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

Abraham Martín, MSc; Santiago Rojas, MSc; Ángel Chamorro, MD, PhD; Carles Falcón, PhD; Núria Bargalló, MD, PhD; Anna M. Planas, PhD

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 un clarified. 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

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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 Interfauna 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.

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 at different points: basal (before treatment), at the moment of dextrose/vehicle administration, after MCAO, and at reperfusion.

Induction of Neutropenia

Rats received either saline (n = 11) or vinblastine (Sigma; 0.5 mg/kg bw; n = 11), and were given antibiotics, as reported. Arterial blood samples were withdrawn to count neutrophils with a blood cell counter (Pentra 120DX, SPS Evolution, HORIBA ABX) at day 0 and 4 days later, before induction of ischemia. Thirty minutes before MCAO all rats received dextrose to induce hyperglycemia. 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 = 12 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 excitations = 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.

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TABLE 1. Physiological Parameters of the Experimental Groups

<table>
<thead>
<tr>
<th>N, n=21</th>
<th>H, n=25</th>
<th>N+M, n=20</th>
<th>H+M, n=22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL): basal</td>
<td>148.9±5.4</td>
<td>149.2±5.5</td>
<td>139.1±7.9</td>
</tr>
<tr>
<td>Glucose (mg/dL): after MCAO</td>
<td>154.0±5.9</td>
<td>331.5±10.1***</td>
<td>169.4±6.5</td>
</tr>
<tr>
<td>Glucose (mg/dL): at reperfusion</td>
<td>139.8±5.3</td>
<td>242.5±15.6***</td>
<td>144.4±5.4</td>
</tr>
<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>101.9±2.6</td>
<td>109.4±4.9</td>
<td>98.0±2.6</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>36.5±0.1</td>
<td>36.7±0.1</td>
<td>36.8±0.1</td>
</tr>
<tr>
<td>pO2 (mm Hg)</td>
<td>112.7±6.9</td>
<td>131.3±9.2</td>
<td>115.1±8.0</td>
</tr>
<tr>
<td>pCO2 (mm Hg)</td>
<td>37.5±2.9</td>
<td>39.0±2.7</td>
<td>34.8±3.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.443±0.013</td>
<td>7.430±0.012</td>
<td>7.437±0.012</td>
</tr>
</tbody>
</table>

The normoglycemic (N) and hyperglycemic (H) groups that also received Metyrapone are identified as N+M and H+M, respectively. Mean blood pressure, rectal temperature, pO2, pCO2 and pH were monitored during MCAO. Values are expressed as the mean±SEM.

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TABLE 2. Physiological Parameters of the Neutropenia Experiment

<table>
<thead>
<tr>
<th>Hyperglycemia Vehicle (n=11)</th>
<th>Hyperglycemia Vinblastine (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood neutrophil counts (% of total leukocyte)</td>
<td>14.3±1.6</td>
</tr>
<tr>
<td>Body weight (g) before treatment</td>
<td>293.2±2.9</td>
</tr>
<tr>
<td>Body weight (g) after Vinblastine</td>
<td>291.2±3.3</td>
</tr>
<tr>
<td>Glucose (mg/dL): before dextrose (basal)</td>
<td>165.8±8.3</td>
</tr>
<tr>
<td>Glucose (mg/dL): after MCAO</td>
<td>335.5±14.5</td>
</tr>
<tr>
<td>Glucose (mg/dL): at reperfusion</td>
<td>256.5±19.0</td>
</tr>
<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>100.1±2.6</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>36.4±0.1</td>
</tr>
<tr>
<td>pO2 (mm Hg)</td>
<td>102.4±10.1</td>
</tr>
<tr>
<td>pCO2 (mm Hg)</td>
<td>42.2±3.6</td>
</tr>
<tr>
<td>pH</td>
<td>7.392±0.016</td>
</tr>
</tbody>
</table>

Hyperglycemia was induced with dextrose in all animals at day 4 after treatment with Vinblastine or vehicle. Mean blood pressure, rectal temperature, pO2, pCO2 and pH were monitored during MCAO. Values are expressed as the mean±SEM.
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; ##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.
2-mm-thick sections that were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 10 minutes at 37°C. Some of the rats previously examined by MRI were killed at 24 hours and used in this study (n=10). Infarct volume was measured with an image analysis system (AIS; Imaging Research Inc).

