Student Name: Alennie Charmaine Lopez

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Project Title: Examination of diabetic brains: Role of thioredoxin system in pathophysiology of diabetic encephalopathy

Primary Supervisor Name: Dr. Eftekhar Eftekarpour
Department: Regenerative Medicine

Co-Supervisor Name:
Department:

Summary (250 words max single spaced):

Background: Dementia is a global epidemic health issue. The prevalence of dementia is increasing at an alarming rate, with an estimate of 66% increase in the number of current patients over the next decade in Canada. Epidemiologic evidences indicate a close relationship between diabetes and dementia. Although the effect of diabetes on eyes, kidneys and peripheral nervous system has been well examined, the molecular link between diabetes and the neuronal loss in the brain has not been well examined.

Rationale: Oxidative stress is a common issue in pathophysiology of dementia and diabetes. Thioredoxin (Trx) system is a major cellular antioxidant that is involved in both diseases. A decrease in protective effect of Trx in these diseases has been linked to over expression of Trx Inhibiting Protein (Txnip). Inhibition of Txnip has been shown to decrease inflammation in diabetic nephropathy; however, the effect of Txnip in the brain and dementia remains to be investigated.

Approach and results: To examine the effect of Txnip in diabetic brain, wild type and Txnip knockout mice were used for this project. Using western blotting and histology we observed that Txnip promotes inflammation in the brain. Downregulation of Txnip attenuated inflammation indicating that Txnip may be the key linking diabetes and dementia. This is the first examination of Txnip in pathophysiology of diabetic encephalopathy.

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Examination of diabetic brains: Role of thioredoxin system in pathophysiology of diabetic encephalopathy

Introduction and Background

Diabetes mellitus (DM) is a chronic condition involving abnormal carbohydrate metabolism that is characterized by hyperglycemia.\(^1,2\) According to a report by the World Health Organization (WHO), the global prevalence of diabetes has increased significantly from 1980 to 2014, rising from 4.7% to 8.5% in the adult population.\(^3\) In 2016, the WHO reported that 7.2% of the Canadian population lived with diabetes,\(^4\) which contributed to 3% of total deaths spanning all ages.\(^4\) There are two main forms of diabetes: Type 1 diabetes mellitus is caused by the destruction of pancreatic beta cells that produce insulin, while type 2 diabetes mellitus (T2DM) accounts for more than 90% of cases and is characterized by varying degrees of insulin deficiency and resistance.\(^1,5\) T1DM accounts for 5-10% of diabetes cases in the US, Canada, and Europe. Poorly controlled diabetes can initiate microvascular and macrovascular changes and can lead to complications including heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage.\(^2,3,6,7\) Fetal death risk is also increased in poorly controlled diabetes in pregnant women, known as gestational diabetes.\(^3\)

It is estimated that around 50% of patients with diabetes will eventually develop peripheral neuropathy.\(^5\) Progressive neuronal damage is seen in people with diabetes and has diverse clinical manifestations. Distal symmetrical polyneuropathy can manifest as numbness, pain, or weakness that typically starts in the feet or hands and proceeds proximally in a "stocking and glove" distribution.\(^9,10\) Autonomic neuropathy, which is also common in diabetic patients, can manifest as one or more of the following symptoms: gastroparesis, constipation, urinary retention, erectile dysfunction, and cardiac arrhythmia.\(^9\) Diabetic neuropathy is caused by a complex interaction of metabolic, vascular, and maybe hormonal factors that affect the balance between nerve damage and nerve fiber repair, usually in favor of the former.\(^11\) Nerve damage due to chronic hyperglycemia is also seen in diabetic retinopathy which is caused by blood-retinal barrier disturbances due to inflammation.\(^12\) The potential effect of diabetes on the central nervous system (CNS) has not been adequately examined.

Dementia is a well-known complication that may arise in diabetes. Several studies have demonstrated that having diabetes has a positive correlation with having all types of dementia and vascular dementia.\(^13-19\) Epidemiological evidence also suggests a link between diabetes and Alzheimer's disease (AD); however, lack of a definitive diagnostic marker for AD in living diabetes patients makes it impossible to draw a solid conclusion. The mechanism of dementia progression in diabetes is an intensely investigated topic.

