Imaging of a Clinically Relevant Stroke Model
Glucose Hypermetabolism Revisited

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Background and Purpose—Ischemic stroke has been shown to cause hypermetabolism of glucose in the ischemic penumbra. Experimental and clinical data indicate that infarct-related systemic hyperglycemia is a potential therapeutic target in acute stroke. However, clinical studies aiming for glucose control in acute stroke have neither improved functional outcome nor reduced mortality. Thus, further studies on glucose metabolism in the ischemic brain are warranted.

Methods—We used a rat model of stroke that preserves collateral flow. The animals were analyzed by 2-[18F]-2-fluoro-2-deoxy-D-glucose positron emission tomography or magnetic resonance imaging during 90-minute occlusion of the middle cerebral artery and during 60 minutes after reperfusion. Results were correlated to magnetic resonance imaging of cerebral blood flow, diffusion of water, lactate formation, and histological data on cell death and blood–brain barrier breakdown.

Results—We detected an increased 2-[18F]-2-fluoro-2-deoxy-D-glucose uptake within ischemic regions succumbing to infarction and in the peri-infarct region. Magnetic resonance imaging revealed impairment of blood flow to ischemic levels in the infarct and a reduction of cerebral blood flow in the peri-infarct region. Magnetic resonance spectroscopy revealed lactate in the ischemic region and absence of lactate in the peri-infarct region. Immunohistochemical analyses revealed apoptosis and blood–brain barrier breakdown within the infarct.

Conclusions—The increased uptake of 2-[18F]-2-fluoro-2-deoxy-D-glucose in cerebral ischemia most likely reflects hypermetabolism of glucose meeting increased energy needs of ischemic and hypoperfused brain tissue, and it occurs under both anaerobic and aerobic conditions measured by local lactate production. Infarct-related systemic hyperglycemia could serve to facilitate glucose supply to the ischemic brain. Glycemic control by insulin treatment could negatively influence this mechanism. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.008407.)

Key Words: diffusion magnetic resonance imaging ■ glucose ■ infarction ■ stroke

Increased local brain glucose metabolism in a rat model of middle cerebral artery (MCA) occlusion was described by Ginsberg et al1 in a seminal article from 1977. Studies using different experimental approaches have investigated the role of regional hypermetabolism of glucose in the pathophysiology after hypoperfusion of the brain.2–11 This phenomenon has been shown in positron emission tomographic (PET) studies of acute human ischemic stroke (IS) and of postasphyctic infants.12,13 Various explanations for the frequent observations of hyperglycemia in IS and the detrimental effects of hyperglycemia on brain tissue have been proposed.14 Higher levels of blood glucose have been shown to be predictive for a more severe stroke and an increased functional impairment in humans, and therefore hyperglycemia is increasingly being considered as a therapeutic target in IS.15 However, in a recent clinical trial in which insulin treatment was used in patients with acute IS, a poorer outcome in the normoglycemic control group was observed.16 A recent review by Cochrane concluded that there is no reliable evidence for glucose control in acute IS.17 Considering that the pathophysiological mechanisms for hyperglycemia in stroke remain largely unknown, further preclinical studies are warranted.

The study of glucose hypermetabolism in experimental stroke has been limited by the lack of clinically relevant stroke models. The frequently used filament MCA occlusion technique for inducing IS in rodents causes obstruction of collateral flow from MCA-adjacent vascular territories. In other words, the filament occludes the anterior cerebral artery and
the posterior communicating artery, which is in the rodent is the main contributor to the posterior cerebral artery. This results in infarctions of entire cortical and subcortical regions. Furthermore, models commonly used for IS provide reperfusion that is unpredictable, which makes imaging studies during reperfusion difficult. In the present study, we used a recently described model for inducing a small focal cortical infarction that preserves collateral flow, and analyzed the recently described model for inducing a small focal cortical and temporal changes in the use of [2-18F]-2-fluoro-2-deoxy-d-glucose ([18F]FDG) in the same animal during occlusion and reperfusion. Using the same experimental protocol as for the small animal PET experiments, we performed high-field magnetic resonance imaging (MRI) to study changes in cerebral blood flow (CBF), diffusion of water (diffusion-weighted imaging), and lactate formation by MR spectroscopy. Further validation was performed with histological analyses of cell death and blood–brain barrier (BBB) breakdown.

This study allowed us to investigate the regional dynamics of [18F]FDG uptake as an indicator of glucose metabolism in relation to CBF, diffusion of water, and immunohistochemical outcome, in a clinically relevant rat model of focal stroke with preserved collateral circulation.

