Inhibition of Protein Kinase Cβ Reverses Increased Blood–Brain Barrier Permeability During Hyperglycemic Stroke and Prevents Edema Formation In Vivo

Marilyn J. Cipolla, PhD; Quillan Huang, BS; Julie G. Sweet, BS

Background and Purpose—We investigated the effect of circulating factors and protein kinase Cβ on blood–brain barrier permeability and edema during hyperglycemic stroke.

Methods—Male Wistar rats that were hyperglycemic by streptozotocin (50 mg/kg) for 5 to 6 days underwent middle cerebral artery occlusion (MCAO) for 2 hours with 2 hours of reperfusion. Blood–brain barrier permeability was measured in middle cerebral arteries that were ischemic (MCAO) or nonischemic (CTL) and perfused with plasma (20% in buffer) from MCAO or CTL animals. A separate set of MCAO vessels was perfused with the protein kinase Cβ inhibitor CGP53353 (0.5 μmol/L) and permeability measured. Lastly, hyperglycemic rats were treated intravenously with CGP53353 (10 or 100 μg/kg or vehicle 15 minutes before reperfusion and edema formation measured by wet:dry weights (n=6/group).

Results—MCAO vessels had increased permeability compared with controls regardless of the plasma perfusate. Permeability (water flux, μm²×10⁶) of CTL vessel/CTL plasma (n=8), CTL vessel/MCAO plasma (n=7), MCAO vessel/CTL plasma (n=6), and MCAO vessel/MCAO plasma (n=6) was 0.98±0.11, 1.13±0.07, 1.36±0.02, and 1.34±0.06; P<0.01). Inhibition of protein kinase Cβ in MCAO vessels (n=6) reversed the increase in permeability (0.92±0.1; P<0.01). In vivo, hyperglycemia increased edema versus normoglycemia after MCAO (water content=78.84%±0.11% versus 81.38%±0.21%; P<0.01). Inhibition of protein kinase Cβ with 10 or 100 μg/kg CGP53353 during reperfusion prevented the increased edema in hyperglycemic animals (water content=79.54%±0.56% and 79.99%±0.43%; P<0.01 versus vehicle).

Conclusions—These results suggest that the pronounced vasogenic edema that occurs during hyperglycemic stroke is mediated in large part by activation of protein kinase Cβ. (Stroke. 2011;42:3252-3257.)

Key Words: blood–brain barrier ■ hyperglycemia ■ protein kinase Cβ vasogenic edema ■ reperfusion injury

Hyperglycemia is common in acute stroke. 1,2 Thirty to sixty percent of patients with stroke have high glucose levels, regardless of pre-existing diabetes, due to a generalized stress reaction and increased levels of glucocorticoids (for review, see Martin and Kent3 and Kruyt et al4). Hyperglycemia during acute stroke is associated with significantly worsened outcome, including larger infarction, edema formation, and a higher risk of mortality.2,5–7 Both diabetic and nondiabetic patients are adversely affected by hyperglycemia, suggesting it is elevated glucose and not diabetic complications that increase stroke damage.2,7

The development of brain edema is one of the most detrimental consequences of stroke and is greatly augmented in the presence of hyperglycemia.6,8,9 Increased blood–brain barrier (BBB) permeability occurs during hyperglycemic stroke and is essential for development of cerebral edema.8,10 The BBB is therefore an important therapeutic target to limit edema formation that can be fatal during hyperglycemic stroke.6,9 Although several mechanisms are thought to contribute to enhanced edema during hyperglycemic stroke, activation of protein kinase C (PKC) in the cerebral endothelium is likely a central mediator of the BBB changes that occur. PKC activity is rapidly increased in endothelium in response to hyperglycemia due to de novo synthesis of diacylglycerol, the primary activator of PKC.11,12 PKC activation can directly affect BBB permeability through its ability to phosphorylate zona occluden-1 (ZO-1) and disrupt tight junctions,13,14 as well as promote calcium/calmodulin-dependent endothelial cell contraction.15 Furthermore, other agents that induce BBB permeability including bradykinin, histamine, and thrombin produce these effects through PKC-dependent mechanisms (for review, see Cipolla9).
Ischemic stroke is also associated with a systemic inflammatory response and release of circulating factors that could increase BBB permeability independent of the effects of either hyperglycemia or ischemia/reperfusion (I/R).\textsuperscript{16,17} Although a cascade of inflammatory events occurs during I/R, release of proinflammatory cytokines could impact BBB integrity and exacerbate edema formation.\textsuperscript{17} Tumor necrosis factor-\(\alpha\), interferon-\(\gamma\), and interleukin-6 are increased in plasma from patients with stroke and in experimental models within 4 to 6 hours of reperfusion.\textsuperscript{16,17} In addition to proinflammatory cytokines, other circulating factors are released during I/R that could increase BBB permeability and promote edema formation, including vascular endothelial growth factor, histamine, and thrombin.\textsuperscript{9}