It is speculated that the effects of diabetes on brain function is mediated by vascular injury, glucose toxicity, hyperinsulinemia, and disturbed metabolism of amyloid beta.\(^14,20\) Because diabetes frequently develops among other risk factors that constitute the metabolic syndrome such as abdominal obesity, dyslipidemia, and hypertension, it is challenging to absolutely determine the extent of diabetic effects on cognition and memory.\(^14,21\) The impact of diabetes on neuronal cell damage in the central nervous system has not been adequately examined. The
available literature indicates the involvement of oxidative stress as a common link between diabetes and dementia.

The CNS has a significantly higher rate of oxygen consumption than the rest of body. Under normal conditions, a fraction of oxygen consumption in mitochondria is used for generation of ROS. Although traditionally ROS generation has been considered a by-product of cellular metabolism and a cause of cellular death, increasing evidence indicate the vital role of these reactive elements in normal physiology of the cell. ROS are inherently responsible for oxidation of their protein substrates: this results in functional changes for these proteins that will affect the cell response in different physiological conditions. This process is known as redox signaling and plays a very important role in the cell.

Redox signaling is highly regulated by the availability of thiol-containing peptides and proteins. Glutathione (GSH), thioredoxin (Trx) and glutaredoxins are among the most abundant thiols in the cell with high capacity to donate electrons from their thiol groups to be used for scavenging the free radicals or reducing the oxidized proteins. These limited supplies of electrons ensure a highly regulated oxidation-reduction balance, known as redox balance, which determines the capacity of the cells to withstand the changes in ROS levels during normal cell metabolism.

Evidence of oxidative stress involvement in diabetes has been linked to the prolonged exposure of the cells to excess glucose. This leads to enhanced generation of ROS in the mitochondria that lead to cellular damage by accumulation of advanced glycosylation end products (AGE) and sorbitol, disruption of the hexosamine and protein kinase C pathway, and activation of the poly (ADP-ribose) polymerase pathway. Lifetime accumulation of the end products from these processes can initiate and progress apoptosis, neuroinflammation, necrosis, and neurovascular impairment.

Amongst the different cellular antioxidant thiols, the involvement of Trx system in diabetes mediated oxidative stress has been partially examined. Thioredoxin 1 (Trx1) is a cytoplasmic protein that contains two thiol groups at its active site. Trx1 is involved in scavenging ROS and can reduce the oxidized proteins by direct protein-protein interaction. Trx1 is oxidized in this process and can only be re-used after receiving electrons from NADPH; a reaction mediated by Trx Reductase-1 (TrxR1). A similar, yet independent system is available in mitochondria and consists of Trx2 and TrxR2. The activities of Trx1 and Trx2 are inhibited by their natural inhibitor known as thioredoxin interacting protein (TXNIP), also known as thioredoxin-binding protein-2 (TBP-2) or vitamin D3-upregulated protein (VDUP). It is an α-arrestin family protein that is induced by glucose influx into the cell. This results in the suppression of further glucose uptake once energy homeostasis is achieved. TXNIP suppresses glucose uptake by directly binding to the glucose transporter-1 (GLUT1). It also induces GLUT1 endocytosis through clathrin-coated pits, and indirectly reduces the levels of GLUT1 mRNA. In contrast, insulin is an effective repressor of TXNIP, and it operates in a negative feedback loop that prevents the production of TXNIP. It has been shown that TXNIP is upregulated in diabetic Psammomys obesus and in β-cells that are exposed to chronic hyperglycemia. TXNIP transcription in these conditions promotes oxidative stress and apoptosis in vascular smooth muscles and INS-1 β-cell lines by binding to Trx1/2 and prevention of their protective function. TXNIP can also promote inflammation in a Trx-independent fashion through induction of inflammasome machinery.
Under oxidative stress conditions TXNIP binds to the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3), also known as NALP3 or cryopyrin. This results in activation of NLRP3 inflammasome and induces the release of inflammatory cytokines. The NLRP3 inflammasome is a major component in the regulation of innate immunity. Unlike other components of the immune system that can detect exogenous pattern recognition receptors, the NLRP3 inflammasome can also be activated by host-derived molecules such as excess ATP, ceramides, reactive oxygen species (ROS), oxidized LDL, uric acid, cholesterol crystals and monosodium urate crystals. NLRP3 recruits the adapter apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) that cleaves pro-caspase-1 to activated caspase-1, which results in the activation of inflammatory cytokines such as interleukin (IL)-18 and IL-1β.

