Prostanoids, Diabetes and the Brain

*Unveiling a Pathophysiological Role for 15-deoxy-Δ^{12,14} – Prostaglandin J2 in Diabetes-related Encephalopathy & Vascular Injury*

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MASTER OF SCIENCE

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2008
“There is a single light of science, to brighten it anywhere is to brighten it everywhere”

Isaac Asimov
(1920-1992)

To my family
My mom, dad, sister
& my friends
Acknowledgements

“…to alleviate pain, restore health, and extend life”, the voices around me were muffled and slowly began to fade away as my eyes fell upon these words. Was the event a moment of epiphany, as if my eyes saw something to which they had previously been blind? It certainly was. As I absorbed these words they appeared conspicuous to me and seemed to take on a challenge, carry a promise, and confer a pledge. It was at that very moment I was suddenly aware of my passion for the sciences, but I was curious about where my future lies with this passion. The pursuit to satisfy my curiosity led to the work on this master’s thesis which commenced in the summer of 2006. Since then this scientific endeavor has been an adventure of unpredictable turns and fascinating discoveries. “...for every door that closes, another opens” strange, but true. And with that I would like to express my deepest gratitude and acknowledge all those who have contributed to the work on this thesis as well as those who have followed me along the way.

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There are several of you over the course of my masters who have contributed positively to this experience and though I may not have mentioned you I would sincerely like to express my gratitude to you all (you know who you are!).

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Abstract

Amongst a host of diabetes-related changes in the central nervous system (CNS) the cerebral microvessels remain a susceptible target for detrimental effects of the disease associated with altered barrier transport and function of the cerebral microvasculature. While oxidative stress and the activation of COX enzymes emerge as two separate pathogenic mechanisms in a variety of CNS-related diseases, a common link between these presumably distinct processes is the oxidation of arachidonic acid and subsequent formation of bioactive prostaglandins. With basal levels of the parent compound, Prostaglandin D$_2$, exceeding those of other prostaglandins in the brain, the liberation of 15-deoxy-$\Delta ^{12,14}$-Prostaglandin J$_2$ (15d-PGJ$_2$) may in turn be warranted.

Irrespective of whether cerebrovascular dysfunction is a cause or consequence of diabetes-related encephalopathies, the current understanding of the molecular mechanisms leading to this detrimental process is limited. The present thesis attempts to characterize the role of one such arachidonic acid metabolite, 15d-PGJ$_2$, as a potential mediator of neurovascular degeneration.

The findings in effect unveil a pathophysiological role for 15d-PGJ$_2$ in a deleterious state of untreated diabetes; corroborated by the Streptozotocin-induced mouse model of diabetes. With a near 8-fold increase in 15d-PGJ$_2$ levels and concomitant reductions of cortical vessel density within the diabetic brain, pharmacological inhibition with the selenocompound SeCl$_4$ appeared to partially rescue cortical capillary density subsequent to reductions in 15d-PGJ$_2$. Furthermore, intracerebroventricular (ICV) administration of pathophysiological doses of 15d-PGJ$_2$ in vivo and treatment of brain explants and aortic rings ex vivo confirmed vaso-obliterative properties and anti-angiogenic potential. Finally, 15d-PGJ$_2$ induced cytotoxicity in vitro was apoptotic in nature, involved the production of reactive oxygen species (ROS) and occurred independently of the DP1/DP2 and PPAR$\gamma$ receptors.

The preceding findings provide novel insights into the upstream molecular mechanisms of oxidative stress on microvascular injury in the diabetic brain, highlighting a pathophysiological role for the cyclopentanone 15d-PGJ$_2$. 
Resumé

Les plus fréquentes complications reliées au Diabète, au niveau du système nerveux central, sont associées aux microvaisseaux cérébraux; cible fragile et compromise. Les effets délétères du diabète causent un endommagement à la fonction de transport de la barrière hémato-encéphalique et des cellules endothéliales. L’augmentation du stress oxydatif et l’activation des cyclooxygenases jouent un rôle primordial dans la génèse de ces complications et entraînent la formation subséquente des prostaglandines. Les mécanismes moléculaires associés à la dysfonction de l’endothélium vasculaire cérébral demeurent indéterminés. Le contenu de cette thèse tentera de caractériser le rôle de la prostaglandine 15-deoxy-$\Delta^{12,14}$-J$_2$ (15d-PGJ$_2$), une potentiel me diatrice de la dégénérescence neuromicrovasculaire.

