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EXTENSION OF THE NEUROPROTECTIVE TIME WINDOW FOR THIAZOLIDINEDIONES IN ISCHEMIC STROKE IS DEPENDENT ON TIME OF REPERFUSION

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Abstract—Stroke is a leading cause of death and disability but has limited therapeutic options. Thiazolidinediones (TZDs), agonists for the nuclear receptor, peroxisome proliferator-activated receptor (PPAR)γ, reduce infarct volume and improve neurologic function following transient middle cerebral artery occlusion (MCAO) in rats. Translation of these findings into clinical therapy will require careful assessment of dosing paradigms and effective time windows for treatment. Understanding the mechanisms by which TZDs protect the brain provides insight into how time windows for neuroprotection might be extended. We find that two TZDs, pioglitazone and rosiglitazone, significantly reduce infarct volume at doses similar to those used clinically (1 mg/kg for pioglitazone and 0.1 mg/kg for rosiglitazone). We also find that pioglitazone reduces infarction volume in a transient, but not a permanent MCAO model suggesting that reperfusion plays an important role in TZD mediated neuroprotection. Since PPARγ agonists reduce inflammation and oxidative stress, both of which are exacerbated by reperfusion, we hypothesized that TZDs would be most effective if administered prior to reperfusion. We administered TZDs 3 h after MCAO and found that infarction volume and neurologic function are significantly improved in animals reperfused at 3 h and 15 min (after TZD treatment), but not in animals reperfused at 2 h (before TZD treatment) when assessed either 24 h or 3 weeks after MCAO. While TZDs reduce intercellular adhesion molecule (ICAM) expression to a similar extent regardless of the time of reperfusion, leukocyte entry into brain parenchyma is more dramatically reduced when reperfusion is delayed until after drug treatment. The finding that delaying reperfusion until after TZD treatment is beneficial despite a longer period of ischemia, is dramatic given the widely held view that duration of ischemia is the most important determinate of injury. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: cerebral ischemia, neuroprotection, pioglitazone, rosiglitazone, PPARγ.

The only Federal Drug Administration (FDA) approved therapy for ischemic stroke is early reperfusion using thrombolytic medication. Although reperfusion is critical to restore blood flow to ischemic tissue, it is also associated with the induction of oxidative stress and a robust inflammatory response that can further exacerbate injury. Numerous agents targeting these processes are protective in animal models; however, translation to effective clinical therapy remains elusive. Treatment of stroke is particularly challenging because of the rapid pace of injury, and it is widely believed that the failure to translate laboratory findings into clinical therapy is due to the difficulty in administering drugs before irreversible injury occurs. Drugs with the most therapeutic potential will be those that can be given to patients quickly, preferably, those that can be administered prior to hospital evaluation. Understanding the time window for therapy will be critical to successful translation of neuroprotective therapy for stroke.

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor (PPAR)γ agonists that we have found, reduce infarct volume and improve neurologic function following cerebral ischemia in rats (Sundararajan et al., 2005; Victor et al., 2006). These findings have been validated by several independent laboratories (Allahtavokoli et al., 2006; Luo et al., 2006; Pereira et al., 2006; Shimazu et al., 2005; Tureyen et al., 2007; Zhao et al., 2005). PPARγ forms a heterodimer with retinod X receptor (RXR) and binds a PPAR response element (PPRE) in the promoter of target genes inducing gene expression. In addition, activated PPARγ suppresses inflammatory gene expression by transrepression of other transcription factors. In the presence of ligand, PPARγ binds small ubiquitin-like modifier (SUMO1) and stabilizes the co-repressor complex on the promoter of pro-inflammatory genes preventing the transcription factor, nuclear factor κB (NFκB), from binding to the promoter and initiating pro-inflammatory gene ex-

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Abbreviations: ANOVA, analysis of variance; AP-1, activator protein 1; CBF, cerebral blood flow; CT, computed tomography; Ct, cycle threshold; Cu/Zn, copper/zinc; DMSO, dimethyl sulfoxide; FDA, Federal Drug Administration; HPF, high powered field; ICAM, intracellular adhesion molecule; -IR, immunoreactivity; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score; MRI, magnetic resonance imaging; NFκB, nuclear factor κB; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PGJ2, 15-deoxy-Δ(12,14)-prostaglandin J2; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; ROS, reactive oxygen species; RXR, retinod X, receptor; SEM, standard error of the mean; STAIR, stroke academic industry roundtable; STAT-1, signal transducers and activator of transcription 1; SUMO1, small ubiquitin-like modifier; TTC, triphenyl tetrazolium chloride; TZD, thiazolidinedione; US, United States.

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pression (Straus and Glass, 2007). In ischemic stroke models, TZD-mediated neuroprotection is associated with reduced inflammatory infiltrate and pro-inflammatory gene expression (Allahtavokoli et al., 2006; Luo et al., 2006; Pereira et al., 2006; Shimazu et al., 2005; Sundararajan et al., 2005; Tureyen et al., 2007; Zhao et al., 2005). In addition, PPARγ agonists reduce the formation of superoxide anion in vascular endothelial cells and increase the expression of the free radical scavengers superoxide dismutase and catalase (Hwang et al., 2007; Shimazu et al., 2005). Reductions in both inflammation and oxidative stress likely contribute to PPARγ agonist mediated neuroprotection.