Materials and Methods

Animal Preparation

All experiments were conducted according to the regulations of the Karolinska Institutet and were approved by the local laboratory animal ethics committee. Male Sprague-Dawley rats (355–450 g; Scanbur, Sollentuna, Sweden) were subjected to microwire occlusion of the M2 branch of the MCA (M2CAO), as previously described (groups 1–6; n=25). Imaging studies and postmortem analyses were performed as shown in Table. Animals were anesthetized using 2% isoflurane (Virbac, Carros Cedex, France) blended with air–O2 (7:3) during surgery and imaging. In group 2, blood glucose levels were assessed immediately before and 60 minutes after retraction of the microwire by analyzing tail artery blood samples with a glucometer (HemoCue 210/201RT; HemoCue, Angelholm, Sweden).

Positron Emission Tomography

Imaging Protocol

In vivo PET investigations were performed on a Focus 120 (CTI Concorde Microsystems, Knoxville, TN) small animal PET scanner. Animals in groups 1 and 2 were placed in the PET scanner with the brain in the field of view within 5 minutes after placement of the microwire in the MCA. [18F]FDG was administered via the tail vein (20–40 MBq, 500 μL). Data were collected continuously during 90 minutes from the time of injection. Next, the microwire was retracted, and a second injection of [18F]FDG was administered via the tail vein (20–40 MBq, 500 μL) followed by data collection every second during 60 minutes from the time of reperfusion. Animals in group 2 also underwent a follow-up MRI 24 hours after M2CAO. Animals in group 3 were placed in the PET scanner with the brain in the field of view 24 hours after 90 minutes of M2CAO.

Quantification Method

A macroparameter analysis method was used in addition to time–activity curves of the standardized uptake values (SUV). A volume of interest (VOI) with a predetermined size was drawn over the infarmediate artery using a PET image summed during the first 5 minutes of data collection. The Patlak method gives the Kᵢ value, the so-called influx constant as a quantification of the net uptake of the radiotracer. The VOIs were chosen using a dual-modality approach, where both a PET and an MRI image were obtained for some animals (n=6).

MRI

Imaging Protocol

The MRI experiments were conducted using a horizontal 9.4-T magnet (Varian, Yarnton, United Kingdom). Within 5 minutes after placement of the microwire in the M2 branch of the MCA, animals in groups 4 and 5 were placed in the MRI scanner and were imaged using diffusion-weighted imaging, arterial spin labeling, and point-resolved spectroscopy. Diffusion tensor images were acquired using multislice 3-shot spin-echo planar imaging sequence with repetition time (TR) 3 s, and echo time (TE) 25 ms; diffusion-sensitizing gradients were applied along 12 directions with 2 diffusion-sensitizing factors b=0 and 1000 s/mm². Perfusion measurements were performed using 3-shot gradient echo-planar imaging with a TE of 10.5 ms and 14 slices of 1-mm thickness with no gap in between. 1H MRI spectra were acquired using point-resolved spectroscopy sequence from a VOI (2.5x1.7x2.5 mm³). Imaging data were collected during 90 minutes. After 90 minutes, the microwire was retracted followed by data collection for an additional 60 minutes.

Immunohistochemistry

The animals in groups 1 to 3 were euthanized at 24 hours after M2CAO, and animals in group 3 were euthanized at 48 to 96 hours after M2CAO. The brains from animals in groups 2, 4, and 6 were removed and snap frozen. The animals in groups 2 and 6 were injected with fluorescein isothiocyanate (FITC)-dextran (4 kDa, Sigma-Aldrich; 500 mL of 50 mg/mL; group 6, n=3) and after 24 hours (group 2, n=4). The animals were euthanized 15 minutes after the injection. Animals in group 6 were euthanized 10 minutes after reperfusion.
after the 90 minutes of M2CAO. Six coronal 2 mm cryosections were taken throughout the brain and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride for photography. To visualize apoptotic staining, the ApopTag Fluorescein or ApopTag Red Detection kits were used. After an overnight incubation, sections were incubated in blocking buffer containing antirabbit and antirat secondary antibodies (1/200, Jackson). All sections were counterstained with the nuclear marker Hoechst (1/5000) and mounted with polyvinyl alcohol/glycerol containing 2.5% DABCO (Sigma).

Fluorescence microscope images were acquired on a Vslide slide scanning microscope (MetaSystems, Altlusseheim, Germany). Whole microscope slides were scanned at x2.5, and tissue was detected based on the Hoechst 33342 signal. Images were stitched to generate a large 4-channel fluorescence image of the entire specimen with microscopic resolution.

Image and Statistical Analysis
PET data were processed with small animal PET manager and evaluated using the Inveon Research Workplace (Siemens Healthcare, Erlangen, Germany) software. MRI data were processed with VnmrJ software (Agilent Technologies, Palo Alto, CA) and evaluated using ImageJ (National Institutes of Health, Bethesda, MD). T2-weighted MR images and PET images from animals in group 1 and 2 were coregistered and analyzed using the Inveon Research Workplace. Three sets of VOIs were defined for PET images. (1) In groups 1 and 2 (n=9), a VOI encompassing the brain regions with elevated [18F]FDG uptake including neighboring regions was manually traced by visual assessment. Next, thresholding was performed to select a final VOI including voxels with elevated [18F]FDG uptake. (2) In group 2 (n=6), 2 sets of VOIs were generated using coregistered images from MRI and PET. One VOI was generated from manually tracing regions of interest matching the final infarct. The other was generated by subtracting the infarct VOI from the VOI containing regions showing elevated [18F]FDG uptake.