Evidence of involvement of TXNIP in diabetic nephropathy and its contribution to induction of inflammation has been previously shown in a streptozotocin (STZ)-induced model of diabetic nephropathy. Genetic inhibition of TXNIP in this model was reported to protect from kidney damage. New reports indicate that TXNIP upregulation is also involved in central nervous system inflammation after brain and subarachnoid hemorrhage, as well as in models of AD.

Based on the pathophysiological and epidemiological evidences linking diabetes and AD, in this project we asked whether diabetic-mediated inflammation may play a role in AD pathogenesis. The involvement of TXNIP in both diseases provides a strong rationale to investigate whether downregulation of TXNIP may have some protective effects against ischemic brain damage and diabetic retinopathy. We therefore hypothesized that diabetic mice will have enhanced levels of inflammation in the brain which will be attenuated after downregulation of TXNIP. If successful, this may provide evidence for TXNIP as a key molecule in diabetic pathophysiology linking diabetes to AD. Considering the high incidence of diabetes and AD, identification of key signaling molecules can be used for novel therapeutic options that may impact the patients’ quality of life and will decrease the financial burden on the public health systems.

Materials and Methods

Animals

Wildtype and TXNIP knockout (Txnip−/−) mice were obtained from the laboratory of Dr. Ivan Fantus, University of Toronto. Animals were housed under standard conditions and provided chow and water ad libitum at the Animal Resource Center, University Health Network (UHN, Toronto, ON, Canada). All the experimental procedures were approved by the Animal Care Committee of the UHN and were followed according to the guidelines of the Canadian Council of Animal Care. Diabetes was induced in at 6–8 weeks of age by intraperitoneal injections of STZ (40 mg/kg in fresh 0.1 M sodium citrate buffer, pH 4.5) daily for 5 days (http://www.diacomp.org), followed 5 weeks later by a second round of five STZ injections. Control (nondiabetic) groups received citrate buffer. Blood glucose levels were monitored with a glucometer (FreeStyle Lite, Alameda, CA) monthly throughout the experimental period. Animals were sacrificed at 16 weeks after the first STZ/Citrate injection. The brains were quickly removed, and the two hemispheres were separated. One hemisphere was snap frozen in liquid nitrogen and the other hemisphere was freshly embedded in the optimum cutting temperature compound (OCT) for histological
assessment. The brains were then transferred on Dry Ice to the University of Manitoba for further analysis. Fourteen animals were used for this project.

**Western blotting**

Western blotting was done according to routine protocols. Fifty micrograms of protein obtained from one hemisphere from each mouse and were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus and transferred onto a PVDF membrane. After blocking for 1 h with 5% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T), membrane was probed with primary antibodies overnight at 4 °C, and HRP- conjugated secondary antibody for 1.5 hours at room temperature. Primary antibodies used were TXNIP (1:1000, Cell Signaling Technology), NLRP3 (1:1000, Cell Signaling Technology), IL-1β (1:1000, AbD Serotec), tumor necrosis factor (TNF) α (1:1000, Cell Signaling Technology), Lamin B1 (1:1000, Santa Cruz Biotechnology), caspase-6 (1:1000, Cell Signaling Technology), thioredoxin reductase (TrxR) 1 (1:1000, Santa Cruz Biotechnology), Trx1 (1:1000, Cell Signaling Technology), TrxR2 (1:1000, Antibodies-Online), receptor for advanced glycation endproducts (RAGE) (1:1000, Santa Cruz Biotechnology), IL-10 (1:500, R&D Systems), caspase-1 (1:250, Abcam), B-cell lymphoma 2 (Bcl-2) (1:1000, Santa Cruz Biotechnology), poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling Technology), ubiquitin (1:1000, Cell Signaling Technology), ionized calcium binding adaptor molecule 1 (Iba1) (1:2000, Wako), ASC (1:1000, Santa Cruz Biotechnology), glial fibrillary acidic protein (GFAP) (1:6000, Sigma-Aldrich), phosphorylated Tau (pTau) (1:500, Santa Cruz Biotechnology), and Tau (1:500, Santa Cruz Biotechnology). Proteins were detected using enhanced chemiluminescence (ECL) prime detection reagent (GE Healthcare Life Sciences). Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also developed to confirm protein loading concentrations. Densitometry measurements were done using AlphaEaseFC (version 6.0.0, Alpha Innotech) and Image J software.

