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Exacerbated Brain Damage, Edema and Inflammation in Type-2 Diabetic Mice Subjected to Focal Ischemia

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Abstract

One of the limiting factors in stroke therapeutic development is the use of animal models that do not well represent the underlying medical conditions of patients. In humans, diabetes increases the risk of stroke incidence as well as post-stroke mortality. To understand the mechanisms that render diabetics to increased brain damage, we evaluated the effect of transient middle cerebral artery occlusion (MCAO) in adult *db*/*db* mice. The *db*/*db* mouse is a model of type-2 diabetes with 4 times higher blood sugar than its normoglycemic genetic control (*db*/+ mouse). Following transient MCAO, the *db*/*db* mice showed significantly higher mortality, bigger infarcts, increased cerebral edema, worsened neurological status compared to *db*/+ mice. The *db*/*db* mice also showed significantly higher post-ischemic inflammatory markers ($ICAM1^+$ capillaries, extravasated macrophages/neutrophils and exacerbated proinflammatory gene expression) compared to *db*/+ mice. In addition, the post-ischemic neuroprotective heat-shock chaperone gene expression was curtailed in the *db*/*db* compared to *db*/+ mice.

Keywords

Hyperglycemia; Diabetes; Inflammation; Infarction; Stroke; Edema

Type-2 diabetes predisposes humans to stroke, and stroke-induced brain damage is known to be exacerbated with poor functional recovery in these patients (Ergul *et al* 2009). Although >30% of stroke sufferers are known to be diabetic, the mechanisms that are responsible for the increased post-ischemic brain damage in this population are understudied. Diabetes occurs in 2 major forms; while type-1 diabetes (early onset) is characterized by insulinopenia caused by the destruction of the insulin producing beta islet cells of the pancreas, type-2 diabetes (adult onset in most cases) results from peripheral insulin resistance. A commonality between the 2 conditions is the hyperglycemia which is detrimental to cognition and other brain functions. Of the ~200 million diabetics worldwide, >90% are type-2 diabetics. The genetically altered *db*/*db* mouse (Hummel *et al* 1966) manifests adult onset type-2 diabetes and hence might be a useful animal model to study mechanisms and to test novel therapeutic compounds to prevent ischemic brain damage in diabetic cohort. In the *db*/*db* mouse, the diabetic gene (*db*) which encodes for a G-to-T point mutation of theleptin receptor, leading to abnormal splicing and defective signaling of the adipocyte-derived hormone leptin is transmitted as an autosomal recessive trait. In these

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mice absence of hypothalamic leptin signaling leads to hyperphagia and obesity culminating in high leptin and insulin levels (Chen *et al* 1996; Lee *et al* 1996). The *db*/*db* mice start to develop diabetic symptoms around 6 weeks and will become frank diabetics by 12 weeks of age (Sharma *et al* 2010).

Using adult *db*/*db* mice and their normoglycemic genetic control (*db*/+ mice), we presently evaluated the effect of transient focal ischemia on the mortality rate, infarct volume, neurological dysfunction, cerebral edema, inflammatory indices (ICAM1 expression, neutrophil infiltration, microaglia/macrophage activation and proinflammatory gene expression) and heat-shock protein (HSP) gene expression.

MATERIALS AND METHODS

Focal Ischemia

All the surgical procedures were approved by the Research Animal Resources and Care Committee of the University of Wisconsin-Madison and the animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals, U.S. Department of Health and Human Services Publication Number. 86–23 (revised*). The *db*/*db* (C57BLKS/J-m+/+*Lepr*$^{db/db}$; type-2 diabetic) and *db*/+ (normoglycemic genetic controls of *db*/*db*) mice (n = 35/genotype) were obtained from the Jackson Laboratories (Bar Harbor, ME). Focal ischemia was induced by transient middle cerebral artery occlusion (MCAO) by an intraluminal suture method as described earlier (Kapadia *et al* 2006); (Tureyen *et al* 2007; Tureyen *et al* 2008). In brief, a mouse was anesthetized with isoflurane (induction: 2%; maintenance: 1.2% in an oxygen and nitrous oxide 50:50 mixture), and the left femoral artery was cannulated for continuous monitoring of arterial blood pressure and to obtain the measurements of pH, P_aO_2, P_aCO_2, hemoglobin and blood glucose concentration (i-STAT; Sensor Devices, Waukesha, WI). The rectal temperature was maintained at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad. After a midline skin incision, the left external carotid artery was exposed, a surgical 6–0 monofilament nylon suture blunted at the end was introduced into its lumen and gently advanced to the internal carotid artery until the regional cerebral blood flow (rCBF) was reduced to ~15% of the baseline (recorded by laser Doppler flowmeter; Vasamedics, LLC, St Paul, MN) as described earlier (Vemuganti *et al* 2004). Following 45 min or 2h of occlusion, the suture was withdrawn (reperfusion was confirmed by laser Doppler), the wound was sutured, mice were allowed to recover from anesthesia and returned to the cage with *ad libitum* access to food and water. Cohorts of mice were sacrificed at 12h (gene expression studies), 1 day (brain water estimation) or 3 days (all other studies) of reperfusion.

