Impairment of Metabolic Recovery With Increasing Periods of Middle Cerebral Artery Occlusion in Rats

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We examined the consequences of reflow on metabolic recovery following increasing periods of focal ischemia. The middle cerebral artery of 21 Sprague-Dawley rats was occluded with a snare ligature for 1, 2, or 6 hours followed by 5, 4, or 0 hours of reflow, respectively (seven rats in each group). All animals were injected with neutral red for visual confirmation that the affected regions were reperfused. The brains were frozen in situ, and the concentrations of adenosine triphosphate, phosphocreatine, glycogen, and lactate were determined in those areas corresponding to the normally perfused medial ipsilateral cortex, the perifocal region, and the ischemic focus. Values for the 6 hours' occlusion with no reflow group served as a control to demonstrate restoration of metabolite concentrations. In both groups with reflow, the levels of high-energy phosphates were greater than control, but this effect of reflow was primarily significant for the group with 1 hour's occlusion \(p<0.05\). The levels of glycogen and lactate provided additional evidence that the extent of metabolite restoration was graded; following 2 hours of occlusion, metabolite recovery was compromised \(p<0.05\). Our data strongly support the concept that the window of opportunity for effective treatment of focal ischemia by reperfusion is narrow (of short duration). (Stroke 1990;21:467-471)

Removing a focal obstruction to brain blood flow, whether by medical or surgical methods, has been considered a useful procedure for the treatment of stroke. Such therapy, however, is not without hazards. Experiments have shown that reperfusion can aggravate brain edema, hemorrhagic infarction, and brain herniation.\(^1-5\) We sought to determine the effects of reperfusion, instituted at different times after the onset of ischemia, on the metabolic processes of both the focal and the perifocal ischemic regions. Results from models of global ischemia suggest that, despite reperfusion, many neurologic signs persist and brain damage develops after relatively brief periods of ischemia.\(^6,7\) The minimum duration for the onset of irreversible brain damage and neurologic dysfunction in models of focal ischemia, however, has not been as clearly defined owing primarily to the inherent variability in these models.\(^8\) Experience from models of global ischemia suggest that reperfusion would be of little value after 15 minutes of ischemia. The purpose of our investigation was to determine if the relation between duration of ischemia and irreversible metabolic derangements applies equally to models of focal ischemia. A preliminary report of this data has been presented.\(^9\)

Materials and Methods

Twenty-three adult male Sprague-Dawley rats weighing 250–300 g were fed ad libitum until 24 hours before the procedure, at which time the food was removed. All rats were housed and cared for at the Department of Animal Facilities, Case Western Reserve University School of Medicine and were treated in strict compliance with the institution's Animal Care and Use Standards, which conform to those of the American Association of Accreditation of Laboratory Animal Care. Anesthesia was induced with 4% halothane, and intubation was performed with a 12-gauge intravenous catheter. The rats were ventilated with a gas mixture of 2% halothane and 70% \(\text{N}_2\text{O}\)/balance \(\text{O}_2\) regulated by a Forreger low-pressure anesthesia delivery machine (Model 100, Smithtown, New York) administered by a Harvard rodent ventilator (Model 880, South Natick, Massa-
chusetts). Rectal temperature was monitored with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) coupled to a 125-W infrared lamp to maintain normothermia.

The proximal middle cerebral artery (MCA) was occluded in all rats using an approach similar to that described by Tamura et al except that a snare was fashioned from 10-0 monofilament nylon suture and a small Silastic tube. The suture was placed proximal to the rhinal branch of the MCA. Blood flow was stopped by tying the suture at the distal end of the Silastic tube. Visual inspection of the artery inside the Silastic tube confirmed occlusion. A small piece of oxidized cellulose was then placed in the bony defect, the muscle was repaired anatomically, and the skin was sutured.

Sixteen rats were briefly intubated a second time after 1 (eight rats) or 2 (eight rats) hours of MCA occlusion; the rats were anesthetized with the same regimen, the snare ligature was cut, and the MCA was examined for evidence of reflow. After anesthesia, each rat's rectal temperature was monitored and normothermia was maintained until the animal was fully awake.