**Myeloperoxidase Assay**

At 24 hours, rats were anesthetized, perfused through the heart with saline, and killed. Brain regions (pial cortex and striatum) ipsilateral and contralateral to MCAO were dissected out, frozen, and kept at −80°C. Myeloperoxidase (MPO) was extracted from tissue. Change in absorbance at 460 nm was measured at 37°C with a spectrophotometer (Ultraviolet 3000; Amersham Pharmacia Biotech). Measures were expressed as units per mg of protein (Bradford assay; Bio-Rad, Hercules).

**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 (DC-120 camera, Kodak) to analyze the intensity of the bands (Kds1D software, Kodak).

**O-Glycosylation**

Brain tissue was obtained at 24 hours for Western blot analysis. O-glycosylation was studied with a mouse monoclonal antibody against O-linked N-Acetylgalactosamine, 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-enhanced 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. 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. Hyperglycemia worsens the cortical diffusion-weighted imaging alteration and this is not attributable to corticosterone.

A, diffusion-weighted imaging and corresponding TTC images obtained at 12 hours of reperfusion showing representative rats for the different treatment groups: normoglycemia (N; n=14), hyperglycemia (H; n=12), hyperglycemia + metyrapone (H+Mety; n=11). Cortical hyperintensities are more manifest in hyperglycemic groups than in the normoglycemic group. B, Cortical apparent diffusion coefficient lesion volume is higher in hyperglycemic than in normoglycemic groups (**P<0.001). Hyperglycemic rats treated with metyrapone show apparent diffusion coefficient lesion volume higher than controls (*P<0.05), but a marked tendency to lesion attenuation compared with hyperglycemic rats not receiving metyrapone. NS means nonsignificant differences between the indicated groups.
Hyperglycemia Enhances Neutrophil Infiltration, but This Effect Does Not Contribute to the Exacerated 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±SD=6.2±1.9 and 5.5±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).
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, and they influenced the rate of hippocampal pyramidal cell disappearance after global ischemia in gerbils. 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. Yet, metyrapone showed a tendency to attenuate the

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, and they influenced the rate of hippocampal pyramidal cell disappearance after global ischemia in gerbils. 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. Yet, metyrapone showed a tendency to attenuate the
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 Sprague-Dawley rats. Differences with this previous study, such as strain of rats, blood pressure, type of ischemia, and also dose of metyrapone, which in that study was 2× higher, might account for discrepancies. In contrast to the view that corticoids worsen the outcome of ischemia, pretreatment with dexamethasone or administration of corticosterone (40 to 80 mg/kg) markedly reduced infarction after hypoxia-ischemia in young rats.

We also found that hyperglycemia exacerbated ischemic damage regardless of raises in neutrophil infiltration induced by this condition at 24 hours. Yet, negative effects of neutrophils are recognized, ie, they generate superoxide production and this effect increases with the duration of ischemia. Our results do not exclude that neutrophils may have some contribution to ischemic damage, and that enhanced neutrophil infiltration might further contribute to worsening secondary damage at later times. Hyperglycemia increased cerebral MMP-9 content after ischemia, which was reduced by preventing neutrophil infiltration. Furthermore, MMP-9 content was correlated with MPO activity, in agreement with previous results showing that infiltrated neutrophils are an important source of MMP-9 in the ischemic brain.

Here, we describe the occurrence of O-linked glycosylation in brain at 24 hours after acute hyperglycemia. O-Glycosylation can cause modification of Ser/Thr residues (O-GlcNAcylation) on a large number of signaling molecules, and a putative role for transiently blocking residue phosphorylation has been proposed. Notably, O-GlcNAcylation prevents Akt activation in response to insulin signaling, and it is increased in human coronary artery endothelial cells exposed to high glucose and in atherosclerotic plaques of diabetic patients. Also, nuclear O-GlcNAcylation impairs cardiomyocyte function under hyperglycemic conditions. Further studies are needed to find out whether O-glycosylation is involved in exacerbating ischemic damage under hyperglycemia.

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.

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References


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