For spectroscopy analyses in animals in group 4 (n=4), 1 voxel was centered in the area displaying restricted diffusion on the diffusion-weighted image acquired at 60 minutes after reperfusion. One VOI was generated by manual tracing of the infarct region showing restricted diffusion. Another VOI was generated by manual tracing of the region showing visualized reduction in CBF outside of the infarct VOI. The Wilcoxon matched-pairs signed-rank test was performed to assess significance levels in time–activity curves and Patlak compartmental analyses from PET, comparing both SUV and macro-parameters in the infarct region to the corresponding region in the contralateral hemisphere after 90-minute M2CAO and after 60 minutes of reperfusion (GraphPad Prism, San Diego, CA). Any value of \( P<0.05 \) was considered significant.

Results
One animal in group 1 did not show any infarct lesion detectable by 2,3,5-triphenyltetrazolium chloride staining. Two animals developed large infarcts covering >2/3 of the MCA territory during in-bore MRI. All 3 animals were excluded from the study. Thus, the failure rate of this method for inducing focal IS was 12% in the present study.

PET Scans of Acute Ischemia

Distribution and Dynamics of [18F]FDG Uptake
The mean blood glucose (mmol/L±SD) in the tail artery before insertion of the microcatheter was 13.0±3.69, and the mean blood glucose in the tail artery at cessation of imaging was 14.5±3.1 (group 2, n=4). The time interval between placement of the microwire tip in the distal MCA and injection of [18F]FDG in the tail vein was 8.2±2.4 minutes (groups 1 and 2, n=9).

First, we analyzed [18F]FDG-PET images summed >90 minutes of occlusion (n=9). By visual assessment, we detected a markedly elevated [18F]FDG uptake in all animals (n=9) in regions within the targeted MCA territory (Figure 1). Next, we coregistered [18F]FDG-PET images with T2-weighted MRI images from follow-up MRI (n=6). The regions showing elevated [18F]FDG uptake encompassed paretal and frontal brain regions including the ischemic region succumbing to infarction in the paretal cortex defined by an increased signal at T2-weighted MRI and a decrease in the apparent diffusion coefficient (ADC) at diffusion-weighted MRI 24 hours after occlusion (Figure 1). Furthermore, we found elevated [18F]FDG uptake in frontoparial peri-infarct regions (Figure 1), showing normal T2 signal and diffusion in MRI acquired at 24 hours. The region showing an elevated [18F]FDG uptake was traced to generate a VOI (ELEV_{voi}) for kinetic and compartmental analysis. The infarct detected by MRI and the region with elevated [18F]FDG uptake outside the final infarct were traced to generate VOIs for kinetic analysis (ISCHEMIC_{voi} and PENUMBRA_{voi}).

Analysis of Whole Region With Elevated [18F]FDG Uptake (ELEV_{voi})
The time–activity curves from the VOI traced around the final infarct showed that the SUV for the ELEV_{voi} had a slower rate than the SUV for the ischemic region (ISCHEMIC_{voi}).

![Figure 1. 2-[18F]-2-Fluoro-2-deoxy-D-glucose positron emission tomography ([18F]FDG PET) images (right column) summed over 90 minutes of occlusion of the M2 segment of the middle cerebral artery (MCA) fused (middle column) with T2-weighted MRI images (left column) acquired 24 hours after occlusion. [18F]FDG uptake is increased in brain regions with subsequent infarct development and in peri-infarct regions. Images show corresponding axial PET and MR sections from the same animal injected with [18F]FDG 10 minutes after occlusion of the M2 segment of the MCA.](image-url)
of increase, reaching maximum tissue radioactivity concentrations at later time points compared with contralateral hemispheres (mean±SD 52.3±13.9 minutes and 13.3±12.5 minutes, respectively (Figure 2A). At the end of 90 minutes of PET registration during M2CAO, the SUV (mean±SD) for the ELEV VOI was significantly higher (P=0.0039) compared with the corresponding contralateral cortex 1.660±0.3615 and 1.264±0.2060, respectively (Figure 2A). At the end of 60 minutes of PET registration during reperfusion, the SUV (mean±SD) for the ELEV VOI was significantly higher (P=0.0117) compared with the corresponding contralateral cortex 1.288±0.3040 and 1.154±0.2799, respectively (Figure 2B). Furthermore, Patlak compartmental analysis of ELEV VOI revealed a significantly increased (P=0.0039) net flux of [18F]FDG to the intracellular compartment during occlusion; K (mean±SEM) for the metabolic lesion was 0.012±0.00067 versus 0.006±0.00085 for corresponding contralateral cortex (Figure 2C). Our findings indicate that the slower inflow of [18F]FDG during occlusion causes a slighter slope of the time–activity curves, although the collateral flow is sufficient to provide [18F]FDG. This results in an elevated [18F]FDG uptake at later time points suggestive of accelerated glycolysis. The smaller differences, although statistically significant, detected during reperfusion may reflect cessation of glycolysis because of infarction.