Nos données révèlent un rôle pathologique de la 15d-PGJ$_2$ dans un modèle animal de diabète induit à la streptozotocine. Les niveaux de 15d-PGJ$_2$ augmentent de 8 fois chez les souris diabétiques par rapport aux souris contrôles et semblent induire une dégénérescence microvasculaire cérébrale. L’inhibition de la formation de 15d-PGJ$_2$ dans ce modèle par SeCl$_4$ prévient partiellement l’induction de ce phénomène. Par ailleurs, l’injection cérébroventriculaire de 15d-PGJ$_2$ in vivo, le traitement d’explants cérébraux et d’anneaux aortiques ex vivo confirment les propriétés anti-angiogéniques. Finalement, nous démontrons que 15d-PGJ$_2$ induit une surproduction d’espèces oxygénées activées suivie d’une mort cellulaire apoptotique indépendante des récepteurs DP1/DP2 et PPAR$\gamma$. À travers cette thèse, nous avons réussi à dévoiler de nouveaux mécanismes induisant les lésions microvasculaires au niveau d’un cerveau diabétique, soulignant le rôle de 15d-PGJ$_2$. 
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<tr>
<td>15d-PGJ₂</td>
<td>15-deoxy-Δ¹²,¹⁴-Prostaglandin J₂</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ASA</td>
<td>acetylsalicyclic acid</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BRB</td>
<td>blood-retinal barrier</td>
</tr>
<tr>
<td>BMEC</td>
<td>brain microvascular endothelial cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CASMC</td>
<td>coronary artery smooth muscle cells</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>COX-1</td>
<td>cyclooxygenase 1 (constitutive)</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase 2 (inducible)</td>
</tr>
<tr>
<td>cPLA₂</td>
<td>cytosolic phospholipase A₂</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>DBD</td>
<td>deoxyribonucleic acid binding domain</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2′-7′-dichlorofluorescin diacetate</td>
</tr>
<tr>
<td>DCF</td>
<td>2′-7′- dichlorofluorescein</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DKA</td>
<td>diabetic ketoacidosis</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethlysulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enznyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell-sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>flourescein isothiocyanate</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLUT2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-s-transferase</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>hematopoietic prostaglandin D₂ synthase</td>
</tr>
<tr>
<td>HBSS</td>
<td>hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethy l pi perazine-N-2-ethane sulphonic acid</td>
</tr>
<tr>
<td>HRMEC</td>
<td>human retinal microvascular endothelial cells</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IxB</td>
<td>inhibitor of nuclear factor kappaB</td>
</tr>
<tr>
<td>IP</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PGG₂</td>
<td>prostaglandin G₂</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PGH₂</td>
<td>prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>prostacyclin</td>
</tr>
<tr>
<td>PGJ₂</td>
<td>prostaglandin J₂</td>
</tr>
<tr>
<td>PGT</td>
<td>prostaglandin transporter</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>lipocalin-type prostaglandin D₂ synthase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappaB</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNase A</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SeCl₄</td>
<td>selenium tetrachloride</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TBE</td>
<td>tris/borate/ ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetra methyl rhodamine iso-thiocyanate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinedione</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
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1. INTRODUCTION
At the turn of the century, a young scientist’s observation that bile salts injected into the brain could cause seizures as opposed to when administered intravenously gave rise to the concept of the blood-brain barrier (BBB). Though the general physiological and anatomical concept of the BBB is familiar to most, the involvement of this structure is often overlooked in the pathogenesis of diabetes. With vascular disease being the main etiology afflicting patients with diabetes, compromised microvascular integrity remains a hallmark of the acute as well as chronic complications of this pathology regardless of whether diabetes is caused by insulinopenia or insulin resistance. Clinically relevant features of diabetes occurring at the level of the microvasculature are often present in organs such as the kidneys as well as the eyes, where an ocular manifestation of the disease is associated with endothelial cell dysfunction, altered vessel structure and design and thus consequential disturbances in blood flow. Given all this, it could be inaccurately assumed that the study of brain microvasculature in diabetes is huge field; in fact the magnitude of impact of diabetes on the brain is still poorly defined. It has often been presumed that brain function and brain microvasculature are preserved in diabetes but an emerging body of evidence is now slowly changing these perceptions. Diabetic patients appear to present with subtle but appreciable neurological deficits as well as an increased incidence of stroke. Furthermore, cerebral edema or brain swelling is the most frequently reported severe consequence of diabetic ketoacidosis (DKA).

With more progressive research in the field, mechanisms of BBB disruption in various CNS-related diseases are now elucidated. Factors known to disrupt the BBB experimentally include arachidonic acid (AA) and the eicosanoids as well as free radicals. It is thus imperative to further investigate the role and interplay between potential molecular mediators, such as bioactive prostaglandins, that could ultimately lead to the degeneration of brain microvessels and subsequently alter cerebral microvascular integrity in the pathogenesis of diabetes. The study described in the present thesis is aimed at investigating the role of one such potential molecular mediator: 15-deoxy-Δ^{12,14-}Prostaglandin J₂.

1.1. Diabetes

1.1.1 Epidemiology and Sequelae of Diabetes

A predominant public health concern causing substantial morbidity, mortality and long-term complications, diabetes mellitus was estimated to affect approximately 171 million
individuals worldwide in the year 2000 (figure 1.1) and yet this estimate is expected to rise to a devastating 4.4% of the world’s population by the year 2030 (Wild 2004). Irrespective of its devastating impact on the global economic front, diabetes is the most common cause of blindness, non-traumatic amputations, and end-stage renal disease in adults, earning its standing as the sixth most common cause of death. Despite an overwhelming prevalence of the disease much remains to be elucidated about the pathogenesis of diabetes including vascular consequences and potential therapeutics for the cause and prevention.

Diabetes is best described as a multi-faceted metabolic disease primarily characterized by the uncoupling of blood glucose and insulin levels with consequential abnormal glycemic excursions. Of the particular manifestations or sequelae of the disease, three main forms preside; type I, type II and gestational diabetes (WHO World Health Organization 1999). The work in the present thesis focuses on an experimental model of type I diabetes with ensuing complications such as diabetic ketoacidosis (DKA).