The brains of all rats were fixed by in situ freezing with liquid nitrogen according to the method of Ponten et al. Briefly, the rats were anesthetized again as described above after 6 hours of occlusion or 4 or 5 hours of reflow, and both the femoral artery and vein were cannulated. Once normocapnia and normoxia were established by blood gas analysis, 2 ml of 2% neutral red was administered to confirm reperfusion of the affected regions. If neutral red was not present in the cerebral cortex ipsilateral to the occlusion in the two groups with reflow, the rat was not included in this study. Only two animals (one subjected to 1 hour and the other subjected to 2 hours of occlusion) early in the series failed to achieve reperfusion secondary to attempts to achieve hemostasis by bipolar coagulation. Blood pressure was continuously monitored, and just before in situ fixation the halothane was discontinued.

We used 21 rat brains (seven in each group) for metabolic analyses. The brains were removed at -20°C, sectioned at thicknesses of 20 µm, and lyophilized for 1 day at -40°C. The freeze-dried sections were dissected, and concentrations of the metabolites were measured as previously described. Three pieces of tissue from the cortex of each brain slice contralateral to the occlusion were dissected and analyzed; the mean of these three determinations served as the value with which to compare metabolite concentrations in samples dissected from the medial to lateral areas of the cortex ipsilateral to the MCA occlusion.

Significant differences (p<0.05) were determined by analysis of variance with Duncan's post hoc analysis for comparisons between groups.

Results

Tissue samples were dissected from the various areas of the cerebral cortex depicted in Figure 1.

While the pattern of altered perfusion elicited by MCA occlusion was not always consistent among rats, the ischemic focus observed in rats with no reflow was generally contained within areas 7 and 8, whereas areas 2 and 3 were served by the anterior cerebral circulation. The perifocal region was generally found in areas 4–6, but this varied among the rats and depended on the duration of reflow.

The concentrations of adenosine triphosphate (ATP) in areas 2–8 gradually decreased from those of area 1, but ATP stores were depleted in area 8 only after 6 hours of MCA occlusion (Figure 2). The ATP levels in areas 2–8 after 5 hours of reflow following 1 hour of occlusion were essentially restored (different from those in the same areas of
the no-reflow group). In contrast, 4 hours of reflow following 2 hours of MCA occlusion only partially restored the ATP levels, and they were significantly different from those in the no-reflow group only in the ischemic focus. In areas 4, 6, and 7 the ATP levels in the group with 5 hours of reflow following 1 hour of occlusion were significantly higher than those in the other two groups, suggesting that there was a residual deficit in energy metabolism following 2 hours, but not 1 hour, of MCA occlusion.

Phosphocreatine (P-creatine) is a high-energy phosphate donor that rapidly regenerates the ATP pool under conditions of metabolic stress. A pattern for P-creatine similar to that for ATP was observed after 6 hours of MCA occlusion (Figure 3). Relative to the no-reflow group, there was an only marginal increase in the concentration of P-creatine after 4 hours of reflow following 2 hours of occlusion, and the difference was significant only in area 7. Although the P-creatine concentration fluctuated across areas in the group with 5 hours of reflow following 1 hour of occlusion, the levels essentially returned to those of area 1 in areas 2–8, and in most areas the values were significantly higher than those in at least one of the other two groups.

The glycogen profile in areas 2–8 was quite different from that for the high-energy phosphates. In areas 2 and 3, the glycogen concentrations were greater than those in area 1, and this effect was evident in all three groups (Figure 4). Reperfusion partially restored the levels of glycogen, but the increases were significant only in areas 7 and 8. Nevertheless, the glycogen concentrations in areas 2–8 of the two reflow groups did not differ significantly from those in area 1.