Analysis of Increased [18F]FDG Uptake in Regions Undergoing Infarction (ISCHEMICOI) and Outside Infarction (PENUMBRAOI)

The SUV (mean±SD) for the ISCHEMICOI was significantly higher (P=0.0313) at the end of occlusion compared with

![Figure 2.](image)

**Figure 2.** A and B, Time–activity curves (TACs) from dynamic [2-18F]-2-fluoro-2-deoxy-d-glucose ([18F]FDG) positron emission tomography during (A) occlusion of the M2 segment of the middle cerebral artery (0–90 minutes) and after (B) reperfusion (90–150 minutes). TACs in A and B are sampled from volumes of interest (VOIs) defined from brain regions displaying elevated [18F]FDG uptake (n=9). Control TACs are defined by corresponding VOIs in the contralateral hemisphere. Mean time to maximum uptake for the lesion and control (tmax; tmax-control) is indicated with longer bars extending above the x-axis, whereas shorter bars extending above the x-axis represent 1 SD. C, Rates of [18F]FDG uptake (K) calculated by Patlak analysis during occlusion and reperfusion in the ischemic hemisphere (dots) and the contralateral hemisphere (squares). Dots and squares represent K values of individual animals, whereas the dotted lines represent the average of the group. A significantly increased net flux of FDG to the brain is detected during occlusion. No significant difference is detected at the group level during reperfusion. Error bars, SD; **P<0.01; *P<0.05. ns indicates not significant; and SUV, standardized uptake value.

![Figure 3.](image)

**Figure 3.** A–D, Time–activity curves (TACs) from dynamic [2-18F]-2-fluoro-2-deoxy-d-glucose ([18F]FDG) positron emission tomography during (A and C) occlusion of the M2 segment of the middle cerebral artery (0–90 minutes) and after (B and D) reperfusion (90–150 minutes). TACs in A and B are sampled from volumes of interest (VOIs) defined from infarct detected by coregistered MRI acquired at 24 hours (n=6). TACs in C and D are sampled from VOIs defined from brain regions displaying elevated [18F]FDG uptake outside of the infarct determined by coregistered MRI acquired at 24 hours (n=6). Error bars, 1 SD; **P<0.01; *P<0.05. ns indicates not significant; SUV, standardized uptake value.
the corresponding contralateral cortex, 1.506±0.3057 and 1.119±0.1290, respectively (Figure 3A). At the end of reperfusion, the SUV (mean±SD) for the ISCHEMIC_VOI was higher although not statistically significant (P=0.0625) compared with the corresponding contralateral cortex 1.698±0.5431 and 1.455±0.3800, respectively (Figure 3B).

The SUV (mean±SD) for the PENUMBRA_VOI was significantly higher (P=0.0313) at the end of occlusion compared with the corresponding contralateral cortex 1.319±0.2899 and 1.130±0.1668, respectively (Figure 3C). At the end of reperfusion, the SUV (mean±SD) for the PENUMBRA_VOI was higher although not statistically significant (P=0.0625) compared with the corresponding contralateral cortex 1.364±0.5181 and 1.259±0.5018, respectively (Figure 3D).[18F]FDG metabolism was thus markedly increased both in the ischemic tissue gradually undergoing infarction (Figure 3A) and in the surrounding penumbra (Figure 3D).

One group of animals was imaged with [18F]FDG at 24 hours after M2CAO (n=3). In all animals, a glucose hypometabolic region was detected in the targeted hemisphere. This region corresponded to the infarct lesions verified by 2,3,5-triphenyltetrazolium chloride staining (Figure I in the online-only Data Supplement).

MRI Scans of Acute Ischemia
To understand the hyperglycolytic response in stroke, we assessed factors contributing to infarct development by MRI during occlusion and reperfusion. Using the same time frame and brain ischemia protocol as for PET, we investigated changes in CBF by arterial spin labeling, restricted diffusion of water by diffusion weighted imaging, and formation of lactate by MR spectroscopy.

