cortisol (mg/dl) | 12.9 ± 1.8           | 9.9 ± 1.4           | 16.9 ± 1.5        | 12.2 ± 1.8 | 12.1 ± 1.0 |
| Growth hormone (ng/ml) | 1.8 ± 0.3       | 1.7 ± 0.2           | 21.3 ± 5.4*       | 3.1 ± 1.0 | 2.9 ± 0.7   |

* p<0.05 vs baseline  
† p<0.05 hypoglycemic vs euglycemic session at time of CBF acquisition
Table 2. Talairach coordinates for areas showing increased or decreased activation during hypoglycemia relative to euglycemia at a threshold of p<0.05, uncorrected.

| Brain Region                        | BA | Talairach Coordinates X | Y | Z | Response to hypoglycemia |
|-------------------------------------|----|--------------------------|---|---|--------------------------|
| Left hypothalamus                   |    | -3                       | -6 | -8 | Activation               |
| Right hypothalamus                  |    | +3                       | -6 | -8 | Activation               |
| Right anterior cingulate cortex     | 24/32 | 4                       | 37 | 4 | Activation               |
| Left caudate                        |    | -10                      | 11 | 8  | Activation               |
| Left putamen                        |    | -23                      | -8 | 8  | Activation               |
| Left superior temporal gyrus        | 22 | -54                      | 0  | -4 | Activation               |
| Left pars triangularis              | 45 | -34                      | 24 | 8  | Activation               |
| Left inferior frontal gyrus         | 47 | -35                      | 38 | -12| Activation               |
| Left visual association cortex      | 18 | 19                       | -74| 28 | Activation               |
| Right pars opercularis              | 44 | 50                       | 3  | 20 | Deactivation             |
| Cerebellum                          |    | 2                        | -48| -16| Deactivation             |
| Right medial frontal gyrus          | 46 | 38                       | 40 | 16 | Deactivation             |
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