The present study had 3 goals. First, we determined the contribution of peripheral circulating factors versus a direct effect of I/R to increased BBB permeability during hyperglycemic stroke. This was accomplished by measuring BBB permeability in nonischemic and ischemic vessels perfused with plasma from hyperglycemic animals that underwent 2 hours of ischemia and 2 hours of reperfusion or plasma from nonischemic controls. We found that the direct effect of I/R on BBB permeability during hyperglycemic stroke was greater than that of plasma. Thus, a second goal of this study was to determine if the direct effect of I/R on BBB permeability during hyperglycemic stroke could be prevented by inhibition of PKC\(\beta\). This isoform of PKC was chosen because it is preferentially elevated in the vasculature by hyperglycemia\textsuperscript{11,12} and hypoxia.\textsuperscript{13} Thus, inhibition of PKC\(\beta\) during hyperglycemic stroke may be an important target to limit the detrimental effects of both hyperglycemia and I/R on BBB permeability. The third goal of this study was then to determine if inhibition of PKC\(\beta\) activation during postischemic reperfusion in hyperglycemic animals could prevent enhanced edema formation compared with normoglycemic stroke.

\subsection*{Materials and Methods}

\textbf{Animal Model of Transient Focal Ischemia}

All procedures were approved by the Institutional Animal Care and Use Committee and complied with the National Institutes of Health guidelines for the care and use of laboratory animals. Male Wistar rats (approximately 300 g; Harlan, Indianapolis, IN) were used for all experiments. Temporary filament occlusion of the middle cerebral artery (MCA) was used to induce I/R in both normoglycemic and hyperglycemic animals, as previously described.\textsuperscript{18} Animals were anesthetized with inhaled isoflurane (1.5\% in air). I/R was confirmed using laser Doppler and any animal that had <50\% drop in cerebral blood flow was excluded from the study. Ischemic animals were exposed to 2 hours of ischemia and 2 hours of reperfusion by suture removal. Sham control animals (CTL) underwent anesthesia and a midline incision without filament occlusion. Animals were made hyperglycemic by a single intraperitoneal injection of streptozotocin (50 mg/kg) 5 to 6 days before middle cerebral artery occlusion, also as previously described.\textsuperscript{18} Glucose was measured on the day of the surgery by a commercially available glucose monitor (Freestyle Lite; Abbott, Abbott Park, IL).

The middle cerebral artery occlusion (MCAO) model was used in hyperglycemic rats to both obtain plasma and distal MCAs exposed to I/R for measurement of BBB permeability, described below. Plasma from hyperglycemic animals was obtained from trunk blood and collected into Vacutainer tubes containing heparin. Blood was centrifuged at 1400 to 1600 g, the plasma removed, aliquoted, and the pooled samples frozen at \(-80\)\(^\circ\)C until experimentation.

\textbf{Measurement of BBB Permeability In Vitro in Response to Plasma, I/R, and PKC\(\beta\) Inhibition}

BBB permeability was measured in isolated and pressurized MCAs obtained from animals that underwent MCAO or sham control (CTL) surgery. MCAs were taken between the M2 and M3 region to eliminate any potential damage from the filament to the BBB during the MCAO procedure. Arteries were dissected from the ischemic side of the brain (MCAO arteries) or the right side for control (CTL arteries). The arteries were mounted on a glass cannula in an arteriograph chamber and perfused with plasma (20\% in buffer) obtained from CTL or MCAO animals, as previously described.\textsuperscript{19} For these experiments, all animals were hyperglycemic (described previously) and the perfusate was matched to the level of glucose from which MCAs were taken. Thus, 4 groups of vessels were compared to assess the contribution of circulating factors versus direct effects of I/R on BBB permeability: control vessel + control plasma (CTL/CTLp;\(n=8\)), control vessel + MCAO plasma (CTL/MCAO;\(p=n=7\)), MCAO vessel + control plasma (MCAO/CTL\(_p\);\(n=6\)), and MCAO vessel + MCAO plasma (MCAO/MCAO;\(p=n=6\)).