Figure 1.1 | Epidemiology of Diabetes mellitus. Global prevalence estimates for diabetes in the year 2000 and projected increases by the year 2030 (Wild 2004; WHO World Health Organization 2007).
Type I Diabetes

Type I diabetes is characterized by a loss of pancreatic beta-cell function and an absolute insulin deficiency. With insulin serving as the primary anabolic hormone that normalizes blood glucose levels, the daily regimen for type I diabetics necessitates a continuous supply of insulin for survival. As a consequence of this deficiency the uncoupling of blood glucose levels and insulin concentration prevents the proper regulation of glycemia (figure 1.2). Deviating from the typically narrow physiological glycemic range (70-130 mg/dl), blood glucose levels in individuals suffering from type I diabetes can extend from hypoglycemia (less than 60 mg/dl) into hyperglycemia (fasting blood glucose greater than 126 mg/dl) (Pickup 1998).

Animal Model of Diabetes

Mouse models of type I diabetes frequently employ the use of streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) administration, a well-established and robust model of experimental diabetes brought about via the selective destruction of the beta cells of the pancreas (Rakieten 1963; Like 1976). Originally identified in the 1950’s as an antibiotic, STZ was first discovered in a strain of the soil microbe Streptomyces achromogenes (Vavra 1959). This discovery was suggestive of its use as a therapeutic agent.
for the treatment of cancers of the beta cells (Murray-Lyon 1968) as well as a diabetogenic agent (Mansford 1968).

STZ is taken up by cells via the glucose transporter 2 (GLUT2) primarily localized in pancreatic beta-cells and exerts its cytotoxic effects mostly by causing the alkylation of DNA (Szkudelski 2001). STZ can exert toxic effects on the brain following an intracerebroventricular (ICV) injection (Blokland 1994), however with the intra-peritoneal (IP) administration method, as employed in the present thesis, any direct effects of STZ on the brain will be limited due to reduced expression of GLUT2 at the blood-brain barrier (Kumagai 1999). In the present model, with the animals receiving no insulin therapy following the induction of diabetes, they consequently developed into a state of diabetic ketoacidosis.

**Diabetic Ketoacidosis**

Diabetic patients are often predisposed to many problems associated with the occurrence of such a multi-faceted metabolic disease. Acute complications that are common but not restricted to type I diabetes, include hyperglycemia, hypoglycemia and diabetic ketoacidosis (DKA). Hyperglycemic and hypoglycemic excursions, resultant of beta cell dysfunction, are frequent sometimes daily occurrences among these patients. DKA is most often observed in children with untreated diabetes type I.

DKA is primarily characterized by hyperglycemia, acidosis (acidity of the blood plasma) and high levels of circulating ketone bodies. A key component that distinguishes DKA is relative insulin deficiency in a setting of elevated counterregulatory hormones such as glucagon, growth hormone and catecholamines. There is now increasing evidence to show that acute cerebral microvascular perturbations can occur in adolescents with metabolic acidosis as a result of DKA (Isales 1999; Hoffman 2002). Though the precise etiology is not fully understood, cerebral edema is reported as the most frequently observed severe complication of DKA and is hypothesized to result either from a breakdown of the blood-brain barrier leading to interstitial brain edema (vasogenic edema) or from swelling of the astrocytes caused by an altered intracellular osmotic balance (cytotoxic edema)(Levitsky 2004).
1.1.2 Diabetes, Microvasculature and the Brain

Vascular complications associated with diabetes can manifest as a number of clinically distinct syndromes. Abnormal glycomic excursions that tend to occur can inflict damage upon both the small blood vessels (microangiopathy) as well as large blood vessels (macroangiopathy) of the body. Diabetic vessels initially endure a series of functional alterations accompanied by structural changes including remodeling processes as observed in both experimental models or human patients (Rumble 1997; Giannattasio 2001). Diabetic microangiopathy may refer to nephropathy resulting in kidney failure, retinopathy which is the leading cause of blindness and neuropathy affecting the nerves leading to conditions such as erectile dysfunction, sensory loss and limb damage.

It could be erroneously assumed that study of the brain microvasculature or the BBB (figure 1.3) in diabetes is a huge field; in fact, the magnitude of the impact of diabetes on the brain is poorly defined. It can be inferred however that a substantial amount of literature warrants still a vascular basis for the functional and structural changes of the CNS reflecting the occurrence of cerebral microangiopathy secondary to chronic hyperglycemia. Diabetes has previously been linked to an increased risk of neurodegeneration (Ristow 2004) and dementia (Ott 1999; Biessels 2006) although its specific consequences on the BBB remain controversial. Some reports associate diabetes with increased BBB permeability (Iwata 1999; Starr 2003; Hovesepyan 2004; Mooradian 2005; Huber 2006) while other studies demonstrate the maintenance of BBB integrity (Ennis 1986; Rechthand 1987; Dai 2002). Diabetic patients found to have the poorest neurocognitive outcomes display signs of cortical atrophy (Schmidt 2004) as well as reduced cerebrovascular reactivity and blood flow especially in the frontal and frontotemporal brain regions (Quirce 1997; Salem 2002). With progressive research in the field, there is accumulating evidence to suggest that diabetes is associated with an increase in proliferative lesions in small cerebral vessels (Gorelick 1999), furthermore, cortical capillaries in experimental models of diabetes exhibit similar microangiopathy to that found in the human retina in diabetes (Taarnhoj 1991). A compelling report on neuropathological damage in type I diabetics found that these patients displayed evidence of cerebral angiopathy, where these vascular changes appeared to be accompanied by diffusely distributed reductions in cortical grey matter and white matter (Reske-Nielsen 1965).
Little is understood about the initial phase in which brain microvessels may destroy, an event presumably occurring subsequent to the progressive development of a deleterious diabetic state such as DKA. It is known that ketosis can accelerate microangiopathy and underlying vascular disease and precipitate neuropathy in patients with long-duration diabetes. Brain autopsy studies of diabetic patients reveal structural abnormalities occurring at the microvascular level as described above, including the thickening of capillary basement membranes and reductions in capillary density (Reske-Nielsen 1965; Johnson 1982). In turn, functional alterations in the cerebrovascular system include decreased blood flow and disturbances of vascular reactivity (Keymeulen 1995).

