Why Does Acute Hyperglycemia Worsen the Outcome of Transient Focal Cerebral Ischemia?  
Role of Corticosteroids, Inflammation, and Protein O-Glycosylation

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Background and Purpose—Hyperglycemia adversely affects the outcome of stroke. Global ischemia data support that the harmful effect of hyperglycemia is mediated by glucose-induced elevated plasma glucocorticoids. Here we sought to evaluate the negative effects of hyperglycemia on transient focal ischemia in the rat, and to test whether these could be prevented by inhibition of either corticosteroid production or neutrophil infiltration.

Methods—Sprague-Dawley rats (n = 217) were used. Ischemia was induced by 1 hour middle cerebral artery occlusion (n = 196). Acute hyperglycemia was induced by IP injection of dextrose 30 minutes before ischemia. Neutrophil infiltration was blocked by neutropenia with vinblastine. Corticosterone synthesis was inhibited by chemical adrenalectomy with metyrapone. We measured MRI lesion and tissue infarct volumes, evaluated the neurological function, brain myeloperoxidase and matrix metalloproteinase-9 activities, and protein O-glycosylation.

Results—Hyperglycemia significantly enhanced MRI diffusion-weighted imaging alterations, increased cortical, but not subcortical, infarct volume, worsened neurological score, and enhanced brain myeloperoxidase and matrix metalloproteinase-9 activities. Metyrapone did not prevent hyperglycemic brain damage despite successful reduction of plasma corticosterone. Yet, metyrapone tended to reduce cortical infarction and apparent diffusion coefficient lesion volume, indicating some negative contribution of corticosterone. Blocking neutrophil infiltration was also ineffective to prevent the harmful effect of hyperglycemia. A new finding was that O-linked glycosylation of cerebral proteins was increased under hyperglycemia.

Conclusions—In transient middle cerebral artery occlusion, the hyperglycemia-exacerbated brain damage cannot be fully explained by the negative effects of plasma corticosteroids or neutrophil infiltration. The contribution of other intrinsic effects of high glucose, such as brain protein O-glycosylation, deserves further investigation. (Stroke. 2006;37:1288-1295.)

Key Words: brain infarction • corticosteroids • glucose • metalloproteinases • neutrophils • rats

In stroke patients, glucose values above 144 mg/dL are associated with a 3-fold increase in mortality and are related to a higher degree of permanent disability. Plasma glucose is an important determinant of brain injury in experimental models of focal cerebral ischemia/reperfusion. Yet, the actual mechanisms involved in these negative effects remain unclarified. Although much attention was focused in the past on the worsening effect of increased lactate production, extracellular lactate accumulation is not a crucial determinant of brain injury. Also, glucose per se, but not lactate, in combination with acidosis mediates the detrimental hyperglycemic effect in organotypic hippocampal slices. Among other putative worsening effects of hyperglycemia is the reported impaired cerebral blood flow restoration at reperfusion. However, no effect of hyperglycemia on cerebral blood flow and still a bad neuropathological outcome has also been reported. Hyperglycemia dramatically enhances neutrophil infiltration in brain after transient focal ischemia. Also, an exaggerated leukocyte-endothelial cell adhesion has been described after mesenterium ischemia in diabetes mellitus. Therefore, hyperglycemia-enhanced inflammatory response to ischemia/reperfusion might contribute to exacerbate the ischemic injury.

More recently, the view is growing that hyperglycemia-induced release of glucocorticoids is responsible for the worsening effect of this condition, as shown in an experimental model of global ischemia in rats. Whether glucocorticoids are...
the main cause of aggravation of ischemic damage by hyperglycemia in transient focal ischemia has not been addressed.

Furthermore, hyperglycemia activates the hexosamine pathway leading to increased O-linked glycosylation, which depends on the action of specific catalytic enzymes. O-glycosylation has been identified as a mediator of pancreatic islet injury induced by hyperglycemia and streptozotin, and in the development of glucose-induced insulin resistance. Whether O-glycosylation occurs in the ischemic brain after acute hyperglycemia has not been investigated.

