Spreading Depression–Induced Gene Expression Is Regulated by Plasma Glucose

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Background and Purpose—Plasma glucose and spreading depression (SD) are both determinants of brain ischemia. The purpose of this study was to examine whether plasma glucose affects SD-induced gene expression in the cortex.

Methods—SD was induced by topical application of KCl. Hyperglycemia and hypoglycemia were induced by intraperitoneal injection of glucose and insulin, respectively. The expression of c-fos, cyclooxygenase-2 (COX-2), protein kinase C-δ (PKCδ), and heme oxygenase-1 (HO-1) was determined by in situ hybridization.

Results—SD alone induced expression of c-fos (by 340%), COX-2 (210%), HO-1 (470%), and PKCδ (410%). Hypoglycemia (2.4 ± 0.9 mmol/L) alone did not induce gene expression, and hyperglycemia (22.1 ± 3.7 mmol/L) alone induced only c-fos by 42%. When hypoglycemia was induced 30 minutes before SD, c-fos induction was enhanced by 145%, but the induction of HO-1 and PKCδ was reduced to 43% and 64%, respectively. When hyperglycemia was induced 30 minutes before SD, c-fos induction was enhanced by 388% and COX-2 expression by 53%, whereas the induction of PKCδ and HO-1 was reduced to 54% and 51%, respectively. The frequency, amplitude, and duration of direct current potentials were unaltered in hyperglycemic SD animals, whereas in hypoglycemic animals the duration was increased by 47%.

Conclusions—While SD induces expression of several genes, the availability of glucose regulates the extent of the gene induction. The effect of glucose is different on early-response genes (c-fos and COX-2) compared with late-response genes. Plasma glucose may contribute to neuronal damage partially by regulating gene expression. (Stroke. 1999;30:114-119.)

Key Words: gene expression ■ hyperglycemia ■ hypoglycemia ■ ischemia ■ spreading cortical depression

Spreading depression (SD) is a wave of ionic transients involving release of K⁺ and uptake of Ca²⁺, Na⁺, and Cl⁻, and it is most likely initiated and propagated by massive presynaptic release of glutamate and activation of N-methyl-D-aspartate (NMDA) receptors subsequent to local brain injury, including focal brain ischemia. In normal brain tissue, SDs repeatedly elicited over a 5-hour period do not lead to neuronal death. However, it is believed that when SD repeatedly collapses ionic gradients, activation of NMDA receptors and gap junctions propagates SD and triggers a massive Ca²⁺ influx, which in energy-compromised neurons is enough to initiate a cell death cascade. This hypothesis is supported by the findings that in focal brain ischemia SD increases the ischemic volume, probably by 23% per SD wave.

Another determinant of brain injury in cerebral ischemia is plasma glucose. In clinical studies diabetic patients experiencing stroke have worse outcomes that those without diabetes, and mortality and morbidity are increased in patients with high plasma glucose levels. In animal models of global brain ischemia, including monkeys suffering from cardiac arrest, glucose loading exacerbates ischemic neuronal injury. In focal ischemia models, acute hyperglycemia is detrimental when ischemia is followed by reperfusion but may be without effect or may even be beneficial when ischemia is permanent. In addition, fasting before transient cerebral ischemia reduces infarct volume.

Even though ischemia decreases both protein and mRNA synthesis, ~100 genes have been found to be induced after cerebral ischemia. The ischemia-inducible genes include immediate early genes, stress proteins, growth factors, adhesion proteins, cytokines, kinases, and genes directly regulating apoptosis. Because it is believed that gene induction contributes to the outcome of brain ischemia, factors regulating these genes need to be determined. The purpose of this study was to examine whether the plasma glucose level affects SD-induced gene expression. We studied c-fos, a prototype of immediate early genes, heme oxygenase-1 (HO-1), also known as heat-shock protein-32, cyclooxygenase-2 (COX-2), an immediate early gene involved in in-
flammmation, and protein kinase C-8 (PKCδ), an injury-inducible kinase.\(^{32}\)

### Materials and Methods

#### Induction of Spreading Depression

Male Wistar rats (weight, 225 to 275 g) were used. All procedures complied with the University of Finland Animal Care and Use Committee. The anesthesia was induced with 5% halothane in N\(_2\)O/O\(_2\) (70:30), and during the operation the halothane concentration was reduced to 1%. The rectal temperature of the animal was maintained between 37.0°C and 37.5°C with a heating pad. A right femoral artery was cannulated for blood gas analyses and plasma glucose recording. After a rat was placed in a stereotaxic frame, a 2-mm craniotomy was made bilaterally 4 mm lateral to the sagittal suture and 4 mm posterior to bregma. Without disruption of the dura, the brain was exposed for 120 minutes to 3 mol/L KCl to induce SD.