Infarct Development
All animals in group 4 (n=6) showed cortical focal infarcts defined by a decrease in the ADC reflecting flux of water to the intracellular compartment (Figure 4B). A decrease in ADC was detected at the first imaging time point 10 minutes after M2CAO. The mean volume (mm³±SD) of the ADC lesion at 10 and 150 minutes after M2CAO was 14.8±17.0 and 25.8±22.8, respectively. Thus, in this model, preserved

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**Figure 4.** MR-based spectroscopy (MRS), diffusion (apparent diffusion coefficient [ADC]), and perfusion (arterial spin labeling [ASL]-perfusion-weighted imaging [PWI]) analysis during occlusion and reperfusion of the M2 segment. A, MRS with volume of interest (VOI) in the ischemic region showing lactate and VOI in the peri-infarct region, with reduced cerebral blood flow (CBF), showing the absence of lactate. B, ADC MRI with corresponding (left) CBF map (right) from the same animal 90 minutes after M2 occlusion. C, Lactate measurements by MRS using a VOI placed in the center of the infarct (top red square in B; n=4). No lactate is detected before occlusion. A significant increase in lactate is detected at the first imaging time point at 30 minutes. This increase is sustained during occlusion and reperfusion. D, CBF measurements from ASL MRI in the ischemic VOI (black line) and in the peri-infarct VOI (grey dotted line; n=4). The ischemic regions of interest (ROIs) were defined by the manifest infarcts, detected by diffusion MRI, and the peri-infarct ROIs were defined by areas showing hypoperfusion on ASL images but without restricted diffusion. CBF values from the VOI over peri-infarct hypoperfused regions are significantly higher than the infarct VOI at 90 minutes (*P<0.024).
collateral flow results in gradual increase of infarction size in the acute phase.

Cerebral Blood Flow
We measured CBF during M2 occlusion and reperfusion to determine the impact of the stroke model on perfusion (group 4, n=4). The mean blood flow ratio±SD between the VOI containing ischemic tissue undergoing infarction and a corresponding VOI in the contralateral cortex during occlusion was 0.054±0.035 (30 minutes), 0.052±0.12 (60 minutes), and 0.056±0.034 (90 minutes; Figure 4D). In the VOI corresponding to peri-infarct regions showing hyperperfusion, the ratio of blood flow±SD during reperfusion was 0.14±0.056 (30 minutes), 0.18±0.12 (60 minutes), and 0.22±0.053 (90 minutes; Figure 4D). The differences in blood flow between the ischemic VOI and the peri-infarct VOI were statistically significant at 90 minutes of M2 occlusion (P=0.024; Figure 4D).

After reperfusion, blood flow in the ischemic VOI and peri-infarct VOI increased to 0.60±0.33 (30 minutes), 0.65±0.12 (60 minutes), and 0.65±0.35 (90 minutes; Figure 4D). The differences in blood flow at 30 and 60 minutes after M2 occlusion were not significant (Figure 4D).

Lactate Formation
The temporal evolution of lactate was measured in a VOI placed in the center of the ADC lesion (group 4, n=4; Figure 4A and 4B). Lactate levels were first elevated at 30 minutes after M2 occlusion and remained unchanged throughout occlusion and reperfusion (Figure 4C). In a separate group of animals (n=3), the peri-infarct region was investigated by lactate MR spectroscopy. No lactate was detected within the peri-infarct region during 90 minutes of occlusion, whereas lactate was detected consistently within the ischemic region succumbing to infarction in the same animals (Figure 4A and 4B). This data, in combination with [18F]FDG PET data, show that increased glycolysis occurs under aerobic and anaerobic conditions.

Immunohistochemistry
Cortical regions from animals in groups 1 and 4 (n=6 and 6, respectively) were analyzed by immunohistochemistry to assess cellular DNA fragmentation and BBB injury. Stainings were compared with the lesion on diffusion-weighted imaging in the corresponding MRI images. Apoptosis defined by terminal deoxynucleotidyl transferase dUTP nick end labeling stainings and morphological criteria corresponded directly to the area of restricted diffusion. DNA-fragmented cells in general, detected by terminal deoxynucleotidyl transferase dUTP nick end labeling stainings, were detected within the lesion defined by T2 MRI (Figure 5A). No terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells were detected outside of the T2 lesion (Figure 5B). Similarly, immunoreactivity for Rat IgG was confined to the T2 lesion (n=6; 24 hours; Figure 5C). Two subsets of animals were intravenously injected with FITC–dextran (4 kDa) either 10 minutes (n=3) or 24 hours (n=4) postreperfusion after M2CAO. In the 10-minute group, FITC–dextran extravasation was strictly confined to the infarct lesion. No FITC–dextran extravasation was detected in the 24-hour group (data not shown).

Discussion
Hypermetabolism of glucose in the ischemic brain has previously been detected by autoradiography and by [18F]FDG-PET. The increase in glucose metabolism has been reported to occur in peri-infarct brain regions in different models of IS in the rat. In contrast to those studies, we used a stroke model in which collateral circulation is preserved to a larger extent. We performed PET/MRI at early time points, and we observed increased [18F]FDG uptake in ischemic brain regions that undergo infarction as well as in brain regions adjacent to the final infarction volume. These findings are important because understanding the glucose metabolism pathophysiology in the context of IS is fundamental for designing optimal treatment regimens.