Methods

Transient Focal Cerebral Ischemia

Adult male Sprague-Dawley rats (Harlan Interfana Ibérica SL; Sant Feliu de Codines, Spain; n = 217) weighing 280 to 320 g were used. Rats had free access to food and water. Animal experiments were conducted with approval of the Ethical Committee of our Institution. Ischemia was produced by 1-hour intraluminal occlusion of the right middle cerebral artery (MCAO) with reperfusion (n = 196), as reported. Sham-operated (n = 10) and nonoperated rats (n = 11) were used as controls.

Induction of Acute Hyperglycemia

Hyperglycemia was induced by IP injection of dextrose (Sigma; 25%; 2.5 mL) 30 minutes before MCAO (n = 19). Normoglycemic rats received vehicle (water; n = 22). Blood samples were withdrawn: before treatment (basal), immediately after MCAO, and at reperfusion, to measure glucose levels (Accu-Check Sensor Glucometer, Roche Diagnostics).

Inhibition of Corticosterone Synthesis

Rats received metyrapone (Sigma; 100 mg/kg; n = 40) or vehicle (n = 52) IP 1 hour before MCAO. Rats were randomly assigned to hyperglycemia (n = 47) or normoglycemia (n = 45) and were accordingly given dextrose or vehicle 30 minutes after metyrapone. Arterial blood corticosterone levels were measured with a Radioimmunoassay assay at different points: basal (before treatment), at the moment of dextrose/vehicle administration, after MCAO, and at reperfusion.

Induction of Neutropenia

Induction of neutropenia was carried out at 24 hours of reperfusion. Rats were killed after 24 hours of reperfusion.

MRI

At 12 hours after MCAO, normoglycemic (n = 14) and hyperglycemic (n = 23) rats, with or without metyrapone (n = 11 and 12, respectively), were anesthetized (ketamine) and introduced in a 1.5T Sigma Horizon LX magnet (General Electrics) provided with a QDWRISt coil. Acquisition parameters for diffusion-weighted imaging were echo time = 91 ms, repetition time = 10 000 ms, field-of-view = 8×8 cm, matrix = 128×128, number of excitation = 4, slice thickness = 2 mm, spacing = 0.5 mm. b values were 0 and 1000 s/mm². Apparent diffusion coefficient maps were produced with Functool2 software (GE), and the threshold was set at 500×10⁻⁶ mm² s⁻¹ to calculate lesion volume.

Assessment of Brain Damage

A simple neurological test in a 0 (normal) to 9 (highest handicap) point-scale was carried out at 24 hours. Rats were anesthetized and killed by decapsulation. The brain was removed and sliced in
Figure 1. Hyperglycemia impairs ischemic brain damage, which is not prevented by metyrapone. A, Plasma corticosterone concentration was measured at times: 0 (basal), 30 minutes (hyperglycemia induction), 60 minutes (MCAO), and 120 minutes (immediately after reperfusion). Measures were carried out in 4 rat groups (n=2 to 4 per group) that were all subjected to ischemia: normoglycemic (N), hyperglycemic (H), normoglycemic + metyrapone (N+Mety), and hyperglycemic + metyrapone (H+Mety). Plasma corticosterone levels increase after ischemia in hyperglycemic rats in relation to basal (1-way ANOVA; *P<0.05). Comparison of the time course between groups (2-way ANOVA) shows higher corticosterone concentrations in hyperglycemic than in normoglycemic (&P<0.05), and lower values in metyrapone groups (#P<0.05). B, At MCAO onset (n=15 to 22 rats per group), plasma corticosterone concentration is significantly reduced by metyrapone (P<0.001). C, Infarct volume is higher in H than in N (**P<0.001). Metyrapone has a nonsignificant tendency to reduce infarct volume in hyperglycemia, but the volume is still higher than in corresponding controls (N+M; #P<0.05). D, Cortical infarct volume is higher in H than N (**P<0.001), and H+Mety than N+Mety (#P<0.05 versus N). E, Hyperglycemia does not significantly affect subcortical infarction. F, Hyperglycemic rats show a worse neurological score (**P<0.01 versus N), even after metyrapone (H+Mety: *P<0.05 versus N; ##P<0.01 versus N+Mety). Data of infarct volume and neurological score were analyzed with the nonparametric Kruskal-Wallis test followed by Dunn Multiple Comparison test. NS means nonsignificant difference between the indicated groups.
Plasma corticosterone levels during and after ischemia in normoglycemic rats were compared with those of hyperglycemic rats treated or not with metyrapone. The synthesis of corticosterone, which is the main plasma corticosteroid in rodents, was inhibited by treating the rats with metyrapone. The effect of treatments at different time points was analyzed with 2-way ANOVA. Bonferroni test was used for post-hoc analyses.

