The robust neuroendocrine counterregulatory responses induced by hypoglycemia protect the brain by restoring plasma glucose, but little is known about molecular responses to hypoglycemia that may also be neuroprotective. To clarify these mechanisms, we examined gene expression in hypothalamus, cortex, and liver 3 h after induction of mild hypoglycemia by a single injection of insulin, using cDNA microarray analysis and quantitative real-time PCR. Real-time PCR corroborated the induction of six genes (angiotensinogen, GLUT-1, inhibitor of xB, inhibitor of DNA binding 1 [ID-1], Ubp41, and mitogen-activated protein kinase phosphatase-1 [MKP-1]) by insulin-induced hypoglycemia in the hypothalamus: five of these six genes in cortex and three (GLUT-1, angiotensinogen, and MKP-1) in liver. The induction was due to hypoglycemia and not hyperinsulinemia, since fasting (characterized by low insulin and glucose) also induced these genes. Four of these genes (angiotensinogen, GLUT-1, ID-1, and MKP-1) have been implicated in enhancement of glucose availability, which could plausibly serve a neuroprotective role during acute hypoglycemia but, if persistent, could also cause glucose-sensing mechanisms to overestimate plasma glucose levels, potentially causing hypoglycemia-induced counterregulatory failure. Although using cDNA microarrays with more genes, or microdissection, would presumably reveal further responses to hypoglycemia, these hypoglycemia-induced genes represent useful markers to assess molecular mechanisms mediating cellular responses to hypoglycemia. 

Diabetes 54:952-958, 2005
**Table 1: Blood glucose**

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1: microarray</strong></td>
<td></td>
</tr>
<tr>
<td>Fed A</td>
<td>7.75 ± 0.11</td>
</tr>
<tr>
<td>Fed B</td>
<td>8.20 ± 0.60</td>
</tr>
<tr>
<td>Fed C</td>
<td>9.51 ± 0.53</td>
</tr>
<tr>
<td>Fed D</td>
<td>10.11 ± 0.34</td>
</tr>
<tr>
<td>Fed E</td>
<td>9.91 ± 0.98</td>
</tr>
<tr>
<td>Hypo A</td>
<td>1.19 ± 0.05*</td>
</tr>
<tr>
<td>Hypo B</td>
<td>1.34 ± 0.08*</td>
</tr>
<tr>
<td>Hypo C</td>
<td>1.73 ± 0.13*</td>
</tr>
<tr>
<td>Hypo D</td>
<td>1.54 ± 0.10*</td>
</tr>
<tr>
<td>Hypo E</td>
<td>1.84 ± 0.18*</td>
</tr>
<tr>
<td><strong>Experiment 2: real-time PCR</strong></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>8.24 ± 0.15</td>
</tr>
<tr>
<td>Fasted</td>
<td>6.54 ± 0.15*</td>
</tr>
<tr>
<td>Hypo</td>
<td>1.17 ± 0.02*</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P ≤ 0.05 by ANOVA.

**Research Design and Methods**

All studies were approved by the appropriate institutional animal review board (Institutional Animal Care and Use Committee). Two-month-old male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and individually housed with free access to food and water under 12:12-h light-dark cycle (lights on at 7:00 A.M.). In the first study, mice were divided into two groups (n = 20 mice per group): euglycemic and hypoglycemic. Hypoglycemia was produced by 2 units/kg body wt insulin injected intraperitoneally into mice previously fasted for 48 h, a protocol that produced blood glucose <2.0 mmol/l from 30 min after injection until the time they were killed 3 h after injection, without producing unconsciousness, seizures, or death. This study was followed by a second study that compared gene expression (assessed by real-time PCR) of euglycemic mice with gene expression in ad libitum–fed mice made hypoglycemic by an intravenous injection of insulin (7 units/kg body wt), which also produced blood glucose <2.0 mmol/l from 30 min to 3 h after injection; gene expression was also assessed in a separate group of fasted mice (for 48 h) (n = 8 per group). In all cases, mice were killed 3 h after injection of insulin (or saline for euglycemic mice). For both studies, mice were killed following a balanced design at the start of the light period (10:00 A.M. to 2:00 P.M.). Mice were killed by decapitation after a brief exposure to carbon dioxide. Hypothalamic and cortical areas, along with peripheral tissues, were quickly removed, frozen on dry ice, and stored at −70°C until extraction of RNA.