TZDs act as insulin sensitizers and two drugs, pioglitazone and rosiglitazone, are FDA approved for treatment of type 2 diabetes. The most serious side effect, congestive heart failure, occurs after several weeks of daily use and is reversed after discontinuation of the drug (Tang and Maroo, 2009). It is unlikely that congestive heart failure would be a consequence of a single dose of TZD. Importantly, both rosiglitazone and 15-deoxy-Δ(12,14)-prostaglandin J2 (PGJ2), an endogenous PPARγ ligand, are beneficial in a rodent hemorrhage model (Zhao et al., 2006, 2007) suggesting that PPARγ ligands might be given safely before differentiating cerebral ischemia and hemorrhage by computed tomography (CT) scanning, thereby allowing TZDs to be given before hospital evaluation.

In the current study we explore optimal TZD dosing and the therapeutic time window of efficacy following middle cerebral artery occlusion (MCAO) using the suture model of proximal MCAO in rats. We confirm previous findings that pioglitazone is protective in transient but, not permanent ischemia. In addition, we formally test the hypothesis that TZDs are most effective when administered prior to reperfusion by administering TZDs 3 h after MCAO and varying the time of reperfusion relative to MCAO. Outcome, assayed by both infarction volume and behavioral function, is improved in drug treated animals that are reperfused after drug treatment despite the longer duration of ischemia. Finally, we examined leukocyte infiltration, a feature of reperfusion injury using real time polymerase chain reaction (PCR) to examine intracellular adhesion molecule (ICAM) mRNA and immunohistochemistry to examine ICAM and myeloperoxidase protein.

EXPERIMENTAL PROCEDURES

Rats

Male Wistar rats, 250–300 g, were obtained from Charles River (Wilmington, MA, USA). Animals were housed and cared for in the Animal Resource Center and allowed free access to food and water before and after surgery. All procedures were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and in accordance with the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Transient middle cerebral artery occlusion

MCAO was performed using the suture method as described by Longa et al., (1989), modified by Kawamura et al., (1994) and previously described by ourselves (Sundararajan et al., 2005). All surgery was performed using continuously inhaled short acting anesthetics delivered by nose cone. Initial experiments used halothane, however, halothane was no longer available in the US when the experiments utilizing 3 week survival were conducted and, therefore, those experiments utilized isoflurane. The tail artery was cannulated to measure arterial blood pressure. A small hole was drilled in the skull using a dental drill at the intersection of the coronal and sagittal sutures on the left over the region of the MCA. A probe holder (PH07-4; Perimed AB, Järfälla, Sweden) was glued into place in order to ensure constant probe position over time. A laser Doppler probe (407; Perimed AB, Järfälla, Sweden) was inserted into the probe holder and cerebral blood flow (CBF) measured using the Periflux 5000 system and Perisoft software (Perimed AB, Järfälla, Sweden). A vertical incision was made in the neck, the common carotid artery was tied with a reversible knot and the external carotid artery was isolated and ligated. A 4.0 nylon suture with a flame rounded tip was advanced from the external carotid artery into the internal carotid artery to occlude the MCA while CBF was monitored. Following suture insertion, the laser Doppler probe was removed, although the probe holder was left in place. The wounds were closed and the inhaled anesthetic gas removed, allowing the animals to regain consciousness within a few minutes. At the time of reperfusion, which varied depending on the experiment, rats were re-anesthetized, the neck wound was reopened, the laser Doppler probe reinserted into the probe holder and suture placement confirmed. CBF was measured and the suture was removed. The ligature on the common carotid artery was released. Reperfusion of the artery was confirmed visually in the neck and using CBF within the brain. The laser dopper probe and the probe holder were removed, all wounds were closed and the animals allowed to recover. Arterial blood pressure and arterial blood gases were monitored and kept within normal parameters throughout the procedure. Temperature was maintained between 36.5 and 37.5 °C during the procedure. In accordance with guidelines Stroke Therapy Academic Industry Roundtable, 1999 (STAIR) only animals with drops of greater than 60% CBF at the time of MCAO were included in the analysis. In addition, animals with no increase in CBF at the time of suture removal (no reperfusion), infarction in the anterior cerebral artery (ACA) distribution (indicating improper suture placement) or obvious sub-arachnoid blood (due to vessel perforation) were excluded from analysis as they represent surgical failures. The number of animals excluded from analysis for surgical failures was equivalent in different treatment groups (Table 1).

Permanent middle cerebral artery occlusion

The procedure for permanent MCAO was identical to that of transient MCAO except that suture was not removed and the MCA, therefore, not reperfused.

Drug treatment

Animals were treated with either dimethyl sulfoxide (DMSO) 0.4 ml/kg, pioglitazone (Takeda Pharmaceuticals, North America, Lincolnshire, IL, USA) or rosiglitazone (GlaxoSmithKline Pharmaceuticals, Harlow, England) dissolved in 0.4 ml/kg DMSO. All drug treatments were administered i.p. While initial experiments utilized a pretreatment paradigm in order to efficiently define optimal dosing and treatment protocols, subsequent experiments examining neuroprotection time windows utilized TZD treatment 3 h after MCAO. Dose response studies tested pioglitazone (0.5, 1, 3.5 and 7 mg/kg) or rosiglitazone (0.05, 0.1, 0.35, 1 mg/kg) administered 24 h before and at the time of MCAO. The maximally effective doses of 1 mg/kg pioglitazone and 0.1 mg/kg rosiglitazone were utilized in all other studies.

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