**Gel Zymography**
Brain tissue was obtained as described above. Samples were subjected to detergent extraction and purification of gelatinolytic activity, and zymography was carried out. A mixture of matrix metalloproteinase-9 (MMP-9) and MMP-2 (CC073, Chemicon) was used as a gelatinase standard. Images of the gels were obtained (Kds1D software, Kodak) to analyze the intensity of the bands (Kds1D software, Kodak).

**O-Glycosylation**
Brain tissue was obtained at 24 hours for Western blot analysis. Gel zymography was studied with a mouse monoclonal antibody against O-linked N-Acetylglucosamine, clone RL2 (Affinity Bioreagents) diluted 1:500. A rabbit polyclonal antibody against actin (Sigma) was used (1:10 000) as a loading control. Band intensity was measured (Kds1D software, Kodak), and expressed as the ratio to the corresponding actin band intensity to correct for any differences in protein loading between lanes.

**Statistical Analyses**
Comparisons between 2 groups were made with the Student t test. One-way ANOVA was used for comparisons between >2 groups. The effect of treatments at different time points was analyzed with 2-way ANOVA. Bonferroni test was used for post-hoc analyses. Samples not conforming normality were analyzed with nonparametric Kruskal-Wallis test followed by Dunn Multiple Comparison test.

**Results**
Plasma Corticosteroids Do Not Account for Hyperglycemia-Enhanced Ischemic Brain Damage
The synthesis of corticosterone, which is the main plasma corticosteroid in rodents, was inhibited by treating the rats with metyrapone 30 minutes before bringing on hyperglycemia. We induced ischemia in 4 groups of rats: normoglycemic, hyperglycemic, metyrapone-normoglycemic, and metyrapone-hyperglycemic. Table 1 shows the physiological parameters of rats. Plasma corticosterone levels increased after ischemia under hyperglycemia in relation to basal (P<0.05; Figure 1A), and were higher in hyperglycemic than in normoglycemic rats (P<0.05). Metyrapone successfully prevented increases in plasma corticosterone levels during and after ischemia in normoglycemic and hyperglycemic rats (P<0.05; Figure 1A and 1B).

Hyperglycemia significantly (P<0.01) increased infarct volume at 24 hours (Figure 1C). This was attributable to larger (P<0.001) cortical (Figure 1D), but not subcortical (Figure 1E), infarct. Metyrapone tended to reduce infarct volume in hyperglycemic rats (Figure 1C), particularly in the cortex (Figure 1D). However, differences between hyperglycemic rats treated or not with metyrapone were not statistically significant, and infarct volume was still higher in hyperglycemic rats treated with metyrapone than in control normoglycemic rats (P<0.05). Also, hyperglycemia worsened the neurological score (P<0.01; Figure 1F), and this was not prevented by metyrapone (Figure 1F).