**Blood chemistry.** Blood glucose was measured by a Glucometer Elite glucose meter (Bayer, Mountain View, CA) (Table 1).

**DNA microarray analysis.** Total RNA was extracted from tissue using TRIzol (Life Technologies, Carlsbad, CA). To minimize individual variation as a source of gene expression variance, 20 mice per condition were used, and 5 μg RNA from 4 mice was pooled into each of 10 samples (five pools representing 20 mice from each condition). Pooled samples from each condition were separately labeled using the Atlas Indirect labeling kit (Clonetech Laboratories, Palo Alto, CA) with Cy3 for euglycemic and Cy5 for hypoglycemic samples; one sample from each condition was then mixed with one sample from the other condition. Thus, the microarray data were based on five paired comparisons of euglycemic versus fasted/hypoglycemic hypothalamic samples.

The DNA microarray and protocol used have been described previously (12–14). Array pre-hybridization was carried out in 6 × sodium chloride–sodium citrate (SSC), 0.5% SDS, and 1% BSA at 42°C for 45 min. The probe was then denatured and hybridized in 24 μl of 50% formamide, 0.12 SSC, 0.5% SDS, 5 × Denhardt’s with 2.4 μg salmon sperm DNA, and 10 μg poly dA at 42°C for 16 h. The arrays were each washed for 10 min in 0.1× SSC, 0.1% SDS, and twice in 0.1× SSC, after which they were scanned using the GMS 418 scanner (Affymetrix, Santa Clara, CA) and analyzed by GenePix (Axon Instruments, Union City, CA).

The microarray itself comprises 956 cDNAs, 155 of which were of cDNAs identified in the Soares Mouse (NMHY) hypothalamic expressed sequence tag (EST) library and chosen after a BLAST search indicated that the EST was previously uncharacterized and expressed primarily in hypothalamic libraries as opposed to other EST libraries. About 100 more of these genes were chosen on the basis of likely metabolic regulation and obtained as IMAGE clones from Research Genetics. Using these chips, a set of validated criteria were developed to minimize false-positive results (13). These criteria included minimum variability of the triplicate spots of each gene on each microarray, a minimum level of expression (>1% of the median gene expression) and a requirement that the induction be at least 30% in every pairwise comparison (5/5) as well as meet the criteria for reproducibility and level of expression as described (13).

**Primer design.** Primers were designed according to criteria outlined in Yuen et al. (14) using MacVector 7.0 software (Accelrys, Norwalk, CT). The design emphasized a bias toward the 3’ end of the transcript, a GC content between 45 and 55%, and a single amplification band between 100 and 300 bp. All primers were tested beforehand with (nonquantitative) PCR to optimize reaction conditions. Primer sets producing multiple bands were discarded and redesigned. Primer sets were successfully designed for 17 genes in this study.

**Real-time PCR.** In brief, the quantitative RT-PCR procedure was as previously described (12,14). Briefly, 5 μg total RNA from each original sample was converted into cDNA, with 100 μg utilized for each individual real-time PCR assay in a 40-cycle three-step PCR using the ABI Prism 7900 thermocycler with 384-well thermal cycler block module and robot arm. Amplification was performed in 20 μmol/l Tris pH 8.4, 5 μmol/l MgCl2, 200 μmol/l deoxyribonucleoside triphosphates, 0.5% (0.05 μl of 100×) SYBR green (Molecular Probes, Eugene OR), 200 μM of each primer, and 0.25 units platinum Taq (Life Technologies In Vitrogen, Carlsbad, CA).