BBB permeability of isolated and pressurized MCAs perfused with different plasmas was accomplished as previously described with modifications.\textsuperscript{19,20} Briefly, arteries were perfused with plasma and mounted within an arteriograph chamber that was superfused with physiological saline solution (PSS) at pH 7.4\pm0.05 and kept at 37\(^\circ\)C. The proximal cannula of the arteriograph chamber was connected to an in-line pressure transducer and servo system that allowed for measurement and adjustment of intravascular pressure. The vessels were only mounted on 1 cannula and tied off at the other end to prevent leaks not due to filtration. MCAs were equilibrated at an intravascular pressure of 60 mm Hg for 3 hours. Intravascular pressure was then increased to 80 mm Hg and the servo controlling pressure was disconnected from the pressure transducer. This allowed measurement of the pressure drop without compensation by the servo system. The measured pressure drop due to filtration of water through the vessel wall in response to hydrostatic pressure was used as a measure of BBB permeability (Supplemental Figure S1, http://stroke.ahajournals.org), as previously described.\textsuperscript{20} The drop in pressure was then converted to flux through the vascular wall as a measure of permeability using a conversion curve that relates the volume of water coming out of the cannula per mm Hg (see Supplemental Figure S3). The MCA was used for these experiments because these vessels have BBB properties,\textsuperscript{21,22} can be exposed to I/R and plasma, and are kept in their physiological, pressurized state. This method of measuring BBB permeability has been used successfully in previous studies.\textsuperscript{19,20}

A separate set of MCAs from hyperglycemic animals that were either CTL or MCAO vessels were perfused with CTL plasma plus 0.5 \(\mu\)mol/L of the PKC\(\beta\) inhibitor CGP35335 (\(n=6\)) to determine if inhibition of PKC\(\beta\) reversed the increase in BBB permeability in MCAO vessels. According to the manufacturer, CGP35335 is a selective inhibitor of PKC\(\beta\) but does inhibit PKC\(\beta\) at 10-fold higher concentrations (IC\(_{50}\) values are 0.41 \(\mu\)mol/L for PKC\(\beta\) and 3.8 \(\mu\)mol/L for PKC\(\beta\)). We therefore chose to use a concentration that was relatively selective for PKC\(\beta\) over PKC\(\beta\). The selectivity of CGP35335 for PKC\(\beta\), inhibition was tested previously.\textsuperscript{12} The efficacy of CGP35335 for PKC-induced permeability was determined in a separate set of experiments in which permeability was measured in response to 0.05 \(\mu\)mol/L indolactam-V, a nonselective activator of PKC, in the absence and presence of 0.5 \(\mu\)mol/L CGP35335. Supplemental Figure S2 shows that this concentration of CGP35335 prevented the increase in permeability due to PKC activation with indolactam-V.

\textbf{Inhibition of PKC\(\beta\) During MCAO and Measurement of Brain Water Content}

Separate sets of animals underwent MCAO for measurement of vasogenic edema using wet and dry weights. All animals underwent
2 hours of ischemia and 2 hours of reperfusion. Ten minutes before reperfusion, hyperglycemic animals were treated intravenously with either 10 μg/kg or 100 μg/kg CGP53353 to inhibit PKCβ during reperfusion. These doses were chosen based on estimates of IC50 values for inhibition of PKCβ1 and PKCβ2. Treated hyperglycemic animals were compared with both hyperglycemic and normoglycemic animals that were infused with vehicle (sterile saline). Blood gases and pH were maintained within normal ranges (Supplemental Table I). At the end of the reperfusion period, the animals were decapitated and the brain removed for measurement of water content, as previously described.18 Thus, 4 groups of animals underwent MCAO and were compared to determine the effect of hyperglycemia and PKCβ inhibition on vasogenic edema: normoglycemic vehicle-treated (n=6), hyperglycemic vehicle-treated (n=6), hyperglycemic+10 μg/kg CGP 53353 (n=6), and hyperglycemic+100 μg/kg (n=6).