MRI studies at 12 hours confirmed the hyperglycemia-induced ischemic damage (P<0.001). Metyrapone again tended to reduce apparent diffusion coefficient lesion volume, but difference was not statistically significant, and the volume was significantly higher in metyrapone-treated hyperglycemic rats than in normoglycemic rats (P<0.05; Figure 2A and 2B). Ischemia induced neutrophil infiltration (P<0.001), as assessed with the MPO assay, and this effect was exacerbated by hyperglycemia (P<0.001; Figure 3A). This enhanced MPO activity was not significantly reduced by metyrapone (Figure 3B). Brain MMP-9 showed 2 bands (band a=95 kDa, and band b=88 kDa), which intensity increased at 24 hours postischemia in normoglycemic rats, as reported.\(^{2,22}\) Hyperglycemia significantly (P<0.01) increased band a intensity (Figure 3C and 3D), but did not affect band b (Figure 3C and 3E). Metyrapone did not significantly reduce the hyperglycemia-induced MMP-9 increase in band a intensity (Figure 3D and 3E), and did not affect band b either (Figure 3D and 3F).

![Figure 2](http://stroke.ahajournals.org/Downloadedfrom/figs/235489648.png)
Hyperglycemia Enhances Neutrophil Infiltration, but This Effect Does Not Contribute to the Exacerbated Ischemic Brain Damage at 24 Hours

The physiological parameters of rats are shown in Table 2. Vinblastine significantly reduced blood neutrophil counts \((P<0.001)\). Accordingly, this treatment successfully reduced postischemic brain MPO in hyperglycemic rats \((P<0.05;\) Figure 4A). However, it did not reduce infarct volume at 24 hours \((Figure 4B)\), and the neurological score was not ameliorated either \((mean \pm SD = 6.2 \pm 1.9 and 5.5 \pm 1.6 in control and neutropenic rats, respectively)\). Neutropenia reduced \((P<0.01)\) brain MMP-9 band \(a\) in hyperglycemic rats \((Figure 4C)\), whereas MMP-9 band \(b\) \((Figure 4F)\) was unmodified by treatments. The cerebral MMP-9 band \(a\) content was correlated with the corresponding MPO activity \((P<0.001;\) Figure 4E).

Hyperglycemia Induces Protein Glycosylation in the Ischemic Brain

The amount of O-glycoproteins was increased in the brain of hyperglycemic rats \((Figure 5A)\). Quantification of one of the major O-glycoproteins showed a significant increase \((P<0.05)\) in the ischemic brain in hyperglycemia \((Figure 5B)\).

Figure 3. Hyperglycemia increases postischemic neutrophil infiltration and MMP-9. These effects are not fully attributable to corticosterone. A, Ischemia increases brain MPO versus control \((nonoperated and nontreated rats, and the contralateral hemisphere of normoglycemic rats; ***\(P<0.001)\). The increase is significantly higher in hyperglycemic than in normoglycemic rats \((###P<0.001)\). B, Normoglycemic and hyperglycemic rats were treated with metyrapone or vehicle. Hyperglycemia enhances ischemia-induced MPO activity \((**P<0.01)\). Metyrapone does not significantly reduce the effect of hyperglycemia. Values are optical density units/mg of protein, and are expressed as percentage of control. C, Hyperglycemia enhances MMP-9 brain content. D, Metyrapone does not significantly reduce MMP-9 induced by hyperglycemia in ischemic rats. Std indicates gelatinase standard. E, Quantification of band intensity in the ipsilateral hemisphere of ischemic rats shows a significant \((**P<0.01)\) increase of band \(a\) intensity in the hyperglycemic group. This effect was not fully prevented by metyrapone. F, Band \(b\) intensity remains unchanged after treatments. Values are optical density units and are expressed as percentage of control. Data were analyzed with 1-way ANOVA test followed by the Bonferroni test. N indicates Normoglycemic; H, Hyperglycemic; N + Mety, Normoglycemic and Metyrapone treatment; H + Mety, Hyperglycemic and Metyrapone treatment; NS, nonsignificant differences between the indicated groups.
Discussion