**Results**

Injection of insulin into fasted mice, the protocol used in the first study, produced between 1.0 and 2.0 mmol/l average plasma glucose for mice in each pool compared with glucose levels >7.5 mmol/l in the ad libitum–fed mice. No mice died or exhibited seizures due to this protocol.

Of the 956 genes on the array, 28 genes (18 increased, 10 decreased) were candidates for regulation (13), as described in RESEARCH DESIGN AND METHODS (Table 2). Twenty-seven of these genes were regulated in all five chip pairs. The final gene, inhibitor of κB, was only regulated in 4/5 but strongly enough that regulation of this gene was also assessed using real-time PCR. To verify regulation, cDNA was made from the original mRNA samples, and primers were successfully designed for 17 of the genes (see Primer Design). Although all 17 of the tested hypothalamic genes met the conservative criteria described above as potentially regulated genes, real-time PCR confirmed regulation (P < 0.05 by t test, n = 24) in only 9 of 17 candidate genes that were tested by real-time PCR (Fig. 1, black bars).

Of nine genes whose regulation with hypoglycemia was corroborated by real-time PCR, seven were upregulated (GLUT4, GLUT1, inhibitor of DNA binding 1 [ID-1], angiotsensin precursor, Ubp41, CBP/p300-interacting trans-activators with glutamic acid [E]/aspartic acid [D]-rich COOH-terminal domain [CITED-1], and mitogen-activated protein kinase phosphatase-1 [MKP-1]) and two were downregulated (CDC-like kinase 1 and signal transducer and activator of transcription 1 [STAT-1]).

To further assess if the regulation was specific to the hypothalimus, real-time PCR was used to express these same genes in cortical mRNA from the same mice. All genes regulated (in either direction) with hypoglycemia in hypothalamic were similarly regulated with hypoglycemia in cortex (P < 0.05 by t test, n = 16) (Fig. 1, white bars), indicating that the regulation was not specific to the hypothalimus.

Since the paradigm in study I entailed producing hypoglycemia by a combination of fasting and insulin injection, in study II, we assessed gene expression after producing hypoglycemia by intravenous insulin injection in ad libitum–fed mice or by fasting only (Fig. 2). For this study, ad libitum–fed C57Bl/6 mice were given either an insulin (7 units/kg i.v., n = 20) or saline (n = 16) injection 3 h before...
they were killed (fed blood glucose 8.24 ± 0.15 mmol/l vs. hypoglycemic 1.12 ± 0.02 mmol/l, P < 0.0001) or after a 48-h fast. Of the nine genes whose regulation by insulin-induced hypoglycemia in fasted mice was confirmed by real-time PCR, five genes (GLUT-1, angiotensinogen precursor, ID-1, Ubp41, and MKP-1) were induced by insulin-induced hypoglycemia alone (P < 0.05, ANOVA followed by Dunnett’s, n = 8). Two of these genes (angiotensinogen precursor and GLUT-1) were regulated by both insulin-induced hypoglycemia and, to a lesser extent, by fasting.

![Graph](image_url)

**FIG. 1.** Quantitative real-time PCR data of regulation of gene expression in hypothalamus (■) and cortex (□) in association with hypoglycemia (produced by and intraperitoneal injection of insulin in fasted mice) expressed as a percentage of euglycemic controls. Hypothalamic samples: n = 24 per group; cortical samples: n = 16 per group (all samples run in quadruplicate). *P < 0.05 by Student’s t test.
and two of the nine genes (GLUT-4 and CITED-1) (Fig. 2) were induced by fasting alone and not by acute hypoglycemia. Of the downregulated genes, the expression of STAT-1 trended toward reduction with fasting and CDC-like kinase 1 trended toward reduction by hypoglycemia, but neither result was statistically significant in this study.

To further assess the tissue specificity of the gene regulation, we assessed the effects of hypoglycemia alone or fasting alone on mRNA from cortex (Fig. 3) and liver (Fig. 4). The regulation of most genes in the cortex was similar to regulation in the hypothalamus, with the exception of angiotensinogen precursor (which was only induced by fasting in cortex not by hypoglycemia) and CITED-1 (which was only regulated by hypoglycemia alone in cortex but not fasting). This suggests that, with the exception of angiotensinogen precursor, most of these central responses to hypoglycemia are regulated throughout the brain and are not specific to the hypothalamus alone.