In this study, the experimental protocol includes PET imaging at earlier time points after vessel occlusion, ie, the tissue that later succumbs to infarction has not yet died at the time of imaging. Furthermore, we apply an occlusion model for focal cortical ischemia that does not impede collateral flow to the same extent as other models. Using MRI, we found significant blood flow differences between the VOI containing the final infarct and the VOI containing the region showing hypoperfusion outside of the ischemic region undergoing infarction, suggesting the presence of an ischemic region and a zone with reduced CBF supported by collateral flow. This would better simulate the situation in human stroke and provide a larger and more differentiated penumbral zone with a greater potential to increase glucose metabolism. Because this occlusion model preserves collateral flow to a larger extent compared with other models of stroke, the increased [18F]FDG...
uptakes in tissue that later undergo infarction could demarcate a penumbral zone, as infarct progression is slower under collateral flow.

Whether a presumed glucose hypermetabolism occurs under aerobic or anaerobic conditions is a subject of controversy. Previous studies of the ischemic rim in rats under conventional MCA occlusion suggest anaerobic as well as aerobic glycolysis. In the present study, we used MR spectroscopy to assess lactate formation in the ischemic region succumbing to infarction and in regions adjacent to the final infarct. We detected lactate in the ischemic region in all animals but not in brain regions directly adjacent to the final infarct in any animal. Considering that [18F]FDG uptake was increased in both the ischemic and peri-infarct regions at the same time points, we conclude that these increases occur under both aerobic and anaerobic conditions. Although accelerated glycolysis is the most plausible mechanism for ischemia-related glucose hypermetabolism, other explanations have been proposed, as, for example, a change in affinity for [18F]FDG over glucose, ie, a changed lumped constant in ischemic and hyperperfused tissues.

In this study, we show evidence for BBB disruption by the detection of rat IgG and terminal deoxynucleotidyl transferase dUTP nick end labeling–positive cells directly in regions directly matching the T2 MRI lesion. This corroborates the definition of ischemic and penumbral VOIs made using in vivo MR and PET. Furthermore, we detected intravenously administered FITC–dextran (4 kDa) within the infarct at 150 minutes after M2CAO but not at 24 hours, indicating BBB disruption within the infarcted tissue itself at 150 minutes after M2CAO with subsequent repair some time before 24 hours after reperfusion. A disruption of the BBB could theoretically change [18F]FDG kinetics by increased diffusion over the BBB. However, this should equally affect the forward and reverse capillary transport of [18F]FDG. In several studies, 2-DG localization has been shown to be independent of BBB disruption in tumors, indicating that the net influx of 2-DG is not increased because the reverse capillary transport is equally increased. Our data support these observations.

Conclusions

We show that focal cortical stroke produces a significantly increased uptake of [18F]FDG during occlusion in ischemic cortical regions succumbing to infarction and in cortical regions adjacent to the final infarct displaying reduction of CBF. We show that lactate formation is detectable within brain regions featuring increased [18F]FDG uptake, misery perfusion, and restricted diffusion of water, although not in regions displaying reduced CBF, demonstrating that increased glycolysis occurs under both aerobic and anaerobic conditions. These findings, in this model of focal ischemia with preserved collateral circulation, strongly suggest that hypermetabolism of glucose occurs in the ischemic penumbra. Hypermetabolism of glucose in this situation could well be a reactive response to increased energy demands, thereby aiming for limitation of the lesion development. This knowledge can be important for the interpretation and design of clinical studies aiming for glucose control in the setting of acute IS.

References


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*Stroke*. published online February 5, 2015;

*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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http://stroke.ahajournals.org/content/early/2015/02/05/STROKEAHA.114.008407

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animal preparation
All experiments were conducted according to the regulations of the Karolinska Institutet and were approved by the local laboratory animal ethics committee. Male Sprague-Dawley rats (355 - 450 g, Scanbur, Sollentuna, Sweden), were subjected to microwire occlusion of the M2 branch of the middle cerebral artery (M2CAO), as previously described (Group 1 - 6, n = 25) 1. Briefly, a microwire is navigated by fluoroscopy from the medial tail artery to a microwire tip position in the M2 branch of the middle cerebral artery (MCA). Imaging studies and post-mortem analyses were performed as shown in Table 1. Animals were anesthetized using 2% isoflurane (Virbac, Carros Cedex, France) blended with air - O₂ (7:3) during surgery and imaging. In Group 2, blood glucose levels were assessed immediately before- and 60 min after retraction of the microwire by analyzing tail artery blood samples with a glucometer (HemoCue 210⁷/201RT, HemoCue, Angelholm, Sweden).