**Immunohistochemistry**

One hemisphere was embedded in optimal cutting temperature (OCT) compound and was sliced to yield 20 micrometer sections of brain that showed the CA1, CA2 and CA3 regions of the hippocampus. Tissues were fixed onto the slides by incubating them in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for fifteen minutes and was washed three times for 10 minutes each in PBS. Tissues were heat fixed at 70 degrees Celsius for ten minutes. The tissues were blocked for one hour in a 2% bovine serum albumin (BSA), 5% skim milk, 0.03% Triton-X in PBS solution. They were incubated overnight with the following antibodies: IL-1β (1:200, Santa Cruz Biotechnology), Ox-42 (1:100, AbD Serotec), and GFAP (1:500, Aves Labs). The slides were washed three times for ten minutes in PBS. Secondary goat anti-mouse Alexa Fluor 647 antibody (1:600 dilution) in 2% BSA, 5% skim milk in PBS solution was added for one hour and slides were kept in the dark. Slides were washed three times for ten minutes. DAPI stain was added for 15 minutes and was washed with PBS twice for ten minutes. Slide cover was mounted with Fluoromount-G (Southern Biotech) and dried overnight in the dark. Slides were viewed in x63 magnification with a confocal microscope.

**Statistical analysis**

Data are expressed as mean ± SEM. Differences among experimental groups were analyzed by one-way ANOVA, followed by Tukey’s post-hoc analysis using the GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Significance was set at P<0.05.
Results

Administration of STZ induces diabetes in the wild type (Txnip+/+) and in our TXNIP knock out animals (Txnip−/−).

At 16 weeks after the first STZ administration, no significant differences were observed in their weights or blood glucose levels. We also examined the levels of TXNIP protein in these animals. Induction of diabetes was associated with increased levels of TXNIP protein level in both animal groups. Administration of STZ in txnip +/- (WT) and txnip −/− (KO) induces diabetes mellitus and is associated with some increase of TXNIP in the brain of WT animals. No significant difference was observed in animal weight between these groups. Using scatter plot, we show the distribution/variation among our samples. (See Figure 1).

Induction of diabetes mellitus is associated with increased inflammasome activation in the wild type (Txnip+/+), but not and in our TXNIP knock out animals (Txnip−/−).

Western blotting was performed to examine the level of NLRP3 protein in the brains of Txnip (+/+ ) and Knockout animals. Induction of inflammasome activation was confirmed by a significant increase in NRLP3 levels in the wildtype animals. No significant changes were observed for the knockout animals. This confirms the TXNIP mediatory role in inflammation induction in the brains of diabetic samples. (See Figure 2).

Activation of NLRP3 results in release of IL-1β.

Immunohistochemistry was used to determine whether increased inflammasome activity is associated with higher levels of IL-1β in hippocampal tissue. The data indicates that induction of diabetes is associated with increased IL-1β in hippocampus. Downregulation of TXNIP attenuated the inflammatory response to levels similar to the wildtype controls. (See Figure 3).

Increased inflammatory response is potentially mediated by microglial cells.

Immunohistochemical staining of Ox-42 was used as a marker for detection of microglia. No apparent changes in astrocyte was observed in the hippocampus. Diabetic animals show increased number of Ox-42 positive microglia. The knockout animals showed fewer number of microglia in this region. Using western blotting, we examined the expression level of Iba-1, a marker of microglia. Our data showed that despite an upward trend in the level of this microglial marker in whole brain homogenates, the data did not reach the significance level. (Figures 4).

Previous reports have suggested an increased astrocyte activation in diabetes. We therefore examined the expression of glial fibrillary acidic protein (GFAP), a well-known marker of astrocytes by immunohistochemistry and western blotting. Our data (Figure 5) did not show any significant difference between diabetic and control animals in both wildtype and knockout groups.
Discussion

In this study we observed that TXNIP protein levels increased in the brain after induction of diabetes using STZ administration in mice. This was associated with increased density of microglia and markers of inflammation in hippocampus. This is in agreement with a previous report that links diabetes induction with hippocampal glia activation and evidence of neuronal loss as well as memory deficits. Although we examined the levels of apoptotic markers (Caspase-3 and Poly (ADP-ribose) polymerase (PARP)), in whole brain homogenates, we did not detect any significant difference between the hyperglycemic and normoglycemic brains.