Infarct volume estimation and immunohistochemistry

Infarct volume was measured as described earlier (Kapadia *et al* 2006; Tureyen *et al* 2007). In brief, *db*/*db* and *db*/+ mice (n =14/group) subjected to 45 min transient MCAO were perfused transcardially with buffered paraformaldehyde at 3 days of reperfusion. Each brain was postfixed, cryoprotected and sectioned (coronal; 40 μm thick at an interval of 320 μm). The serial sections were stained with Cresyl violet and scanned using the NIH Image program. The volume of the ischemic lesion was computed by the numeric integration of data from 4 serial sections in respect to the sectional interval. To account for edema and differential shrinkage resulting from tissue processing, the injury volumes were corrected by using the Swanson formula: corrected injury volume = contralateral hemisphere volume − (ipsilateral hemisphere volume-measured injury volume) (Swanson *et al* 1990). Parallel sets of sections from each mouse were immunostained with antibodies against ICAM1 (1:1,000; BD Bioscience Pharmingen, San Jose, CA) and OX42 (CD11b; 1:1,000; BD Pharmingen, San Jose, CA) as described earlier (Kapadia et al., 2006). The $OX42^+$ cells were counted in

3 to 4 X300 fields in the ipsilateral cortex of each mouse. To ensure that the homologous areas of injury was sampled between animals, sections between the coordinates +1 to +1.5 from Bregma in all animals as described earlier Kapadia.

Neurological evaluation

Post-ischemic neurological deficits were evaluated on a 5-point scale before transient MCAO and at 3 days of reperfusion by an investigator blinded to the study groups as described earlier (Longa *et al* 1989); (Kapadia *et al* 2006; Tureyen *et al* 2007; Tureyen *et al* 2008). The number of mice used for the neuroscoring included those used for infarct measurement and MPO activity (n = 14 for the *db*/*db* and 17 for the *db*/+ groups). A score of 0 suggests no neurological deficit (normal), 1 suggests mild neurological deficit (failure to extend right forepaw fully), 2 suggests moderate neurological deficit (circling to the right), 3 suggests severe neurological deficit (falling to the right), and 4 suggests very severe neurological deficit (the mouse could not walk spontaneously; depressed level of consciousness).

Myeloperoxidase (MPO) Assay

MPO activity in brain tissue reflects the neutrophil extravasation. MPO activity was estimated as described previously (Weston *et al* 2007) in a cohort of *db*/*db* and *db*/+ mice (n = 5/genotype) subjected to 45 min transient MCAO and 3 days of reperfusion. In brief, a mouse was transcardially perfused with isotonic saline, the brain was sliced in matrix to generate 1-mm sections. A section from the coordinates between +1 mm to −1 mm was quickly stained with TTC to confirm infarction. From the adjacent sections the ipsilateral and the contralateral cortex (~50 mg each) were dissected. The wet weight was noted and the tissue was homogenized in ice-cold 5 mM phosphate buffer (pH 6), centrifuged at 30,000*g* for 30 min at 4°C. The pellet washed briefly with buffer and suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St Louis, MO). The suspension was subjected to 3 freeze–thaw cycles with sonication (10 sec) between cycles followed by incubation (at 4°C for 20 min) and centrifugation (at 12,500*g* for 15 min at 4°C). A 50 μl of the supernatant was mixed with 1.45 ml of 50 mM phosphate buffer (pH 6.0) containing *o*-dianisidine dihydrochloride (0.167 mg/ml; Sigma, St Louis, MO) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was recorded for 3 min at 15 sec intervals. MPO activity was calculated using human MPO (Dako, Carpentaria, CA) as a standard. One unit of MPO activity was defined as the amount that degrades 1 μmol of peroxide/min at 25°C.

Brain water content

The brain water content was measured as previously described (Titova *et al* 2008). Briefly, the ipsilateral and the contralateral cortex were dissected from a cohort of *db*/*db* and *db*/+ mice (n = 5/genotype) killed at 1 day of reperfusion following 45 min MCAO. Tissue samples were weighed to the nearest mg to obtain the wet weight. The tissue was dried at 100°C for 1 day and weighed again to determine the dry weight. Brain water content (%) was calculated as [(wet weight-dry weight)/wet weight] × 100.

Real-time PCR

Gene expression analysis was conducted using quantitative real-time PCR as described earlier (Kapadia *et al* 2006; Tureyen *et al* 2007). A cohort of *db*/*db* and *db*/+ mice subjected to 45 min transient MCAO were killed at 12h of reperfusion (n = 4/group). From each mouse, total RNA was extracted from the ipsilateral and the contralateral cortex using the Trizol reagent (Invitrogen, Carlsbad, CA). 1 μg of RNA from each sample was reverse transcribed with oligo(dT)15 and random hexamer primers using M-MuLV reverse

transcriptase (Life Technologies, Rockville, MD). 10 ng of cDNA and gene-specific primers were added to SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq DNA polymerase, dNTPs with dUTP and optimal buffer components; Applied Biosystems) and subjected to PCR amplification in a Perkin-Elmer TaqMan 5700 Sequence Detection System (1 cycle at 50°C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min). PCR reactions were conducted in duplicate. The amplified transcripts were quantified with the comparative C_T method using 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal controls as described earlier (Kapadia *et al* 2006; Tureyen *et al* 2007). The following transcripts known to be upregulated after focal ischemia were estimated: interleukin (IL)-1β, IL-6, macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), P-selectin, E-selectin, HSP27, HSP70 and HSP32 (heme oxygenase-1; HO1). The real-time PCR primers were designed using the Primer Express software (Applied Biosystems) based on the GenBank accession numbers and are same as in our previous papers (Kapadia *et al* 2006; Tureyen *et al* 2007).