In the liver, only GLUT-1, angiotensinogen precursor, and MKP-1 exhibited regulation similar to the brain, suggesting that these genes may be part of a global response to hypoglycemia and not merely a central one. There were also two significant differences from the central regula-
therefore hypothesize that at least some member of the Klf4 of another member of the family with the cDNA of Klf4; we apparent regulation of Klf4 might reflect cross-hybridization ubiquitously expressed family of transcription factors, the hypoglycemia. We speculate that since Klf4 is from a large microarray analysis, was the gene most highly induced with kroppel-like factor 4 (Klf4) gene, which, according to the confirmation was especially surprising in the case of the demonstrated to produce reliable prediction of regulation studies. Therefore, it can be anticipated that in using such high-density arrays far more hypoglycemia-regulated genes, and more refined analysis hypothalamus most likely precluded discovery of some manipulations (15). Examining gene expression in whole hypothalamic nuclei obtained by microdissection. The hypothalamus consists of a highly complex set of cells and circuits, and even anatomically close areas can exhibit opposite responses to nutritional manipulations (15). Examining gene expression in whole hypothalamus most likely precluded discovery of some hypoglycemia-regulated genes, and more refined analysis using microdissection is a goal for future studies. Furthermore, the present study entailed the use of a cDNA microarray that consisted of only 956 genes, far less than can be currently manufactured using oligonucleotide arrays. Therefore, it can be anticipated that in using such high-density arrays far more hypoglycemia-regulated genes may be discovered, and this too is a goal for future studies.

Although 17 tested genes met the criteria previously demonstrated to produce reliable prediction of regulation (13), real-time PCR corroborated the regulation of only about half of these genes. This relatively low rate of confirmation was especially surprising in the case of the kruppel-like factor 4 (Klf4) gene, which, according to the microarray analysis, was the gene most highly induced with hypoglycemia. We speculate that since Klf4 is from a large ubiquitously expressed family of transcription factors, the apparent regulation of Klf4 might reflect cross-hybridization of another member of the family with the cDNA of Klf4; we therefore hypothesize that at least some member of the Klf4 family is regulated by hypoglycemia, but which family member is regulated is currently unknown.

The present studies indicated that microarrays can be used to detect even relatively small changes in gene expression (as in the roughly 50% changes observed with CTED-1, MKP-1, STAT-1, and CLK-1) and very-low-abundance mRNAs (as in GLUT-4). The fold regulations in this study ranged between 1.4 and 2.6, yet these small changes detected and corroborated. On the other hand, even when highly conservative criteria are used to infer the reliability of regulation, this study also demonstrates the necessity of corroborating the putative regulation by another method (in this case by real-time PCR) using standard statistical comparisons (in the present study by t tests). In the present study, highly conservative criteria were used that had produced reliable concordance with real-time PCR in previous studies (13), but in the present study, even these highly conservative criteria produced concordance with real-time PCR in only about half of the genes examined. These results suggest that microarray results that are not corroborated by an independent method must be interpreted with caution.

Strikingly, four (angiotensinogen, GLUT-1, ID-1, and MKP-1) of the induced six genes are implicated in enhancing glucose metabolism and could therefore plausibly mediate both neuroprotection and counterregulatory failure. The induction of the angiotensinogen expression is of particular interest because angiotensin II (ATII; the active derivative of angiotensinogen) stimulates regional blood flow (16) and may therefore mediate effects of hypoglycemia to induce increased vasodilation (10). In turn, sustained vasodilation would plausibly lead to elevated glucose availability and thus counterregulatory failure due to hypothalamic overestimation of plasma glucose. Furthermore, ATII induces the expression of GLUT-1 (17) and MKP-1 (18), which some data suggest can act as a switch during hypoglycemia to stimulate phosphatidylinositol 3-kinase–mediated glucose metabolism (19,20). Clearly, the induction of GLUT-1, whose induction by chronic hypoglycemia had been previously reported (21), would plausibly serve a neuroprotective role by increasing the transport of glucose into the brain and by doing so lead to counterregulatory failure, as previously proposed (22). To our knowledge, this is the first report that a single episode of mild hypoglycemia, which is sufficient to produce counterregulatory failure (22,23), rapidly induces GLUT-1 expression.