Measurement of PKCβ Activation in the Cerebral Circulation During Hyperglycemia

To determine if hyperglycemia increased PKCβ activation in the cerebral circulation in our model, we examined phosphorylation of PKCβ using immunoblotting. Cerebral vessels were isolated from brain tissue and processed for Western analysis, as described in supplemental materials.

Drugs and Solutions

In vitro BBB permeability experiments were conducted in a bicarbonate-based PSS; the ionic composition was (mmol/L): NaCl 119.0, NaHCO3 24.0, KCl 4.7, KH2PO4 1.18, MgSO4 1.17, CaCl2 1.6, EDTA 0.026, and glucose 5.5. PSS was made each week and stored without glucose at 4°C; glucose was added to the PSS (Ellisville, MO).

Data Calculations and Statistical Analysis

BBB permeability was compared as water flux over time between the different plasmas and vessel combinations using 2-way analysis of variance with 4 treatment groups and a post hoc Student-Newman-Keuls test for multiple comparisons. To determine the effect of PKCβ inhibition on BBB permeability, the flux at each time point was compared among CTL, MCAO, and MCAO+CGP53353 all perfused with CTL plasma using 1-way analysis of variance with 3 treatment groups and a post hoc Student-Newman-Keuls test for multiple comparisons. Percent water content was compared between ipsilateral and contralateral sides of the brain using paired t test. Percent water content was compared between groups for both contralateral and ipsilateral sides of the brain separately using analysis of variance with 4 treatment groups and a post hoc Student-Newman-Keuls test for multiple comparisons.

Results

Role of Circulating Factors and I/R in BBB Permeability During Hyperglycemic Stroke

Circulating factors can increase BBB permeability independent of I/R.19 We therefore compared BBB permeability in nonischemic MCAs perfused with plasma from CTL (normoglycemic) hyperglycemic animals or hyperglycemic animals that underwent MCAO (Figure 1). MCAO plasma tended to increase BBB permeability in CTL vessels but this was not statistically significant. In contrast, MCAO arteries that were exposed to I/R were significantly more permeable compared with CTLv/CTLp vessels, regardless of the plasma perfusate. Furthermore, the combination of MCAO plasma to the MCAO vessels did not have an additive effect on BBB permeability. Thus, there was no significant interaction between plasma and vessel type.

Role of PKCβ in Enhancing BBB Permeability During Hyperglycemic Stroke

Because plasma did not have a significant effect on BBB permeability, we chose to focus on other mechanisms that might account for increased permeability after I/R. Thus, we used vessels perfused with only CTL plasma for subsequent studies. Figure 2 shows that BBB permeability was significantly increased in MCAO vessels compared with CTL vessels. Perfusion of MCAO vessels with the PKCβ inhibitor CGP53353 reversed the increase in BBB permeability. In fact, permeability of the MCAO vessel in which PKCβ was inhibited had similar permeability as CTL vessels.

Role of PKCβ in Enhancing Vasogenic Edema During Hyperglycemic Stroke

The in vitro studies described above suggested there was a direct effect of I/R on the BBB during hyperglycemic stroke to increase permeability that was reversed by inhibition of PKCβ. Because increased BBB permeability is a primary contributor to vasogenic edema formation,9 we next wanted to determine if in vivo treatment with the PKCβ inhibitor CGP53353 could prevent increased edema formation during hyperglycemic stroke. Figure 3 shows the percent water content of contralateral and ipsilateral cerebral cortex after MCAO in normoglycemic and hyperglycemic animals. No-
Hyperglycemia increases PKCβ activity in endothelium and is thought to underlie many of the vascular complications associated with diabetes.11,12 To further evaluate involvement of PKCβ in mediating increased permeability and vasogenic edema in hyperglycemic animals, we measured levels of phosphorylated PKCβ in isolated cerebral vessels by Western blot. Figure 4 shows that phosphorylated PKCβ was significantly increased in the cerebral circulation at 3, 7, and 14 days of hyperglycemia compared with normoglycemic controls (0 days of hyperglycemia). Thus, PKCβ activation occurs during hyperglycemia in the cerebral circulation. These findings are consistent with our pharmacological and physiological data supporting the concept that PKCβ is a key contributor to increased permeability and vasogenic edema in hyperglycemic animals during I/R.