Statistical analysis

The data are expressed as mean ± SD. Comparisons among groups were performed by one-way ANOVA with Tukey-Kramer multiple comparisons post-test.

RESULTS

Body weight and blood glucose levels

At 5 weeks of age, both *db*/*db* and *db*/+ cohorts showed similar body weight (Fig. 1A; n = 35/group). By 14 weeks of age, the body weight increased by 173% in the *db*/*db* mice and by 99% in the *db*/+ mice (Fig. 1A). Thus, at 14 weeks of age the body weight of *db*/*db* mice was 74% higher than *db*/+ mice (n = 35/group; $p<0.05$) (Fig. 1A). The blood glucose level was 21% higher in the *db*/*db* mice over the *db*/+ mice at 5 weeks of age (n = 35/group) (Fig. 1B). At 14 weeks of age, the *db*/*db* mice showed a 267% higher blood glucose levels than the *db*/+ mice (n = 35/group; $p<0.05$) (Fig. 1B).

Mortality following transient MCAO

When mice were subjected to a 2h transient MCAO, within 1 day of reperfusion the *db*/*db* mice showed a 75% rate of mortality compared to a 13% mortality in the *db*/+ cohort (n = 8/genotype) (Fig. 2A). When the duration of MCAO was decreased to 45 min, the *db*/*db* mice showed a 31% mortality compared to 8% mortality observe in the *db*/+ cohort (n = 13/genotype) (Fig. 2A). Hence, we used 45 min MCAO duration in the remainder of the experiments. In the 45 min MCAO group, the rCBF (Fig. 2B) and the other physiological parameters (pH, P_ao_2, P_aco_2 and hemoglobin; data not shown) were not significantly different between the *db*/*db* and *db*/+ cohorts.

Infarction and neurological dysfunction following focal ischemia

Following 45 min transient MCAO and 3 days of reperfusion, the infarct volume was observed to be 54.5% higher in the *db*/*db* compared to the *db*/+ mice (n = 9 for *db*/*db* and 12 for *db*/+ cohorts; $p<0.05$) (Fig. 3A). The average post-ischemic neuroscore at 3 days of reperfusion following 45 min transient MCAO was also observed to be 88% higher ($p<0.05$) in the *db*/*db* (3.35 ± 0.45; n =14) over the *db*/+ (1.76 ± 0.047; n = 17) cohorts (Fig. 3B). This indicates a very severe neurologic deficit in the *db*/*db* group compared to the mild neurological deficit in the *db*/+ group. Cresyl violet stained sections from representative mice from the *db*/*db* and *db*/+ cohorts subjected to 45 min transient MCAO and were shown in Fig. 3.

Post-ischemic brain water content

We measured brain water content which reflects edema in *db*/*db* and *db*/+ mice subjected to 45 min MCAO and 1 day of reperfusion (n =5/group). Compared to the respective contralateral cortex, the ipsilateral cortex of both genotypes showed a significant increase in the water content (Fig. 4A). However, the *db*/+ mice showed a greater percent increase compared to *db*/+ mice (Fig. 4A). In the *db*/*db* mice the contralateral cortex also showed significantly increased water content compared to the contralateral cortex of *db*/+ mice (Fig. 4A).

Post-ischemic inflammatory markers

The MPO activity which indicates the presence of neutrophils was negligible in the contralateral cortex of *db*/*db* and *db*/+ mice subjected to transient MCAO and 3 days of reperfusion (n =5/group) (Fig. 4B). The ipsilateral cortex of both genotypes showed significantly elevated MPO activity compared to the respective contralateral cortex but the fold increase was significantly higher in the *db*/*db* cohort (*db*/+ by 10.4 fold and *db*/*db* by 18.3 fold; n =5/group) (Fig. 4B). In addition, OX42 immunostaining (a measure of activated microglia/macrophages) and the ICAM1 immunostaining (indicates increased extravasation of neutrophils and macrophages into brain parenchyma) were higher in the ipsilateral cortex of the *db*/*db* compared to *db*/+ mice (n = 5/genotype) (Fig. 5).

At 12h of reperfusion following 45 min transient MCAO, the ipsilateral cortex of both *db*/+ and *db*/*db* mice showed significantly increased expression of several inflammatory genes compared to the respective contralateral cortex (n = 4/genotype; Table 1). However, *db*/*db* group showed significantly higher increases over *db*/+ group in the levels of pro-inflammatory cytokines IL-6 and IL-1β (by 84% and 146%, respectively; $p<0.05$), chemokines MIP-1α and MCP-1 (by 135% and 65%, respectively; $p<0.05$), adhesion molecules P-selectin and E-selectin (by 64% and 96%, respectively; $p<0.05$) (Table 1).