The left hemisphere was exposed to 0.9% NaCl and served as a control. Thirty minutes before the SD experiment, hypoglycemia was induced by an injection of insulin (Actrapid, Novo Nordisk; 2.7 IU/kg IP) after overnight fasting, and hypoglycemia was maintained between 37.0°C and 37.5°C with a heating pad. A right femoral artery was cannulated for blood gas analyses and plasma glucose recording. After a rat was placed in a stereotaxic frame, a 2-mm craniotomy was made bilaterally 4 mm lateral to the sagittal suture and 4 mm posterior to bregma. Without disruption of the dura, the brain was exposed for 120 minutes to 3 mol/L KCl to induce SD in the right hemisphere. The KCl application was terminated by carefully placing a dry piece of a filter paper for 10 seconds on the dura. The left hemisphere was exposed to 0.9% NaCl and served as a control. Thirty minutes before the SD experiment, hypoglycemia was induced by an injection of insulin (Actrapid, Novo Nordisk; 2.7 IU/kg IP) after overnight fasting, and hypoglycemia was induced by an injection of 50% d-glucose (2.6 to 3.4 mL IP). Normoglycemic rats were injected with saline (3 mL IP). Three hours after the cortical KCl application was stopped, the rats were rapidly reanesthetized, and the brains were processed for in situ hybridization. For c-fos immunostaining, the brains were processed 4 hours after cortical KCl application was stopped, and for COX-2, HO-1, and PKCδ immunostaining, the brains were processed 15 hours after KCl application.

#### In Situ Hybridization

Briefly, we used oligonucleotide probes for c-fos (5'-GCAGCGG-GAGGATGACGCTCTGAGTCCGC-GTTGAAACCCGAGAA-3'), COX-2 (5'-TTATTTGAGATGAGACTGAATTGAGGC-ACTGTTGTAGATGA-3'), HO-1 (GCAATCTTCTTCAGGA-9'), PKCδ (AGACAGCTG-TCTTCTTGCATCCCATTGATATT-3'), and control oligonucleotides with the same length and GC ratio similar to the corresponding antisense oligonucleotides but without homology to any known sequences. The probes were 3' end-labeled with \(^{35}\)S-dATP (New England Nuclear, Boston, Mass), and 10-µm-thick coronal sections, thawed on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, Pa), were hybridized as described previously.\(^{33}\) The specificity of the oligonucleotide probes with the use of rat brain tissue has been shown in Northern blotting in our previous studies.\(^ {32,33}\) To obtain optical density measurements of the sections, a digital image analysis system was used (MCID 4, Imaging Research). The gray levels corresponding to the \(^{14}\)C plastic standards (Amersham) lying within the exposure range of the film were determined and used as a fourth-degree polynomial approximation to construct a gray level to activity transfer. Densitometric measurements were done from 4 sections (at -1.0±0.2, -2.5±0.2, -3.0±0.2, and -4.0±0.2 mm from bregma) for each animal.

#### Statistical Analysis

The data between control and hypoglycemic animals and between control and hyperglycemic animals were assessed with Student’s \(t\) test. A value of \(P<0.05\) was considered significant.

#### Cortical Direct Current Potential

In a separate set of 5 to 13 animals per group, cortical direct current (DC) potentials were measured bilaterally during the 60-minute period of KCl exposure. Animals were prepared as described earlier, but 2 additional burr holes were made over the frontal cortex (1 mm anterior to the bregma and 3 mm lateral to the midline) for the recording electrodes. DC potentials were recorded by using an extracellular low-resistance needle electrode, inserted 1 mm deep into the cortex. Signals were led through a DC amplifier to an instrumentation tape recorder. The data were assessed with Student’s \(t\) test.