That these effects of ATII could persist after restoration of euglycemia is suggested by the observation that in addition to its acute effects to increase glucose transport and metabolism and regional blood flow, ATII can also promote angiogenesis by inducing vascular endothelial growth factor (VEGF) (24–26). Furthermore, the effects of VEGF to promote angiogenesis appear to depend on ID-1 (27) and also entail an induction of GLUT-1 (28). These data suggest the hypothesis that hypoglycemia-induced secretion of VEGF (29) is mediated by the release of ATII and that the induction of GLUT-1 and ID-1 are in turn mediated by the release of VEGF, whereas the induction of MKP-1 is mediated directly by the release of ATII. The functional significance of this mechanism is suggested by the observation that VEGF levels are positively correlated
with neurocognitive function during hypoglycemia (29), indicating that VEGF acutely increases nutrient availability. Increasing cerebral blood flow may improve glucose uptake in some brain regions through recruitment of capillaries with an otherwise slow perfusion rate (30), and several studies have shown that under normal conditions, capillary perfusion in the brain is heterogeneous, with some areas receiving less than maximal perfusion (31). However, since increased permeability and blood flow have an additive effect on local cerebral glucose uptake (22), the combined effect of ATII to increase both GLUT-1 and vascular permeability could produce a particularly profound effect on local cerebral glucose uptake. Increased capillary density is strongly correlated with both increased GLUT-1 density and increased local cerebral glucose uptake, suggesting that angiogenesis could be a long-term neuroprotective mechanism against recurrent hypoglycemia (32). Thus, taken together, these data support the hypothesis that angiotensinogen II, acting through several gene products, including GLUT-1, MKP-1, ID-1, and VEGF, acts to coordinate a neuronal response to hypoglycemia by increasing local blood flow, glucose transport, and angiogenesis. While this response to acute hypoglycemia is plausibly neuroprotective, persistent expression of these genes (as might occur if associated with angiogenesis) would plausibly lead to counterregulatory failure through overestimation of blood glucose concentrations. It should be noted that since hypoglycemia was induced by insulin injection, it remains a formal possibility that the induction of these genes is due to elevated postinjection hyperinsulinemia rather than hypoglycemia. However, since the same genes were induced by fasting, which is characterized by low insulin and low glucose, it seems much more likely that glucose, rather than insulin, constitutes the relevant signal. Supporting this hypothesis, in contrast to other genes, STAT-1 was induced in liver during insulin-induced hypoglycemia but inhibited in brain and liver by fasting. These data are consistent with previous reports that insulin induces STAT-1 in peripheral tissues but not in the brain (33). On the other hand, the observation that fasting induced the same genes as insulin-induced hypoglycemia, even though the levels of glucose in fasted mice were not low enough to produce counterregulatory failure (34), the combined effect of ATII to increase both GLUT-1 and MKP-1 in the liver, and release of ATII from the liver might then lead to an induction of GLUT-1 and MKP-1 in other tissues. On the other hand, other mechanisms mediating effects of hypoglycemia, including mechanisms involving ATP-sensitive K⁺ channels or adenosine, are also plausible. Assessment of these and other glucose-sensing mechanisms, using the novel molecular mechanisms of hypoglycemia discovered in the present studies, are currently underway.

REFERENCES

2. Sokoloff L: The brain as a chemical machine. Prog Brain Res 94:19–33, 1992
19. Kusari AB, Byon J, Bandyopadhyay D, Kenner KA, Kusari J: Insulin-induced mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) attenuates insulin-stimulated MAP kinase activity: a mechanism for the...