HSP gene expression after focal ischemia

The expression of the neuroprotective HSPs HSP27, HSP70 and HSP32/HO-1 are known to be upregulated after focal ischemia (Dhodda *et al* 2004). The ipsilateral cortex of both *db*/*db* and *db*/+ mice subjected to 45 min transient MCAO and 12h reperfusion showed significantly increased mRNA levels of HSP70, HSP27 and HSP32/HO-1 compared to the respective contralateral cortex (n =4/group; Table 1). However, the increased gene expression of all the 3 HSPs was significantly less in the *db*/*db* compared to *db*/+ mice by 62 to 78% (Table 1).

DISCUSSION

Results of the present study show that transient focal ischemia induces bigger infarcts, worsened neurological status, increased cerebral water accumulation, higher inflammation and curtailed induction of HSPs in type-2 diabetic *db*/*db* mice compared to the normoglycemic *db*/+ mice. Furthermore, type-2 diabetic mice manifested a significantly higher rate of mortality following focal ischemia.

One of the limiting factors in stroke therapeutic development is the use of healthy rodents to understand the mechanisms of post-ischemic brain damage. As >30% of the stroke sufferers are diabetic with several metabolic changes in the body, it is important to understand the pathophysiologic characteristics and further test the new stroke drugs in diabetic subjects. The present study reiterates this by showing that type-2 diabetic mice respond very differently to focal ischemia with increased edema, inflammation and mortality. Our studies show that the *db*/*db* mouse manifests many other characteristics of human diabetics

following stroke. The *db*/*db* mice is known to develop neuropathy (Calcutt *et al* 1988), nephropathy (Sharma *et al* 2003), retinopathy (Clements *et al* 1998) and impaired wound healing (Kumari *et al* 2007) similar to human type-2 diabetic patients. Hence, these mice might be well-suited to study the effects of stroke with all the confounding pathological features of diabetes. One of the limitations of the present study is that we haven't studied the effect of focal ischemia in prediabetic (5 weeks old) db/db mice as those were too small in size to induce MCAO.

One of the hallmarks of diabetes is recurrent hyperglycemia which might be a contributor of the presently observed exacerbated post-ischemic brain damage in diabetic mice. However, a previous study from our laboratory observed no neuroprotection when focal ischemia was induced in *db*/*db* mice in which the blood glucose levels were controlled with a 3 week metformin treatment (Tureyen *et al* 2007). Surprisingly, treating *db*/*db* mice with transcription factor PPARγ agonist rosiglitazone for 3 weeks (which also controlled the blood glucose levels) prior to the induction of focal ischemia led to a moderate decrease in the infarct volume (Tureyen *et al* 2007). Thus, normalizing blood glucose level might not be protective and rosiglitazone-induced neuroprotection can be attributed to its anti-inflammatory effects. Recent studies also showed that treatment of obese, diabetic *ob*/*ob* mice with PPARγ agonist darglitazone for 7 days before the induction of hypoxic-ischemia reduced the infarct size and exacerbated the inflammatory response at 8h and 24h after ischemia onset (Kumari *et al* 2010). It is well known that post-treatment with PPARγ agonists rosiglitazone and pioglitazone significantly decreased CNS damage following focal ischemia, traumatic brain injury and spinal cord injury in normoglycemic rodents (Okada *et al* 2002; Park *et al* 2007; Sundararajan *et al* 2005; Tureyen *et al* 2007; Yi *et al* 2008; Zhao *et al* 2006). However, disappointingly rosiglitazone post-treatment had no protective effect in *db*/*db* mice subjected to transient MCAO (Tureyen *et al* 2007). This lack of efficacy with the acute post-treament might be due to low levels of PPARγ in diabetics and as rosiglitazone treatment induces PPARγ expression, the long-term pretreatment might have allowed the drug action. Darglitazone is a much more robust PPARγ agonist with 7 times more efficacy than rosiglitazone and hence might be a better choice as a therapeutic, but it is not known if this drug can protect the diabetic brain if given after stroke treatment.

The majority of the experimental studies to date that evaluated the effect of diabetes in stroke outcome used type-1 diabetes models. The post-ischemic brain damage was also observed to be exacerbated in type-1 diabetic rodents following global or focal ischemia (Elewa *et al* 2009; Ergul *et al* 2009; Kamada *et al* 2007; Kusaka *et al* 2004; Shen *et al* 2010). Studies that used type-2 diabetic animals to understand the mechanisms of stroke-induced brain damage were much fewer. Vannucci et al., (2001) showed that female *db*/*db* mice are more resistant to hypoxic ischemia than male *db*/*db* mice. A recent study showed that exacerbated post-ischemic pathological symptoms observed in *db*/*db* mice can be alleviated by knocking-out the enzyme aldose reductase which is the first enzyme in the polyol pathway that converts excess glucose to sorbitol and further metabolizes to fructose (Yeung *et al* 2010). In addition to *db*/*db* mice, the effect of focal ischemia was studied in few other rodent models of type-2 diabetes. Mayanagi et al. (2008) showed that treating 8 week old obese diabetic *ob*/*ob* mice with rosuvastatin induces neuroprotection after focal ischemia. The *ob*/*ob* and *db*/*db* mouse models are different in the leptin status, the former lacks leptin while the latter lacks leptin receptor (Halle and Persson, 2002). While *db*/*db* and *ob*/*ob* mice are obese, type-2 diabetes models, the Goto-Kakizaki (GK) rat is a lean type-2 diabetes model (Goto *et al* 1976). The GK rat is an important resource for experimental studies as many type-2 diabetics in the world are not obese. Furthermore, in diabetic population in addition to infarction, edema and hemorrhagic transformation (HT) are also major problems following stroke (Ergul *et al* 2009). In the present study, we observed exacerbated edema and infarction, but not HT in *db*/*db* mice subjected to 45 min transient