#### Immunocytochemistry

The perfusion-fixed brains were cut at a 50-µm thickness on a Leica VT1000M vibratome. The free-floating sections were incubated for

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**Figure 1.** In situ hybridization autoradiographs show expression of c-fos, COX-2, PKCδ, and HO-1 in the hypoglycemic, normoglycemic, and hyperglycemic rat brain 3 hours after cortical SD, which was induced by topical application of 3 mol/L KCl on the right cortex. Physiological saline was topically applied on the left cortex, which served as a control. The expression of c-fos mRNA is enhanced by both hypoglycemia and hyperglycemia, and COX-2 mRNA is enhanced by hyperglycemia. The expression of PKCδ and HO-1 mRNA is suppressed by both hypoglycemia and hyperglycemia.
48 hours at 4°C with the primary PKCδ (rabbit, GibcoBRL, Life Technologies, Gaithersburg, Md, 1:250; and rabbit, Santa Cruz Biotechnology, Santa Cruz, Calif, 1:1000), c-fos (rabbit, Santa Cruz Biotechnology, 1:1000), HO-1 (rabbit polyclonal, StressGen, Victoria, British Columbia, Canada, 1:2000), or COX-2 (Transduction Laboratories, Lexington, Ky, 1:300) diluted in 0.1 mol/L sodium phosphate buffer, pH 7.4, containing 0.3% Triton X-100 and 1% bovine serum albumin. The bound antibody was visualized with the avidin-biotin-peroxidase method (Vectastain Kit, Vector Labs, Burlingame, Calif) with 3,3′-diaminobenzidine used as the peroxidase substrate. Control staining included incubations with the primary antibody preabsorbed with the antigen peptide and incubations without the primary peptide.

Results

Plasma Glucose Levels and Blood Gas Values

There were no statistically significant differences in PaO₂, PaCO₂, pH, or rectal temperature between the animal groups. The plasma glucose levels are shown in the Table.

In Situ Hybridization

The basal cortical expression of c-fos, COX-2, and PKCδ was very slight but detectable, whereas no expression of HO-1 was seen in the control cortex. In unoperated animals, hypoglycemia or hyperglycemia did not alter the gene expression, with the exception of increased c-fos expression in hyperglycemic rats by 41.7% (not shown). Three hours after the KCl cortical application was finished, the expression of all the genes studied was significantly increased (Figures 1 and 2A) compared with the contralateral control cortex: c-fos by 340%, COX-2 by 210%, HO-1 by 470%, and PKCδ by 410%. Hyperglycemia enhanced the SD-induced expression of c-fos by 388% and COX-2 by 53%, whereas hyperglycemia reduced the SD-induced expression of PKCδ to 54% and HO-1 to 51% (Figures 1 and 2B). Hypoglycemia enhanced the SD-induced c-fos expression by 145%, did not alter COX-2 expression, and reduced the SD-induced expression of PKCδ to 64% and HO-1 to 43% (Figures 1 and 2B). When the plasma glucose values and mRNA quantitation values were plotted against each other, the deviation of the plasma glucose levels from the normoglycemic value correlated positively with c-fos mRNA levels, whereas a negative correlation with HO-1 and PKCδ mRNA levels was observed (Figure 3).

Immunocytochemistry

Compared with the control brain, SD induced strong immunoreactivity of c-fos and COX-2 proteins and, to lesser extent, of PKCδ throughout the cortex, as previously described (S. Miettinen, MSc, et al, unpublished data, 1998, and References 33, 36, 37). A less intense but readily detectable induction of HO-1 immunoreactivity was also seen (Figure 4). The expression of c-fos, COX-2, and PKCδ was exclusively neuronal, whereas HO-1 immunoreactivity was seen in astrocyte-like cells. No attempt was made to detect differ-
Hyperglycemia suppresses the mRNA induction of brain-derived neurotrophic factor, a neurotrophic early-response gene, in global ischemia without altering the time course. In addition, our preliminary studies suggest that the time course of PKCδ and HO-1 induction is not significantly altered by hyperglycemia in SD.

Hyperglycemia did not cause significant changes in the parameters of DC potentials, whereas hypoglycemia increased the duration of DC potentials by 47%. This is in agreement with the results of Gidö et al., who reported increased duration of calcium transients and DC potential shifts in hypoglycemic SD rats. Our results on hyperglycemic rats differ, however, from the study by Nedergaard and Astrup, who found that the amplitude but not frequency and duration of DC potential is attenuated to 15% by hyperglycemia of 32 mmol/L. Therefore, plasma glucose levels <20 mmol/L may not significantly suppress KCl-induced DC potentials. Consequently, we suggest that the altered SD-induced gene expression we observed during hyperglycemia is not due to suppression of depolarization waves, whereas the attenuation of PKCδ and HO-1 and enhancement of c-fos and COX-2 gene expression caused by hypoglycemia could be attributed to delayed restoration of ionic gradients.