**Figure 1.3 | The Blood-Brain Barrier.** The cerebral microvascular endothelium is distinguished by the blood-brain barrier consisting of (a) capillary endothelial cells surrounded by (b) basement membrane or basal lamina, (c) tight-junctions restricting paracellular permeability, in concert with (d) astrocyte foot processes providing cellular linkage to neurons.

Among the leading causes of such vascular complications associated with diabetes oxidative stress emerges as a pivotal factor (Giugliano 1996). Reactive oxygen species (ROS) have been shown to be involved in regulating the activation of various signal transduction pathways affecting processes such as cell growth and apoptosis which in turn may contribute to the redesign of vessel structure and function (Irani 2000).
1.1.3 Diabetes and Oxidative stress

The term ‘oxidative stress’ refers to a cellular state subject to excessive levels of ROS (detailed in section 1.3.1). The involvement of oxidative stress in the progression of diabetes and its complications is a widely accepted phenomenon (Baynes 1991; Baynes 1999) and the evidence for oxidative damage in diabetes dates as far back as 1979 (Sato 1979). Sato et al. suggest that high levels of plasma lipid peroxide may cause an increase in lipid peroxide levels in the intima of the blood vessel. Ongoing reports since have confirmed these findings and a more recent study demonstrated that untreated fresh red blood cells from diabetic patients displayed elevated levels of lipid peroxidation products and that the increase in the level of oxidative stress was significantly related to hyperglycemia, using glycated hemoglobin as a marker of hyperglycemia (Dominguez 1998). This increase could be reversed when hyperglycemia was controlled with insulin (Jain 1989) substantiating the prevalence of oxidative stress in a state of untreated diabetes such as DKA. The origin of oxidative stress could be from a variety of mechanisms. A growing body of evidence suggests that diabetes is accompanied by an increase in the production of free radicals (Baynes 1991; Chang 1993; Young 1995; Baynes 1999) and or impaired antioxidant defenses (Halliwell 1990; McLennan 1991; Saxena 1993). Mechanisms by which increased oxidative stress may be involved have been partly elucidated and include the activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C (Maritim 2003). In diabetic vessels hyperglycemia itself can present as a direct contributor to the formation of ROS, however, it can also facilitate the formation of other ROS-releasing compounds.

Oxidative Stress in Diabetic Ketoacidosis

The underlying mechanisms by which ketoacidosis promotes vascular disease in type I diabetic patients still remain elusive however several lines of evidence highlight a frequent association with oxidative stress. It has been found that diabetic patients with excessive levels of ketone production exhibit decreased levels of the anti-oxidant GSH (glutathione) as opposed to diabetics with normal levels of ketone bodies (Jain 1989). This suggests that elevated ketone body levels can influence cellular GSH production in vivo. As mentioned previously, ketoacidosis occurs secondary to a relative or absolute deficiency of insulin leading to intense lipolysis. This lipolysis in turn releases large amounts of free fatty acids that can reach the
hepatic mitochondria and result in the overproduction and release of large amounts of acetoacetate, β-OH (beta-hydroxy butyrate) and acetone into the blood. Jain et al. observed that increasing concentrations of acetoacetate caused a significant increase in lipid peroxidation and the viscosity of red blood cells as well as a significant depletion of GSH content. Other studies have shown that acetoacetate levels similar to those frequently encountered in diabetic patients can cause ROS production in monocytes (Jain 2003). Though the exact mechanism remains elusive, this increased cellular oxidative stress maybe attributed to oxygen radical production by acetoacetate or other effects of acetoacetate on enzymes or signal transduction pathways within the cell. Thus, suggesting that ketoacidosis is associated with increased oxidative stress in diabetics.

Elevated levels of oxidative stress in ketotic patients can play a significant role in the development of vascular inflammation and contribute to the increased incidence of vascular disease and complications associated with type I diabetes. Reactive oxygen free radicals can in turn participate in enzymatic and non-enzymatic processes including free-radical mediated peroxidation of arachidonic acid generating bioactive eicosanoids such as cyclopentanone prostaglandins.

1.1.4 Diabetes and Inflammation

While there are several aspects of diabetes that may contribute to a disturbance in the pro-oxidant and anti-oxidant balance, favoring the former, several reports are also suggestive of a pro-inflammatory response. Hyperglycemia has been linked to the activation of the MAP kinase and PKC pathways, both of which can stimulate cytokine production and promote inflammation (Wilmer 2001; Devaraj 2005). Furthermore advanced glycation end-products that can accumulate following periods of prolonged hyperglycemia can also promote inflammation (Schmidt 2000; Vlassara 2002). Though the precise etiology of microvascular diabetic complications is not fully understood there is evidence to show that the initiation and or activation of several pathways such as the polyol pathway, the PKC pathway, and the subsequent formation of inflammatory mediators such as TNF-α (tumor necrosis factor-alpha), resultant of metabolic disturbances often seen in diabetes, maybe especially involved in the initiation of events linked to inflammation and apoptosis (De Vriese 2000; Dagher 2004; Xu 2004).