The present work shows that the exacerbated ischemic damage by acute hyperglycemia cannot be fully explained by enhanced neutrophil infiltration and plasma corticosteroids, and that hyperglycemia induces formation of O-linked glycoproteins in the ischemic brain. Nonetheless, we found indication that increased levels of plasma corticosteroids under hyperglycemia might negatively contribute to brain damage. Plasma corticosteroids were significantly involved in ischemic damage in rat global ischemia,\textsuperscript{12–14} and they influenced the rate of hippocampal pyramidal cell disappearance after global ischemia in gerbils.\textsuperscript{25} Here, in transient focal ischemia, metyrapone treatment did not show a clear benefit against hyperglycemic damage, suggesting that corticosterone is not the main determinant of exacerbated damage in this experimental model, conforming with previous studies.\textsuperscript{26} Yet, metyrapone showed a tendency to attenuate the

Figure 4. Neutropenia prevents neutrophil infiltration but does not ameliorate ischemic brain damage in hyperglycemic rats at 24 hours. A, Ischemia-induced MPO brain activity increase (1-way ANOVA and Bonferroni test, \(*\*P<0.001\)) is prevented by vinblastine (\(*P<0.05\)) in hyperglycemic rats. B, Vinblastine does not reduce infarct volume in hyperglycemic rats. C, Brain MMP-9 content is reduced after vinblastine in hyperglycemic rats. Two MMP-9 bands, \(a\) and \(b\), corresponding to 95 and 88 kDa, are detected. Gelatinase standard: std; molecular weight standard: std mw. D, Band \(a\) intensity is reduced after vinblastine (\(t\) test; \(*\*P<0.01\)). Values are optical density units expressed as percentage of the H group mean. E, MMP-9 (band \(a\)) values correlate with the corresponding MPO (linear regression analysis; \(r^2=0.512; P<0.0001\)). Points represent values for hyperglycemic rats receiving vinblastine (filled squares) or not (empty circles), and are expressed as percentage of the H group mean. F, Band \(b\) intensity is not modified by treatments.
worsening effect of hyperglycemia on lesion volume, thus suggesting that corticosterone might further contribute to impair ischemic damage.

Metyrapone was reported to reduce infarct volume at 24 hours after permanent focal ischemia in normoglycemic spontaneously hypertensive rats. In contrast, here we did not observe a significant protective effect of metyrapone in normotensive rats. In contrast, here we did not observe a significant protective effect of metyrapone in normoglycemic rats. Nonetheless, our results indicate that corticosterone release might further exacerbate ischemic damage in hyperglycemic conditions. Various effects of high glucose might account for the intrinsic damaging effect of hyperglycemia. Among them, we identified O-linked glycosylation of proteins as a putative deleterious factor to be further investigated.

Summary

The results support that acute hyperglycemia intrinsically aggravates ischemic damage in transient MCAO in rats, besides plasma corticosterone and neutrophil infiltration. Nonetheless, our results indicate that corticosterone release might further exacerbate ischemic damage in hyperglycemic conditions. Various effects of high glucose might account for the intrinsic damaging effect of hyperglycemia. Among them, we identified O-linked glycosylation of proteins as a putative deleterious factor to be further investigated.

Acknowledgments

Supported by National Grants Comisión Interministerial de Ciencia y Tecnología (CICYT) (SAF2002-01963 and SAF2005-05793-C02-01) and Fondo de Investigaciones Sanitarias (FIS) (FIS041104-0). A. Martin and S. Rojas had PhD fellowships from FIS and CICYT, respectively. We thank Noelia Montoya (supported by CICYT) and Eugenia Gómez (supported by IDIBAPS) for skillful technical assistance, and Pere Palau and César Garrido for the MRI technical work. We are indebted to Dr Joan Salom and Dr Ramon Deulofeu for assessment in the neurological test, and the biochemical assays, respectively.

References

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Stroke. 2006;37:1288-1295; originally published online April 6, 2006; doi: 10.1161/01.STR.0000217389.55009.f8
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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