To our knowledge, this study is the first to examine the effect of STZ diabetes in the brains of TXNIP knockdown mice. Although constitutive downregulation of TXNIP in this model did not prevent induction of hyperglycemia, the expression of inflammation was attenuated in this region as suggested by a significant decrease in markers of inflammation and the apparent decrease in the number of microglia. The pro-inflammatory role of TXNIP has been previously shown in diabetic nephropathy in this animal model. Our data also suggest that TXNIP upregulation and elicitation of inflammatory reaction is a total body response that can affect the brain structure and function.

These data suggest the therapeutic capacity of targeting TXNIP as a potential treatment for diabetes as well as AD. Several factors can affect the TXNIP levels: hyperglycemia, Ca\textsuperscript{2+} influx, and excessive oxidative stress. This indicate that targeting any of these factors can be used to prevent the induction of inflammatory response in the brain and therefore decrease the diabetes associated complications including dementia.

In this study we also examined the response of Trx system to diabetes and tested the levels of Trx1, Trx2, TrxR1, and TrxR2 in whole brain homogenates using western blotting (Figure 6). No significant differences were observed under these experimental conditions.

Limitations

We are aware that sample size was the major limitation and the cause of inconsistency in the results. Using whole brain reagents instead of site-specific dissection of the brain regions is another source of inconsistency. Our histological examination clearly showed that attempt should be made for a focused examination of hippocampus is required for future molecular biology examination of these samples.

Future direction

Research in diabetes-mediated brain damage or diabetic encephalopathy is its infancy stage. Future research will examine site-specific analysis of apoptosis, autophagy and inflammation in the cortex as well as hippocampus. The effect of diabetes on hippocampal
neurogenesis and stem cells remains to be examined. Our results indicating the elevated levels of IL-1β is specifically interesting, as previous report indicate the role of this inflammatory mediator in neuronal loss/decreased neurogenesis associated with dementia. Future research must include using genetic models of cell-specific knockdown of TXNIP to adequately examine the contribution of neuronal/glial Trx system in diabetes.

**Student contribution**

Under the supervision of the principal investigator and senior graduate students, the student has performed all the molecular biology and histology as well as imaging and analysis for this project.

**References**


Figures

*Figure 1*) Administration of STZ in *txnip +/-* (WT) and *txnip -/-* (KO) induces diabetes mellitus (A) and is associated with some increase of TXNIP in the brain of WT animals (B). No significant difference was observed in animal weight between these groups (C). Using scatter plot here we show the distribution/variation among our samples.

*Figure 2*) Western blotting was performed to examine the level of NLRP3 protein in the brains of *Txnip +/-* and Knockout animals. Induction of inflammasome activation was confirmed by a significant increase in NRLP3 levels in the wildtype animals. No significant changes were observed for the knockout animals. This confirms the TXNIP mediatory role in inflammation induction in the brains of diabetic samples.
Figure 3) Confocal microscopic images depicting the expression of IL-1β in CA2 region of hippocampus. Immunostaining was performed in parallel between the diabetic and control samples and a uniform imaging protocol was used for all tissues. The photographs were quantified using Image J software. Our data indicates that induction of diabetes is associated with increased IL-1β in hippocampus. Downregulation of TXNIP attenuated the inflammatory response to levels similar to the wildtype controls. (*p<0.05, ** p<0.005) (magnification x630)
Figure 4) Representative confocal microscopical images depicting immunohistochemical staining for Ox42, a marker for glial cells in the CA2 region of hippocampus. Diabetic animals show increased number of Ox42-positive microglia. The knockout animals showed fewer number of microglia in this region. (magnification x200). Using western blotting, no significant difference was observed in the levels of Iba-1, a marker of microglia in whole brain homogenates.

Figure 5) Confocal images depicting immunohistochemical staining for astrocytes as marked by GFAP. No apparent changes in astrocyte was observed in the hippocampus (magnification x200).
Figure 6) Induction of diabetes did not affect the level of members of Trx system in the whole brain homogenate. Down regulation of Txnip also did not affect these proteins.