Inflammation encompasses a complex cascade of events involving white blood cells, plasma endothelium and tissue. The acute inflammatory phase entails the activation of
Introduction

platelets, macrophages and mast cells resultant of tissue injury. These cells in turn may communicate with endothelial cells via a variety of mediators including prostaglandins and subsequently alter vascular tone and permeability resulting in tissue edema. Furthermore the formation of inflammatory cytokines such as TNF-α have been shown to stimulate the formation of COX (cylooxygenase)-mediated products in various cell types primarily via the induction of COX-2 expression in damaged tissue (Fu 1990; Isakson 1994). In this context, inflammation can serve as a stimulus triggering the initiation of the arachidonic acid cascade and subsequent formation of COX-mediated derivatives such as the prostaglandins in the pathogenesis of diabetes. Of relevance, endogenous prostaglandin synthesis has been demonstrated to be elevated in the diabetic state where an increase in the synthesis of prostaglandin E-like material during platelet aggregation has been found (Halushka 1977), prostaglandin E and prostaglandin F₂ production have been reported to be significantly elevated in blood samples from children with diabetes mellitus (Chase 1979), furthermore, elevations in urinary L-PGDS (lipocalin-type prostaglandin D₂ synthase) and prostaglandin D₂ was demonstrated in patients during the early onset of diabetes mellitus (Hirawa 2001).

1.2. Prostaglandins

Cerebrovascular arachidonic acid metabolism plays a significant role in the regulation of normal circulatory physiology (Siesjo 1982; White 1982) and more specifically in the cerebrovascular response to injury (Dux 1982; Chan 1984; Asano 1985; Koide 1985; Kontos 1985; Dempsey 1986).

1.2.1 Eicosanoids Derived by the COX Pathway

Eicosanoids are a class of 20 carbon backboned oxygenated fatty acids, found widely in a variety of microorganisms, plants and animals. In humans, eicosanoids are local hormones that can act as autocrine and paracrine mediators typically derived via COX-mediated and oxidative stress-mediated oxidation of arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid). Arachidonic acid is a crucial component of the eukaryotic cell membrane where it resides in esterified form in membrane phospholipids. Major constituents of cell membranes are phospholipids which provide the essential milieu of cellular membranes and act as barriers for the entry of compounds into cells. Cellular phospholipases,
such as phospholipase A₂ (PLA₂) cleave unesterfied free AA yielding pivotal intermediates such as prostaglandins which in turn give rise to the formation of a wide spectrum of cellular lipid mediators (figure 1.4). The most relevant of compounds to human physiology and CNS-related pathophysiology are the cyclopentanone prostaglandins (Brash 2001; Musiek 2005) in particular 15-deoxy-Δ¹²,¹⁴-PGJ₂ (15d-PGJ₂). No specific synthase for 15d-PGJ₂ has been identified to date but rather it is often referred to as the ultimate metabolite of prostaglandin D₂ (PGD₂) and thus its synthesis greatly relies on the enzymatic generation of PGD₂.

**Figure 1.4 | Arachidonic Acid Metabolism and Prostaglandin Synthesis.**

### 1.2.2 Prostaglandin D₂

Prostaglandin D₂ is a major prostanoid that has is abundantly generated in the CNS of mammals (Abdel-Halim 1980; Narumiya 1982; Hiroshima 1986) including humans (Ogorochi 1984). This conventional eicosanoid has been shown be involved in the regulation of various physiological processes including the maintenance of body temperature (Ueno 1982; Moritomo 1988; Sri Kantha 1994), the induction of sleep and sedation (Laychock 1980), nociception (Minami 1996) and hormone release (Kinoshita 1982; Nishi 1984; Terao 1995). Two distinct PGD₂ synthases can be selectively expressed in various cell types; lipocalin-type PGD synthase (L-PGDS) is a secreted protein most prominently expressed in
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brain tissue (also known as GSH-independent enzyme) while hematopoietic PGD synthase (H-PGDS), often referred to as the spleen-type enzyme (or GSH-dependent enzyme) is characterized as such due to its localization in hematopoietic organs.

Lipocalin-type PGD Synthase

The production of PGD$_2$ in the brain is largely elicited by the action of L-PGDS which was originally purified from the rat brain as a monomeric glycoprotein (Urade 1985). Previous studies have shown that inorganic quadrivalent selenium (Se$^{4+}$) compounds are non-competitive reversible inhibitors of L-PGDS however they do not affect H-PGDS (Islam 1994). The tertiary structure of L-PGDS reveals a unique free sulphydryl (SH) group of cysteine residue 65 not found in other lipocalins (Hayaishi 2000). It resides in the hydrophobic pocket and is considered to be active site of the enzyme (figure 1.5).

The reversible inhibitor of L-PGDS (SeCl$_4$) appeared to interact with the free sulphydryl group of the cysteine residue 65 in the active site since the inhibition could be reversed by the addition of excess amounts of SH compounds such as glutathione or dithiothreitol (DTT) (Matsumura 1991).

Figure 1.5 | Tertiary Structure of Mouse Lipocalin-type PGD Synthase.
**Hematopoietic PGD Synthase**

The production of PGD$_2$ in peripheral tissues is catalyzed by H-PGDS, originally purified from the rat spleen (Urade 1987) and identified as a cytosolic GSH-dependent enzyme. The tertiary structure of H-PGDS identified via x-ray crystallography revealed a unique prominent cleft at the active site of the enzyme not observed among other members of the GST-family (Urade 1999).