Discussion

There were several major findings of the present study. First, we investigated the effect of circulating factors present during hyperglycemic stroke on BBB permeability compared with direct effects of I/R by measuring permeability of MCAs that were either nonischemic or exposed to I/R and perfused with plasma from control versus MCAO animals. We found that although MCAO plasma had little effect on permeability in nonischemic vessels, I/R significantly increased vascular permeability. Second, the effect of I/R on BBB permeability during hyperglycemic stroke was reversed by inhibition of PKCβ with CGP53353, suggesting that activation of PKCβ is an underlying mechanism by which permeability was increased. Activation of PKCβ in cerebral vessels during hyperglycemia was confirmed by Western blot of phosphorylated PKCβ. Lastly, treatment to inhibit PKCβ during postischemic reperfusion in hyperglycemic animals prevented increases in brain water content. Thus, it appears that PKCβ inhibition may be an important therapeutic target for prevention of fatal edema formation that is relatively common during hyperglycemic stroke.

The findings from this study demonstrate that inhibition of PKCβ reversed the increased permeability of hyperglycemic MCAs that were exposed to I/R. These results suggest that increased BBB permeability can be a rapid and reversible process involving PKCβ activation. For example, ZO-1 has been shown to be a substrate for PKC and thus PKCβ activation could directly affect the integrity of the tight junction through phosphorylation.14 Kim et al showed using a cell culture model that hypoxia-induced increased BBB permeability and disruption of occludin and ZO-1 was attenuated by PKCβ inhibition.13 Alternatively, PKCβ activation could affect endothelial calcium to enhance contractile properties, a primary means of increasing paracellular permeability.15 Although the regulation of endothelial cell calcium is not well understood, activation of transient receptor potential V4 channels is thought to be a primary contributor.23 These channels are expressed in cerebral endothelium and are activated by PKC.23 Lastly, PKCβ inhibition could protect the BBB through decreasing oxidative stress. PKC activation increases superoxide production through a direct effect on NADPH oxidase.24 In a similar study, 3 days of hyperglycemia before MCAO caused increased edema and BBB perme-
ability that was prevented in a transgenic rat overexpressing superoxide dismutase, suggesting superoxide production is a primary means of enhancing stroke damage during hyperglycemia. It is worth noting that normoglycemic animals did not exhibit increased edema formation at this time period of I/R (Figure 3), demonstrating the significant effect of hyperglycemia on the BBB. These results support the concept that hyperglycemia-induced PKC activation has a greater role in edema formation during I/R than ischemia alone.

An inflammatory reaction occurs peripherally and centrally in response to stroke and includes release of proinflammatory cytokines that can affect BBB integrity. Circulating factors have been shown to have a significant effect on BBB properties that could enhance the detrimental effects of I/R. In the present study, we found that nonischemic arteries perfused with plasma from hyperglycemic MCAO animals did not significantly increase BBB permeability. However, MCAs were exposed to plasma for only 3 hours and it is possible that longer exposure to the plasma would have had a greater effect on barrier properties. Another limitation of this approach was that plasma was diluted to 20% before perfusion because of limited quantities. Physiologically, normal plasma concentration in blood is approximately 55% and thus a more concentrated plasma may have a greater effect. However, a previous study found that 20% plasma from pre-eclamptic women significantly increased BBB permeability suggesting this concentration is sufficient to affect barrier properties under certain conditions.

Conclusions
In the present study, we found that during MCAO, hyperglycemia significantly increased BBB permeability and edema formation compared with normoglycemic stroke. The effect on barrier properties appeared to be predominantly due to direct effects of I/R and not circulating factors. In addition, increased BBB permeability and edema formation during hyperglycemic stroke was reversed by inhibition of PKCβ, suggesting that this pathway may be an important therapeutic target for limiting edema formation.

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Disclosures
None.