MCAO. We also failed to observe any HT in the *db*/*db* mice that died within the first 12h after 2h MCAO. On the other hand GK rats show increased cerebrovascular matrix metalloprotease activity and tortuosity, and hence focal ischemia in these animals induces HT (Elgebaly *et al* 2007; Ergul *et al* 2007; Ergul *et al* 2009; Li *et al* 2010). However, due to high collateral circulation the GK rats develop smaller infarcts (Ergul *et al* 2007). Thus, *db*/*db* mice and GK rats together offer a unique resource to study post-stroke brain damage and therapies that need to be tested to minimize infarction and HT in diabetic patients. A recent study showed that in high fat diet combined with streptozotocin rat model of type-2 diabetes, exacerbated post-ischemic brain damage and deteriorated cognitive impairment are associated with the β-secretase (BACE1) activation and increased Aβ generation (Zhang *et al* 2009).

Uncontrolled inflammation during the acute period after stroke is thought to be a major mediator of cerebrovascular failure and brain damage (del Zoppo 2009). Increased expression of cell adhesion molecules enabling the extravasation of WBCs and further induction of pro-inflammatory transcription factors and other inflammatory genes are thought to be the major mediators of post-ischemic inflammation (Yi *et al* 2007). We currently observed that the gene expression of pro-inflammatory cytokines, chemokines and adhesion molecules was much higher in the *db*/*db* mouse brain compared to normoglycemic *db*/+ control at 12h of reperfusion following transient MCAO. Thus, the exacerbated inflammation might be a contributing factor to the increased post-stroke brain damage observed in the diabetic brain. Furthermore, the macrophages and neutrophils will release the oxygen and nitrogen free radicals which are extremely toxic to neurons. Interestingly, *db*/*db* mice are known to manifest systemic inflammation as well as an impaired ability to curtail inflammation (Dula *et al* 2010; Li *et al* 2009; Lu *et al* 2004; Meng *et al* 2010).

Following transient focal ischemia, HSPs are known to be induced in brain as an endogenous neuroprotective response. HSP70 induced in neurons acts as a molecular chaperone that mediates the ischemic tolerance, prevents protein aggregation/denaturation and attenuates the excitotoxic neurotransmitter release after ischemia (Dhodda *et al* 2004; Takagi *et al* 1994). HSP27 induced in astrocytes is also thought to be neuroprotective by mediates protein chaperoning in addition to increasing intracellular glutathione levels, reducing the reactive oxygen species and preventing the apoptosis (Currie *et al* 2000; Garrido *et al* 1999; Mehlen *et al* 1996). HSP32/HO-1 is a stress-inducible astroglial form of HO which is known to prevent the cell death by regulating the cellular iron levels and by controlling the inflammation (Baranano and Snyder 2001; Ferris *et al* 1999; Lee and Chau 2002). We presently observed that the post-ischemic induction of HSP70, HSP27 and HSP32/HO1 was significantly curtailed in the brains of *db*/*db* mice indicating a disability to prevent cell death and failure to induce plasticity in the diabetic brain. The presently observed inability to induce neuroprotective HSPs following focal ischemia in type-2 diabetic mice was not reported earlier and this might be an important feature that might be responsible for the increased ischemic brain damage in these animals.

Unfortunately, many diabetic patients also show chronic hypertension. Elevated blood pressure is known to be associated with increased HT and edema after stroke and treatment with the anti-hypertensive drug candesartan was shown to curtail ischemic brain damage in type-1 diabetic rodents (Kusaka *et al* 2004). The *db*/*db* mice show mild hypertension, but its contribution to the presently observed exacerbated ischemic brain damage observed in these animals is not known. Hypercholesteremia is another complication associated often with type-2 diabetes and previous studies showed that treatment of GK rats subjected to focal ischemia with atorvastatin reduces HT (Elewa *et al* 2009). Thus, a combination therapy that can curtail multiple pathophysiological mechanisms including, but not limited to

inflammation, hypertension, hyperglycemia, edema and oxidative stress might be a good choice for preventing post-stroke brain damage in diabetics.