**PGD$_2$ Receptors**

PGD$_2$ can bind two unrelated receptors: DP1 and DP2 (also known as CRTH2) (Alfranca 2006). The DP1 receptor is comprised of seven hydrophobic transmembrane domains and is a member of the G-protein coupled rhodopsin-type receptor family (GPCR). DP1 is thought to be coupled to $G_s$ whereby activation of the receptor can lead intracellular cAMP (cyclic adenosine monophosphate) accumulation, however functional coupling of DP1 to $G_q$ leading to PLC activation and calcium mobilization (Tokuda 1999) has also been reported. The DP2 / CRTH2 receptor is also a member of the GPCR family of receptors coupled to $G_i$ whereby activation of the receptor results in the inhibition of the cAMP response and subsequent increases in Ca$^{2+}$. A number of endogenous ligands that retain some affinity for the CRTH2 receptor include: PGD$_2$, 13-14-dihydro-15-keto-PGD$_2$ (DK-PGD$_2$), Δ$^{12}$-PGD$_2$, Δ$^{12}$-PGJ$_2$, 9α11β PGF$_2$, 11-dehydro-thromboxane B$_2$ (Pettipher 2007).

**1.2.3 Cyclopentanone Prostaglandins**

Eicosanoids are typically classified into two major groups according to their mechanism of action: conventional eicosanoids such as PGD$_2$ and cyclopentanone prostaglandins such as 15d-PGJ$_2$, as mentioned previously. Cyclopentanone prostaglandins are unique products of COX-mediated AA metabolism owing to an unsaturated carbonyl moiety in their cyclopentanone ring structure, a property rendering them highly reactive species. The presence of the cyclopentanone ring system allows these prostaglandins to react covalently via Michael addition reactions with nucleophiles such as the free sulfydryls of glutathione and cysteine residues in the cellular proteins (Fukushima 1992; Rossi 1996; Bui 1998). The reactive center characteristic of cyclopentanone prostaglandins is not present in synthetic PPAR$_\gamma$ ligands and therefore has been postulated to account for some of the
PPARγ-independent actions of PGJ2 and its metabolites as well as related cyclopentanone prostaglandins such as prostaglandin A1 and prostaglandin A2 (Rossi 1996; Bui 1998). Unlike conventional prostaglandins, cyclopentanone prostaglandins do not interact with specific membrane-bound receptors but alternatively are taken by cells via an active transport mechanism with partial accumulation in the nucleus (Narumiya 1986; Narumiya 1987).

1.2.4 15-Deoxy-$\Delta^{12,14}$-Prostaglandin $J_2$

15d-PGJ2 Synthesis

First identified in 1983 as a degradation product of PGD2 (Fitzpatrick 1983) as with other prostaglandins the synthesis of 15d-PGJ2 commences with the initial generation of AA via the activation of cellular phospholipase A2 (figure 1.5). Following the sequential action of the cyclooxygenases COX-1 and COX-2 (also known as PGH synthase 1 and 2), AA is converted to PGG2 with its subsequent reduction to PGH2 (Ueno 2001). PGH2 is a relatively unstable intermediate and is enzymatically converted to a series of biologically active prostanoids. As described previously, the formation of PGD2 is elicited by the actions of two distinct PGD2 synthases (L-PGDS & H-PGDS) and can be metabolized via enzymatic reactions to 11β-PGF$_2\alpha$ and 13,14-dihydro-15-keto-PGD2. Alternatively PGD2 can convert to PGJ2 and its highly reactive metabolites $\Delta^{12}$-PGJ2 and 15d-PGJ2 (Straus 2001) via sequential non-enzymatic dehydration reactions (losing molecules of water), a process that is catalyzed by the presence of serum albumin. PGJ2 undergoes further dehydration via the loss of the 15-hydroxyl group coupled to migration of the 13,14 double bond to yield 15d-PGJ2. Furthermore, this spontaneous conversion has been demonstrated to occur in vitro (Fitzpatrick 1983; Kikawa 1984; Fukushima 1992) and in vivo (Hirata 1988; Shibata 2002). The high reactivity of 15d-PGJ2 is often attributed to the cyclopentanone ring in its structure (figure 1.6). With no specific synthase for 15d-PGJ2 identified to date its synthesis greatly relies on the enzymatic generation of PGD2.

![Figure 1.6 | Structure of 15-deoxy-$\Delta^{12,14}$-PGJ2](image)
Introduction

**Extracellular and Intracellular 15d-PGJ2 Targets**

Prostaglandin activities are generally modulated via the activation of seven-transmembrane domain G-protein coupled receptors such as the DP (1&2), EP, FP, IP, and TP receptors for PGD₂, PGE₂, PGF₂α, PGI₂, and TXA₂, respectively (Coleman 1994). Accumulating evidence indicates that 15d-PGJ₂ may exert weak agonistic activity on the DP₁ receptor coupled to Gₛ (Wright 1998; Vaidya 1999; Zhang 2002). Furthermore 15d-PGJ₂ has also been shown to activate the DP₂ receptor coupled to Gᵢᵢ with consequential increases in intracellular calcium levels (Monneret 2002; Monneret 2003; Powell 2003).