References


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by

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Supplemental Figures

Supplemental Figure S1

**Supplemental Figure S1**: A, Measured drop in pressure used to calculate vascular permeability as flux of water through the vascular wall in response to hydrostatic pressure. Middle cerebral arteries were either nonischemic or exposed to ischemia and reperfusion (I/R) by MCAO (2 hours of ischemia and 2 hours of reperfusion) and perfused with plasma (20% in buffer) from animals that were either I/R or sham controls. Plasma had little effect on permeability whereas I/R significantly increased permeability of MCA. ** p<0.01 vs. CTLv/CTLp. B, Measured drop in pressure used to calculate vascular permeability as flux of water through the vascular wall in response to hydrostatic pressure. Middle cerebral arteries that were either exposed to ischemia and reperfusion (I/R) by MCAO or sham surgery (Sham) were perfused with sham control plasma only. The effect of PKCβ inhibition was assessed in MCAO vessels by perfusing with CGP53353 (0.5 µg/mL). Inhibition of PKCβ with CGP53353 reversed the increase in permeability in MCAO vessels. **p<0.01 vs. CTL and MCAO vessels.
Supplemental Figure S2: Conversion graph used to calculate the flux through the vascular wall. The curve was made by measuring the volume of fluid (buffer) that flowed out of the cannula at each pressure and was used to convert the drop in pressure to flux through the vessel wall in response to hydrostatic pressure.
Supplemental Figure S3: Effect of 0.5 \( \mu \text{mol/L} \) CGP53353 on PKC-induced permeability. PKC was activated in the cerebral endothelium by perfusing with 50 nmol/L Indolactam-V (IL-V), a non-selective activator of PKC. CGP53353 in addition to IL-V prevented the increase in permeability suggesting that this concentration of CGP53353 was effective at preventing PKCbeta-induced permeability. *p<0.05 vs. CTL.
Supplemental Methods: Analysis of PKC activity in the cerebrovasculature

Only nonischemic animals were used for determination of PKCβ activity in cerebral vessels from normoglycemic and hyperglycemic animals. Animals were made hyperglycemic by STZ injection (50mg/kg ip) and used after 3, 7 or 14 days and compared to normoglycemic (0 days). Cerebral vascular tissue was isolated from the brain parenchyma. Briefly, rat brains were removed from the skull, the brainstem removed and the remaining tissue homogenized with a Dounce tissue grinder in an ice-cold phosphate buffered saline (PBS, 0.01mmol/L, pH=7.4). The homogenized brain was then centrifuged at 2000g for 10 min. at 4°C. The supernatant was discarded and the pellet washed by resuspension in PBS then re-centrifuged at 2000g for 10 min. The supernatant was again discarded, the pellet resuspended in PBS, gently layered on top of a dextran solution (15%, MW 38,400) and centrifuged at 4000g for 20 min. The pellet was collected, resuspended in dextran and centrifuged again for 20 min. at 4000g. The final pellet was poured into a petri dish for collection of cerebral vessels that were stored at -80°C until Western analysis of protein content. Frozen microvessel samples were homogenized separately in 20% (wt/vol) ice-cold buffer containing: 10mM Tris-HCl (pH=7.4), 1% SDS, 1mM sodium vanadate, 10μg/mL leupeptin, and 1mM phenylmethylsulfonyl fluoride (PMSF). After homogenization, total protein concentration was determined using Coomassie Plus Protein Assay Kit (Pierce Biotechnology) and a spectrophotometer. Protein from each sample was loaded for electrophoresis into a 4-20% TrisGlycine gel (Invitrogen) and subsequently transferred to an Immun-Blot PVDF membrane (Bio-Rad). After the transfer, the membrane was blocked for 1 hour in Aquablock (East Coast Biologics) and then incubated over night at 4°C in antibody against phospho-PKCβ (Santa Cruz). The following morning, the membrane in primary antibody was incubated at 37°C for 30 minutes, washed 3 times for 15 minutes in 1x PBS and TWEEN-20 (PBST), and subsequently incubated for 1 hour in secondary antibody. After 3 washes in PBST and 1 wash in PBS, the membrane was scanned and densitometric analysis performed using the Odyssey Infrared Imaging System.

Supplemental Table. Physiologic characteristics of animals from which plasma and middle cerebral arteries were taken for permeability and edema measurements. *p<0.01 vs. normoglycemic; † p<0.05 vs. normoglycemic.

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<th>Normoglycemic MCAO Vehicle (n=6)</th>
<th>Hyperglycemic MCAO Vehicle (n=18)</th>
<th>Hyperglycemic MCAO 10 µg/kg CGP53353 (n=6)</th>
<th>Hyperglycemic MCAO 100 µg/kg CGP53353 (n=6)</th>
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