The most obvious explanation for differential regulation of gene expression would be distinct activation of transcription factors in response to plasma glucose. All the genes studied have an activator protein-1 binding site in the 5' flanking region. Activator protein-1 binding activity is easily achieved by strong depolarization and is likely to contribute considerably to SD-induced gene expression. cAMP-responsive element (CRE) is found in the regulatory region of c-fos and COX-2, but is not present in the rat PKCδ or rodent HO-1 gene. CRE binding activity also occurs after glutamate treatment and by stimulation of NMDA receptors, and high glucose has been shown to stimulate fibronectin gene expression through CRE in mesangial cells, suggesting that plasma glucose could regulate the SD-induced c-fos and COX-2 expression by increasing CRE binding activity. In addition, a serum response element (SRE) is present in the promoter of the c-fos gene, and the NMDA receptor–mediated Ca++ influx has been reported to induce c-fos via SRE- and ELK-1–dependent mechanisms. An ELK/SRE-dependent pathway also enhances COX-2 expression induced by the v-src oncogene, whereas no SRE is present in the rodent PKCδ or HO-1 genes. Altogether, the difference in the influence of plasma glucose between SD-induced early-response genes (c-fos, COX-2) and late-response genes (HO-1, PKCδ) could be due to CRE and SRE/ELK-1 binding sites in c-fos and COX-2 genes. Binding sites for nuclear factor-κB, an oxidative stress–responsive transcription factor that can be activated by several glutamate receptors, are found in promoters of rodent PKCδ, HO-1, and COX-2, but it is unclear whether nuclear factor-κB is activated in SD or in the perifocal area after focal brain ischemia (J. Koistinaho, MD, PhD, et al, unpublished data, 1998, and Reference 53).

The expression of SD-induced PKCδ and HO-1 genes was decreased by both hypoglycemia and hyperglycemia. Transcriptional activation of these late-response genes but not of
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early-response genes requires protein synthesis, which in general is reduced during SD. It is possible that when combined with a moderate hypoglycemia, compromised energy sources and reduced protein synthesis reach a level low enough to restrict protein synthesis—dependent mRNA induction of late-response genes, such as HO-1 and PKC. According to this hypothesis, COX-2 and c-fos would not be expected to be downregulated, which is in agreement with the present study. Instead, delayed restoration of Ca\(^{2+}\) gradient may have enhanced the CRE-mediated and protein synthesis—dependent induction of immediate early genes, which is also supported by the hyperglycemia-enhanced c-fos expression in the present study.

Altogether, the results show that after a noninjurious cortical stimulation, long-term alterations requiring increased gene expression of at least some early-response genes and enzymes are influenced by plasma glucose levels. Even though it is likely that the observed alterations are reflected in corresponding protein levels, the protein levels were not studied in the present studies. Therefore, the possibility that the alterations in gene expression may not be strictly followed by translation of the message cannot be ruled out.

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Cerebral ischemia triggers a massive genomic response that leads to induction of genes normally not transcribed, as well as modulation of the expression of constitutive genes. The role that these genes and their products play in the mechanisms of ischemic brain injury remains one of the challenges facing investigators in the field. In the accompanying article, Koistinaho et al studied whether variations in plasma glucose modulate the pattern of gene expression following cortical SD. Cortical SD by itself is not injurious, but, after cerebral ischemia, cortical SD–like events at the periphery of the infarct are thought to contribute to brain damage. The authors found that changes in plasma glucose have profound effects on the mRNA expression of the early gene c-fos, of the inflammatory gene COX-2, of the heat-shock protein HO-1, and of PKCδ, an injury-induced kinase. The findings demonstrate that systemic variables, such as plasma glucose, have a profound effect on the molecular events triggered by cortical SD and probably on postischemic gene expression as well.

It is well known that hyperglycemia enlarges ischemic infarcts in animal models of transient cerebral ischemia and worsens the outcome of human stroke. The mechanisms of this effect are not entirely clear. Hyperglycemia–induced hypermetabolism, worsening of acidosis, vascular factors, and increased production of reactive oxygen species may contribute to the effect. The findings of the present study raise the possibility that alterations in gene expression also play a role in the deleterious effect of hyperglycemia. For example, hyperglycemia enhances cortical SD–induced expression of COX-2, a prostaglandin-synthesizing enzyme whose reaction products contribute to ischemic brain injury.

The excellent study of Koistinaho et al represents the starting point for future investigations. For example, the effects of hyperglycemia on cortical SD–induced gene expression should be validated in models of cerebral ischemia. Furthermore, it should be determined whether the effects of hyperglycemia on injury-induced gene expression are transcriptional, translational, or posttranslational.

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