Contrary to other prostaglandins however, no specific receptor for 15d-PGJ₂ has been discovered, instead it has been shown to mediate its effects via interactions with intracellular targets including nuclear receptors, the best-studied of which is the nuclear receptor PPAR-γ (peroxisome proliferator-activated receptor gamma) (Forman 1995; Kliwer 1995; Lehmann 1995; Nosjean 2002; Ide 2003; Scher 2005). Although it is mostly well-known for its PPAR-γ-dependent responses, the inflammatory activity of 15d-PGJ₂ appears to be elicited largely independent of these nuclear receptors. Multiple mechanisms since have been put forth to elucidate the diversity of actions of 15d-PGJ₂ (figure 1.7) of these mechanisms, the involvement of two main signaling pathways takes precedence: the NF-κB pathway and the extracellular signal-regulated kinase (ERK) signaling pathway. Briefly, the transcription factor NF-κB is sequestered in the cytosol in inactive form by its repressor protein I-κB. Upon cellular signaling, I-κB is phosphorylated by I-κB kinase (IKK) resulting in the degradation of I-κB and the translocation of NF-κB to the nucleus where it regulates the expression of genes implicated in inflammatory processes (Ghosh 1998), 15d-PGJ₂ inhibits NF-κB action by blocking I-κB kinase activity either by covalently binding to and inactivating IKK (Rossi 1997; Castrillo 2000; Rossi 2000; Straus 2000), by directly interacting with NF-κB (Ricote 1998), by inhibiting NF-κB binding to DNA through the covalent modification of the p65 and p50 NF-κB subunits (Straus 2000; Cernuda-Morollon 2001), or via binding to and activating PPAR-γ with resultant PPAR-γ-induced synthesis of I-κB (Castrillo 2000).

The mitogen-activated protein kinases (MAPK) are serine/threonine kinases constituting three main families including ERK, c-Jun N-terminal kinase (JNK) and p38 (Johnson 2002). ERK activation is largely dependant upon the activation of Ras, a low molecular weight GTPase, of which three main forms have been identified (H-Ras, N-Ras, C-Ras).
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K-Ras) (Leon 1987; Hall 1998). 15d-PGJ₂ has been shown to interact with H-Ras via the formation of a covalent adduct with the cysteine 184 residue of H-Ras (Oliva 2003). Furthermore 15d-PGJ₂ has also been shown to interact with JNK though the mechanism is not fully understood (Wilmer 2001).

![Diagram of 15d-PGJ₂ Action](image)

**Figure 1.7 | Known Mechanisms of 15d-PGJ₂ Action.** 15d-PGJ₂ may serve as an agonist for the DP receptors while cytosolic 15d-PGJ₂ may activate the Ras/Erk MAP kinase pathway, suppress NF-κB activation via the inhibition of IKK (I-κB kinase) activity and or NF-κB binding to DNA in the nucleus. Additionally 15d-PGJ₂ can bind and activate PPAR-γ which in turn can stimulate the synthesis of the NF-κB inhibitory subunit IκB among other processes.

**Biological Effects of 15d-PGJ₂**

An assessment of the literature displays the varied biological activity of 15d-PGJ₂. With evident effects on multiple physiological targets, the possibility of concerted actions with other prostaglandins or cellular mediators seems probable. The wide array of diversified biological effects include 15d-PGJ₂’s anti-viral effects (Santoro 1997), anti-tumoral activities (Fukushima 1990), anti- and pro-inflammatory actions, anti-proliferative actions, the modulation of heat shock response (Maggi 2000; Vanaja 2000), the induction of...
oxidative stress (Kondo 2001; Koh 2005; Kang 2006; Ray 2006) and apoptosis of several cancerous (Keelan 1999; Chen 2002; Clay 2002; Chen 2002; Pignatelli 2005) and non-cancerous cell lines including, endothelial cells (Bishop-Bailey 1999), neurons (Rohn 2001; Kondo 2002; Smith 2003; Yagami 2003), microglia (Bernardo 2003), and most recently, oligodendrocyte precursor cells (Xiang 2007) via PPAR-\(\gamma\)-dependent and independent mechanisms.

**Effective Concentration of 15d-PGJ\(_2\)**

Most frequently described for its pro-apoptotic effects and its ability to inhibit cell growth (Tanikawa 1998), 15d-PGJ\(_2\) displays the most potent anti-proliferative properties among the prostaglandins. However, the nature of its effects appears to be cell type- and dose-dependent. The intracellular accumulation of 15d-PGJ\(_2\) *in vivo* has been demonstrated (Shibata 2002) and concentrations have been measured recently in biological fluids at picomolar amounts (Bell-Parikh 2003). Prostaglandins have normally been reported to occur in bodily fluids in the picomolar-to-nanomolar range (Fukushima 1990) however, their concentrations may be greatly exaggerated during an allergic response reaching micromolar concentrations at sites of inflammation (Offenbacher 1986). For the most part studies to date have investigated the effects of 15d-PGJ\(_2\) at micromolar concentrations, interestingly in a more recent report, 15d-PGJ\(_2\) was demonstrated to exhibit biphasic effects that are concentration-dependent (Emi 2004). At 3\(\mu\)M, it was shown to be an inducer cell proliferation, while at 10\(\mu\)M it appeared to induce apoptosis.

**Putative 15d-PGJ\(_2\) Transport**

There is evidence to suggest that cyclopentenone prostaglandins are taken up by cells via an active transport mechanism and can then accumulate intracellularly (Narumiya 1986) with nearly 50% of the compound transported to the nucleus (Narumiya 1987). Prostaglandin transport has been thought to occur via the prostaglandin transporter (PGT). This serves as a key physiological process since at normal pH levels prostaglandins exist as charged anions that diffuse poorly across the plasma membrane (Chan 1998; Schuster 1998). PGTs have been identified in various tissues (table 1.1) including endothelial cells, liver, kidney, lung,
smooth muscle (Lu 1996) and more recently have been shown to exhibit widespread
distribution in the CNS with highest expression in the cortex, followed by the cerebellum and
hippocampus, with the least expression in the brainstem or diencephalons (Scafidia 2007). In
addition to PGT another organic anion transporter (OAT) also has the ability to transport
prostaglandins (Nishio 2000).