PET

Imaging protocol
In vivo PET investigations were performed on a Focus 120 (CTI Concorde Microsystems, Knoxville, USA) microPET scanner. Animals in groups 1 and 2 were placed in the PET scanner with the brain in the field of view within five min after placement of the microwire in the MCA. [¹⁸F]FDG, administered via the tail vein (20-40 MBq, 500 µL), was an aliquot obtained from daily productions for clinical PET at the Karolinska University Hospital and had passed all quality requirements for administrations in humans. Data were collected continuously during 90 min from the time of injection. Next, the microwire was retracted and a second injection of [¹⁸F]FDG was administered via the tail vein (20-40 MBq, 500 µL) followed by data collection every second during 60 min from the time of reperfusion histogrammed as the 90 min scan but 9 frames x 300 s and physical half-life compensation following the original injected dose. Animals in Group 2 also underwent a follow-up MRI 24 h after M2CAO. Animals in Group 3 were placed in the PET scanner with the brain in the field of view 24 h after 90 min M2CAO. Data were acquired in list mode and were corrected for random coincidence, dead time and decay and histogrammed 6 frames x 10 s, 10 frames x 60 s and 15 frames x 300 s. Images were reconstructed by standard 2D filtered back projection using a ramp filter.

Quantification method
In order to estimate the radiotracer uptake, a macro parameter analysis method was used in addition to time-activity curves of the standard uptake values (SUV) ². Since [¹⁸F]FDG is trapped irreversibly intracellularly in tissues low in dephosphatases, the Patlak data-driven method was chosen ³. A data-driven kinetic model does not assume a specific compartment set-up a priori. A macro parameter requires a mathematical input function, in this case, from a volume of interest (VOI) with a pre-determined size was traced over the innominate artery using a PET image summed during the first five min of data collection. The Patlak method gives the $K_i$ value, the so-called influx constant as a quantification of the net uptake of the radiotracer. The VOIs were chosen using a dual-modality approach, where both a functional (PET) and an anatomical (MRI, 9.4T) image were obtained for some animals (n = 6).
MRI

Imaging protocol
The MRI experiments were conducted using a horizontal 9.4 T magnet (Varian, Yarnton, UK) equipped with a 12 cm inner diameter gradient system with maximum gradient strength of 600 mT/m. A birdcage resonator was used for excitation (Rapid Biomedical GmbH, Würzburg-Rimpar, Germany) and a ‘rat head’ 4-channel phased array surface coil (Rapid Biomedical GmbH, Würzburg-Rimpar, Germany) served as the receiving coil. For perfusion measurement, an arterial spin labeling surface coil (Rapid Biomedical GmbH) was additionally introduced and placed 2 cm away from the head coil underneath the neck area. Within 5 min after placement of the microwire in the M2 branch of the MCA, animals in Groups 4 and 5 were placed in the MRI scanner. The physiological state of the anaesthetized rats was monitored and maintained during scanning (SA-instruments, Stony Brook, NY, USA). Imaging data were collected during 90 min. Next, the microwire was retracted followed by data collection for an additional 60 min.

T2 and Diffusion tensor imaging
Diffusion tensor images were acquired using multi-slice three-shot spin-echo echo-planar imaging (EPI) sequence in transverse plane (14 slices of 1mm thickness) with repetition time (TR) 3 s, echo time (TE) 25 ms, field of view $32 \times 32$ mm$^2$, matrix size $96 \times 96$, in-plane resolution of $33 \times 33$ μm, diffusion gradient duration ($\Delta$) 2.3 ms and diffusion gradient separation (δ) 6.5 ms. Diffusion sensitizing gradients were applied along 12 directions with two diffusion sensitizing factors $b = 0$ and 1000 s/mm$^2$.

Arterial spin labeling
Perfusion measurements were performed using three-shot gradient EPI with field of view $32 \times 32$ mm$^2$, matrix size $96 \times 96$, TR 6.15 s, TE 10.5 ms and 14 slices of 1 mm thickness with no gap in between. Continuous arterial spin labeling was accomplished by applying an off-resonance radio frequency (RF) power to the arterial spin labeling (ASL) coil in the presence of a 1 Gauss/cm gradient during TR. The labeling plane was located 2.4 cm upstream from the imaging slice package and a transit time (TI) of 2 ms was assigned to the labeled blood moving away from the tagging plane. A pair of tagged and control images were acquired for each slice. To acquire the control image, the tag position was reversed by switching the sign of the labeling offset-frequency, resulting in the tagging plane being moved downstream to the imaging plane.