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Abbreviations used

db	diabetic gene
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GK	Goto-Kakizaki
HO1	heme oxygenase-1
HSP	heat shock protein
HT	hemorrhagic trnasformation
ICAM1	intracellular adhesion molecule
IL	inerleukin
MCAO	Middle cerebral artery occlusion
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
ob	obese gene
PPAR	peroxisome proliferator-activated receptor
rCBF	regional cerebral blood flow
TTC	triphenyl tetrazolium choloride
WBC	white blood cell

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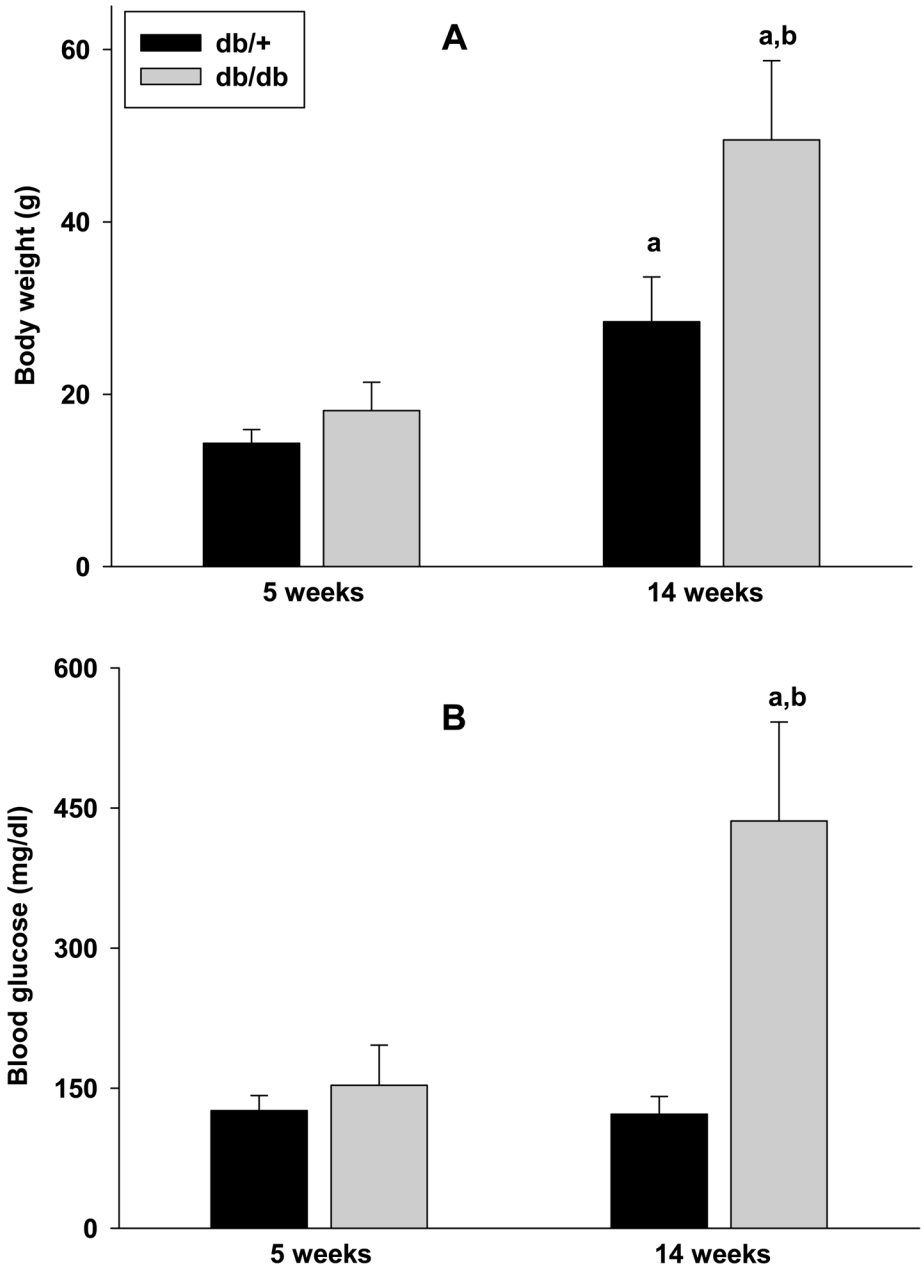

Fig. 1.

Body weights (A) and blood glucose levels (B) of *db*/*db* and *db*/+ mice at 5 weeks and 14 weeks of age. Values are mean ± SD (n = 35/group). Statistics: [a]p<0.05 compared with the respective 5 week old group, [b]p<0.05 compared with the 14 week old db/+ group.