Relative to area 1, the lactate levels increased almost 10-fold in the ischemic focus of the no-reflow group (Figure 5). While there were significant decreases relative to the no-reflow group in lactate concentrations after 4 or 5 hours of reflow, the concentrations remained elevated relative to area 1 in most samples. Of note in the group with 5 hours of reflow was the elevation in areas 5 and 6, that is, the perifocal region, areas that by all other metabolite concentrations were normalized.

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**Figure 3.** Graph of concentrations of phosphocreatine in various areas of rat cerebral cortex following reversible focal ischemia. ●, 6 hours of unilateral middle cerebral artery occlusion followed by no reflow; ▲, 2 hours of occlusion, followed by 4 hours of reflow; ■, 1 hour of occlusion followed by 5 hours of reflow. Each point is mean of seven rats. 1, significantly (p<0.05) different from ●; 2, significantly different from ▲.

**Figure 4.** Graph of concentrations of glycogen in various areas of rat cerebral cortex following reversible focal ischemia. ●, 6 hours of unilateral middle cerebral artery occlusion followed by no reflow; ▲, 2 hours of occlusion followed by 4 hours of reflow; ■, 1 hour of occlusion followed by 5 hours of reflow. Each point is mean of seven rats. 1, significantly (p<0.05) different from ●; 2, significantly different from ▲.

**Figure 5.** Graph of concentrations of lactate in various areas of rat cerebral cortex following reversible focal ischemia. ●, 6 hours of unilateral middle cerebral artery occlusion followed by no reflow; ▲, 2 hours of occlusion followed by 4 hours of reflow; ■, 1 hour of occlusion followed by 5 hours of reflow. Each point is mean of seven rats. 1, significantly (p<0.05) different from ●.
Discussion

Models of focal ischemia are, for a number of reasons, more clinically relevant than models of global ischemia to the study of stroke in humans. Our understanding of the pathogenetic events in focal ischemia is lacking, owing primarily to the inherent variability of such models. With the advent of novel procedures to remove obstructions to blood flow in the brain, it is crucial to examine experimentally the risks and benefits of such strategies, especially with respect to the duration of ischemia. A priori, there is no particular reason to expect that brain cells could tolerate longer periods of focal than global ischemia. Experience from models of global ischemia has shown that neurologic dysfunction is manifested during recirculation after 10–15 minutes of ischemia and that the dysfunction increases in intensity after longer periods of ischemia. Similarly, the apparent risk of reperfusion of the ischemic region would be expected to increase after longer periods of focal ischemia. This is supported by several studies that have shown that reflow can actually exacerbate the damage to the brain following focal ischemia. The mechanisms for the enhanced injury induced by reperfusion and the duration of ischemia necessary to elicit the response have received only limited attention.

The ability of ischemic tissue to recover function requires the generation of energy to reestablish the electrolyte imbalances between the intracellular and extracellular compartments that arise during the insult. It has been shown with models of global ischemia that the restoration of energy-related metabolite concentrations during reflow correlates well with the evolution of brain damage. In both gerbils and rats, the rate of restoration of concentrations of high-energy phosphates and glucose-related metabolites during reflow decreases with increasing periods of ischemia. The metabolite concentrations were eventually restored after 2–4 hours of recirculation, and full restoration was compromised only after periods of ischemia that were incompatible with survival of the animal. From these studies on reflow following global ischemia, it is apparent that the concentrations of select metabolites or their rates of restoration might be valid predictors for evaluating outcome following focal ischemia. Measurement of a single metabolite could, however, lead to an erroneous conclusion. For example, the concentrations of both P-creatine and glucose increase during reperfusion to greater than control in animals destined to exhibit neurologic signs of brain damage. A similar effect has been noted during reflow following 1 hour of unilateral ischemia in gerbils. For this reason, we examined a profile of four metabolites in various areas of the cortex following focal ischemia.