Spectroscopy
$^1$H MRI spectra were acquired using point resolved spectroscopy (PRESS) sequence from a volume of interest (VOI) ($2.5 \times 1.7 \times 2.5$ mm$^3$). Two separate VOIs were centered in the area displaying restricted diffusion and in the peri-infarct region on the diffusion tensor images. The water suppressed spectrum was acquired with TR 3s, a total TE 15.66 ms, 3 ppm offset from water signal, spectral width 8013Hz, 4096 complex data points and 32 scans, with a total acquisition time of 1.42 min. The water signal was suppressed with variable power RF pulses and optimized relaxation delays (VAPOR). A water reference scan was recorded per each water-suppressed spectrum for quantification and eddy current compensation purposes. The gradient polarity was reversed to compensate for the chemical shift displacement artifacts due to spectral lipid contamination.
Immunohistochemistry

The animals in groups 1-3 were sacrificed at 24 h after MCAO and animals in group 4 were sacrificed 48-96 h after M2CAO. Animals in group 6 were sacrificed 10 min after reperfusion, following the 90 min of M2CAO. The brains from animals in group 1 and 3 were removed immediately after sacrifice. Six coronal 2 mm cryosections were taken throughout the brain and immersed in a 2 % solution of 2,3,5-triphenyltetrazoliulm chloride (TTC) in normal saline at 37°C for 30 min, after which the sections were fixed in 10 % phosphate buffered formalin for photography. The brains from animals in groups 2, 4 and 6 were removed and snap frozen in isopentane-dry ice. The animals in groups 2 and 6 were injected with fluorescein isothiocyanate (FITC)-dextran (4kDa, Sigma-Aldrich; 500 ml of 50mg/ml) in phosphate-buffered saline via the tail vein immediately after reperfusion (group 6, n = 3) and after 24h (group 2, n=4). The animals were sacrificed 15 min after the injection. Coronal 14 µm cryosections were taken at -15°C throughout the infarct and peri-infarct regions using a Leica cryostat (CM3000, Leica Instruments GmbH, Nussloch, Germany). The sections were thaw mounted and stored at -20°C prior to use.

To visualize apoptotic staining, the ApopTag Fluorescein or ApopTag Red Detection kits were used following the protocol suggested in the kit. Briefly, sections were placed in fresh 4% paraformaldehyde solution, post-fixed in ethanol:acetic acid solution and incubated in TdT solution for one hour at 37°C. Stop/wash buffer was added and the slides were finally incubated with Anti-Digoxigenin Conjugate solution for 30min. Then rabbit anti-Glut1 primary antibody (Atlas Antibodies) diluted 1/1000 in 0.3% TX-100, 0.1% NaN₃ in phosphate buffered saline was added. Following an overnight incubation, sections were washed in Tris-HCl buffered saline (pH 7.4) containing 0.5% tween, blocked (Perkin Elmer) and incubated in blocking buffer containing anti-rabbit and anti-rat secondary antibodies (1/200, Jackson) followed by several washes in Tris-HCl buffered saline containing 0.5% tween. All sections were counterstained with the nuclear marker Hoechst (1/5,000) and mounted with polyvinyl alcohol/glycerol containing 2.5% DABCO (Sigma).

Fluorescence microscope images were acquired on a Vslide slide scanning microscope (MetaSystems, Alltlussheim, Germany) equipped with a CoolCube 1 camera (12 bit grey-scale), 2.5x, 5x, 10x and 20x objectives and filter sets for DAPI (EX350/50 - EM470/40), FITC (EX493/16 – EM527/30), Cy3 (EX546/10 – EM580/30), Cy3.5 (EX581/10 – EM617/40) and Cy5 (EX630/20 – 647/long pass). Whole microscope slides were scanned at 2.5x and tissue was detected based on the Hoechst 33342 signal. After generating a position map all tissue covered areas were scanned using 20x primary objective. Individual field of view images were stitched to generate a large 4-channel fluorescence image of the entire specimen with microscopic resolution.

Image and Statistical analysis

PET data were processed with MicroPET manager and evaluated using the Inveon Research Workplace (Siemens Healthcare, Erlangen, Germany) software. MRI data were processed with VnmrJ software (Agilent Technologies, Palo Alto, CA, USA) and evaluated using ImageJ (National Institutes of Health, Maryland, USA). MRI and PET images from animals in group 1 (n = 6) were co-registered using the Inveon Research Workplace and exported to ImageJ for analysis. ROIs were manually traced for the brain regions showing altered metabolism and infarct.

The Wilcoxon matched-pairs signed rank test was performed to assess significance levels in time activity curves and Patlak compartmental analyses from PET, comparing both SUV and macro parameters in the infarct region to the corresponding region in the contralateral
hemisphere at 90 min of imaging (GraphPad Prism, San Diego, CA, USA). Any value of p < 0.05 was considered significant.


Supplemental Figure I

$[^{18}F]$FDG uptake reduction in the established infarct 24 hours after ischemia compared to contralateral side. (B) Standardized uptake value (SUV) ratio between infarct region of interest (ROI) and corresponding contralateral ROI showing a lower uptake of FDG in the infarct at 24h after reperfusion (n = 3). (A) FDG PET image and (C) corresponding TTC staining from the same animal showing the decreased uptake of $[^{18}F]$FDG in the infarct.