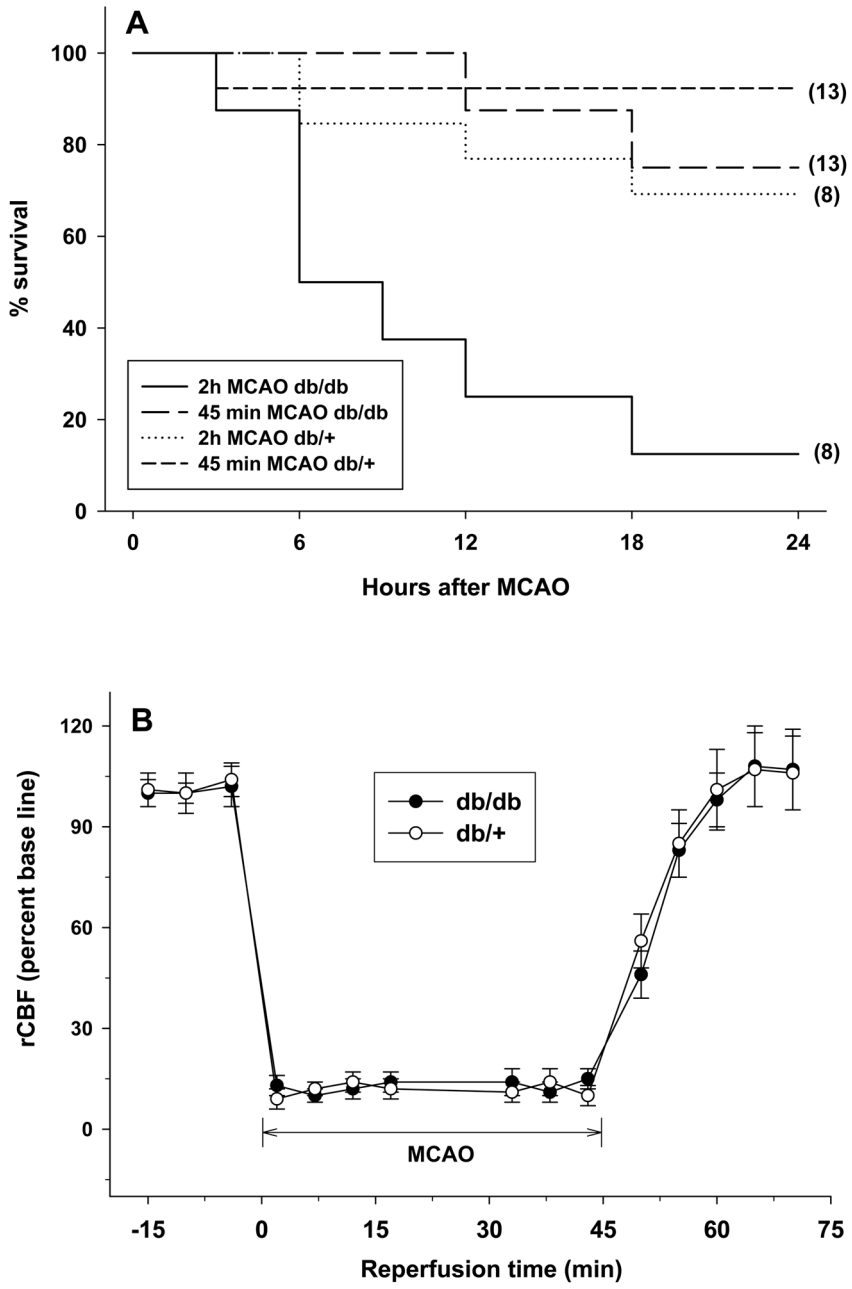

Fig. 2.

Survival rates (A) in 14 week old *db*/*db* and *db*/+ mice within the first 24h of reperfusion following transient MCAO. In panel A, the solid line is 2h MCAO in *db*/*db*, dotted line is 2h MCAO in *db*/+, long dashed line is 45 min MCAO in *db*/*db* and short dashed line is 45 min MCAO in *db*/+ cohorts. The number in the parenthesis after each line is the number of mice used for that group (A). The rCBF was not significantly different between *db*/*db* and *db*/+ mice subjected to 45 min transient MCAO (B). Values are mean ± SD (n = 13/group).

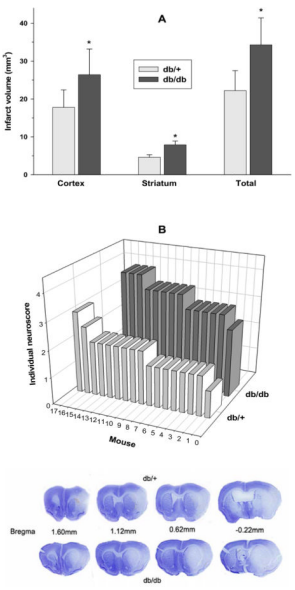

Fig. 3.

The *db*/*db* mice showed significantly bigger infarcts (A) and worsened neurological function (B) compared to *db*/+ mice following 45 min transient MCAO (A). Mice were tested at 3 days of reperfusion for both measures. *p<0.05 compared to *db*/+ group (one-way ANOVA followed by Tukey-Kramer multiple comparisons post-test). Bars in A represents mean ± SD (n = 9 for the *db*/*db* and 12 for the *db*/+ groups). The number of mice used for infarct measurement in the 2 groups was not equal as 3 mice in the *db*/*db* and 1 mouse in the *db*/+ groups died before 3 days of reperfusion. Bars in B represent individual neuroscore of each animal (n = 14 for the *db*/*db* and 17 for the *db*/+ groups). The number of mice for the neuroscoring includes those used for infarct measurement and MPO activity. The mean ± SD neuroscore was 88% higher (p<0.05) in the *db*/*db* (3.33±0.46) compared to *db*/+ (1.76 ±0.47) cohort. The bottom images are Cresyl violet-stained serial section from representative mice from *db*/*db* and *db*/+ cohorts subjected to 45 min transient MCAO and 3 days of reperfusion.