Our results suggest that restoration of the concentrations of high-energy phosphates, glycogen, and lactate subsequent to reperfusion depends on the duration of the ischemic episode. While the recovery of energy metabolite concentrations appears to be complete after 5 hours of reflow following 1 hour of MCA occlusion, recovery is compromised after 4 hours of reflow following 2 hours of MCA occlusion. Since ATP and P-creatine concentrations could be restored only if reperfusion had been established, the increase in concentrations of these metabolites in the group with 4 hours of reflow following 2 hours of occlusion over those in the no-reflow group indicates that reperfusion of the affected region had occurred, and this was confirmed by the neutral red data (not shown). Even though these ischemic periods are substantially longer than those normally used in models of global ischemia, there appears to be a biochemical lesion arising after 2 but not 1 hours of ischemia. Levels of ATP and P-creatine were depressed after 4 hours of reflow, a time sufficient for near-total restoration of the concentrations of these metabolites following 15 and 30 minutes of global ischemia in rats and gerbils, respectively. The high-energy phosphate data in the group with 4 hours of reflow following 2 hours of occlusion suggest that the insult in the ischemic focus was equal to or greater than that following a similar period of global ischemia. Despite the disparity in metabolite concentration recovery after reflow following 1 and 2 hours of MCA occlusion, it is not clear if the functional or histopathologic outcome between the two groups would differ with longer periods of reflow; ultimately, these are the final criteria for establishing the efficacy of reperfusion.

The depression of high-energy phosphate recovery after 4 hours of reflow following 2 hours of MCA occlusion could be related to disruption of mitochondrial function. It has been reported that mitochondrial function is relatively insensitive to prolonged periods of global ischemia. In one study, however, State 3 respiratory activity in isolated mitochondria after 1 hour of global ischemia decreased by almost 50% after 2 hours of global ischemia in gerbils, and this corresponds well with the decrease in high-energy phosphate restoration after focal ischemia that we saw. It is also possible that reperfusion imposes an additional insult on the mitochondria, and this might further compromise their function. Reintroduction of oxygen and glucose to a tissue deprived of energy elicits a number of responses, including lipid peroxidation and the accumulation of calcium, that could directly affect mitochondrial function and ultimately the viability of the brain.

The fate of the perifocal region may differ from that of the ischemic focus since previous studies have shown that energy metabolism in this region deteriorates gradually, even though the perifocal region does become infarcted by 1 day after MCA occlusion. Since the size of the perifocal region varied among the rats in the two reflow groups, it is difficult to conclude if the restoration of ATP and P-creatine concentrations was less affected by the duration of the MCA occlusion. Since there appeared to be a trend toward greater energy reserves in the perifocal
region, it is possible that the additional risk of reperfusing ischemic tissue may be offset by saving the perifocal region from further metabolic and structural deterioration.

The observed elevation of lactate levels may indicate a persisting acidosis, which would further hinder the normalization of metabolism. In most models of global ischemia, lactate concentration is generally restored after 4 hours of recirculation.\(^6\) It is presently unclear why the lactate levels in our investigation remained elevated after 4 and 5 hours of reflow following 2 and 1 hours of occlusion, respectively.

The elevation of glycogen concentration in areas 2 and 3 (served by the anterior cerebral circulation) indicates that the effect of ischemia is not limited to the ischemic focus and perifocal region, but extends to areas beyond the MCA perfusion territory. If this is a type of metabolic diaschisis secondary to loss of input from the ischemic regions, then reflow apparently has not restored function in the affected regions. Alternatively, it is possible that the elevation in glycogen concentration is attributable to paralysis of the tissue, thus decreasing its energy demands, resulting in an accumulation of glycogen.\(^23\) Since the ultimate aim of our research was to minimize the extent of brain damage following focal ischemia, it becomes clear that the different conditions of the various compartments affected by focal ischemia may require different therapeutic approaches. For example, treatment for the dysfunctional tissue within the anterior cerebral circulation, where the energy status of the tissue is normal, would differ markedly from that for tissue in the ischemic focus exhibiting mitochondrial dysfunction.

In summary, the ability of reperfusion to restore metabolic viability is impaired with increasing periods of focal ischemia. This biochemical impairment of energy metabolism may contribute to the irreversible brain damage arising in the ischemic focus.

References


Key Words • cerebral ischemia • metabolism • reperfusion • rats
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