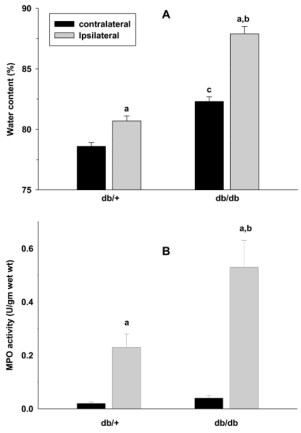

Fig. 4.

The cerebral water content was observed to be significantly higher in the ipsilateral cortex in comparison with the respective contralateral cortex in both *db*/+ and *db*/*db* cohorts subjected to 45 min transient MCAO and 1 day of reperfusion (A). The ipsilateral cortex of *db*/*db* group also showed significantly increased water content compared to the ipsilateral cortex of the *db*/+ mice. The MPO activity which is an indicator of the presence neutrophils was observed to be significantly higher in the ipsilateral cortex compared to the respective contralateral cortex of both *db*/+ and *db*/*db* mice subjected to transient MCAO and 3 days of reperfusion (B). In addition, the ipsilateral cortex of *db*/*db* showed significantly higher MPO activity than the ipsilateral cortex of the *db*/+ mice (B). The values are mean ± SD (n = 5/group). Statistics: $^{a}p<0.05$ compared with the respective contralateral cortex, $^{b}p<0.05$ compared with the ipsilateral cortex of *db*/+ and $^{c}p<0.05$ compared with the contralateral cortex of *db*/+ (one-way ANOVA followed by Tucky-Kramer multiple comparisons post-test).

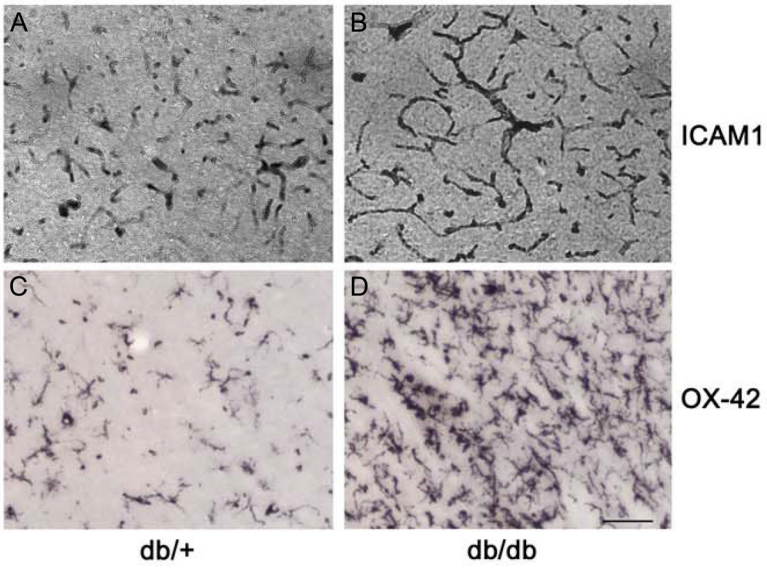

Fig. 5.

ICAM-1 immunopositive capillaries (A and B) and the OX-42 immunopositive activated microglia/macrophages (C and D) were higher in the ipsilateral cortex of *db*/*db* (B and D) compared to the *db*/+ (A and C) mice subjected to 45 min transient MCAO and 3 days of reperfusion. Scale bar is 100 μm. The images were taken from the ipsilateral cortex from the coordinates +1.0 from Bregma. The $OX42^{+}$ cells were observed to be significantly higher (by 67% ± 11%; $p<0.05$ by Student's t test) in the ipsilateral cortex of db/db (85 ± 15 cells/high power field) compared to db/+ (44 ± 8 cells/high power field) mice (n = 3/genotype).

Table 1

Post-ischemic cortical inflammatory gene expression in the db/db and db/+ mice

Transcript	GenBank#	Δfold over contralateral cortex		
		db/+	db/db	% difference
IL-1β	NM_008361	3.6±0.4	7.4±0.8	+146*
IL-6	NM_031168	5.8±0.8	9.8±1.0	+84*
MIP-1α (CCL3)	NM_011337	4.1±0.7	8.3±1.0	+135*
MCP-1 (CCL2)	AF065933	8.1±1.1	12.7±1.6	+65*
E-selectin	NM_011345	3.5±0.5	5.1±0.6	+96*
P-selectin	NM_011347	6.6±1.1	10.2±1.1	+64*
HSP70	NM_010478	106±12	41.2±9.1	−62*
HSP27	U03561	39.1±7.3	12.3±2.6	−71*
HO-1 (HSP32)	NM_010442	24.2±4.1	5.1±1.9	−78*

18S rRNA and GAPDH used as house-keeping controls showed no difference in expression between the two genotypes subjected to sham-operation or transient MCAO. IL, interleukin; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; HSP, heat shock protein; HO, heme oxygenase. The tissue was obtained from the ipsilateral cortex at 12h of reperfusion following a 45 min transient MCAO. The Δfold is in comparison to the respective contralateral cortex. The percent difference = (Δfold over sham in MCAO of db/+ group − 1) −(ΔFold over sham in db/db group − 1)/(fold over sham in MCAO of db/+ group − 1)*100.

* $p<0.05$ (*db*/+ versus *db*/*db* by one-way ANOVA followed by Tucky-Kramer multiple comparisons post-test).