Table 1.1 | Tissue Distribution of the Putative PGJ₂ Transporter, PGT (adapted from (Nosjean 2002))

<table>
<thead>
<tr>
<th>Transcript length</th>
<th>Tissue distribution</th>
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<tbody>
<tr>
<td><strong>Human</strong> (Lu 1996)</td>
<td></td>
</tr>
<tr>
<td>1.8– 2.0 kb</td>
<td>Skeletal muscle, prostate, testis, ovary, small intestine and colon</td>
</tr>
<tr>
<td>2.5– 2.9 kb</td>
<td>Heart and skeletal muscle</td>
</tr>
<tr>
<td>4.0 kb</td>
<td>Ovary</td>
</tr>
<tr>
<td>4.4– 5.1 kb</td>
<td>Heart, whole brain, placenta, lung, liver, skeletal muscle, kidney, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes</td>
</tr>
<tr>
<td>8.8 kb</td>
<td>Testis and colon</td>
</tr>
<tr>
<td>10.1 kb</td>
<td>Testis</td>
</tr>
<tr>
<td>2.1, 3.5, 4.8 and 7.5 kb</td>
<td>Most CNS regions</td>
</tr>
<tr>
<td><strong>Mouse</strong> (Pucci 1999)</td>
<td></td>
</tr>
<tr>
<td>4.4 kb</td>
<td>Lung &gt; liver &gt; kidney</td>
</tr>
</tbody>
</table>

15d-PGJ₂ and the Brain

The brain tissue is a prime location where the secreted protein L-PGDS catalyzing the
formation of PGD₂ (Igarashi 1992) exists in abundance. This conventional eicosanoid
therefore, is one of the most abundantly produced prostaglandins in brain (Abdel-Halim
1977; Narumiya 1982; Ogorochi 1984) and can be present in higher concentrations than
other prostaglandins under physiological conditions. Bacterial lipopolysaccharide (LPS), is a
potent pro-inflammatory factor that can also induce abundant PGD₂ or 15d-PGJ₂ formation
in microglia cultures (Gebicke-Haerter 1989; Bernardo 2003), and in the CSF and spinal cord
following systemic administration (Mouihate 2004; Grill 2006). 15d-PGJ₂ in particular was
demonstrated to be produced by microglia upon activation (Minghetti 1995). Furthermore,
increased 15d- PGJ₂ - like immunoreactivity in spinal cord sections from ALS (Amyotrophic Lateral Sclerosis) patients has been demonstrated (Kondo 2002). In effect, when taken together these data strongly support the possibility that the levels of PGD₂ derivatives may reach functionally significant amounts in the brain.

15d-PGJ₂ in diabetes and diabetic ketoacidosis

The profile of prostanoids in the pathogenesis of diabetes and more specifically DKA is varied where an elevation in plasma (Axelrod 1982), kidney (Schambelan 1985; Miltényi 1990) and adipose tissue (Chatzipanteli 1996) prostaglandin production has been previously demonstrated. Also, diabetes increases the risk of cerebrovascular events and has been associated with brain ischemia. It has been established that prostaglandins are upregulated in the ischemic brain (Nogawa 1997) and that inhibition of their production can be neuroprotective (Nogawa 1997; Nakamaya 1998; Manabe 2004). Although the production of 15d-PGJ₂ in diabetic ketoacidosis has not been extensively studied, there are several lines of evidence suggestive of the possibility; hypercapnic acidosis increases prostaglandin production (Wagerle 1988; Willis 1999) more specifically acidosis can stimulate K⁺ channel openings (Faraci 1994; Kontos 1996; Kinoshita 1997; Xu 2000) in endothelial cells leading to an increase in the activity of phospholipase A₂ (Hyslop 1993).

1.3. Free Radicals & Oxidative Stress

With the discovery of free radicals, a little over 50 years ago, (Commoner 1954) the field of free radical biology largely remained a proprietary domain of physical chemists. Recent advances in the field since have implicated free radicals in several biological and pathophysiological processes where they serve as important intermediates involved in cytotoxicity, regulation of vascular tone, and neurotransmission.

Free radicals can be described as atoms or molecules whose electronic configuration consists of one or more unpaired electrons in their external orbits. The presence of unpaired electrons increases their chemical reactivity rendering them highly unstable (Slater 1984). Equilibria involving protons and electrons often influence the reaction kinetics and properties
of free radicals. In order to decrease energy levels free radicals gain one or more electrons from neighboring molecules thus exhibit properties of an oxidant or oxidizing agent. A crucial balance between oxidant and antioxidant systems must exist in aerobic organisms, in order to minimize or repair any free radical induced damage owing to their highly reactive nature. An imbalance in these systems can result in an impaired ability to readily detoxify the reactive intermediates and subsequently lead to state of oxidative stress (Sies 1997). The pathophysiological sequelae of oxidative stress is not fully understood however, the significance of oxidative stress has become increasingly recognized such that it is now considered to be a component of virtually every disease process (Hensley 2000). Notably, the inseparable relationship of oxidative stress to inflammation has become irrefutable along with the recognition that certain reactive oxygen species (ROS) function as messenger molecules to propagate inflammatory signals.

1.3.1 Reactive Oxygen Species

Reactive oxygen species (ROS) (table 1.2) and increased products of peroxidation, with vasoconstrictor and cytotoxic properties, can ultimately lead to the degeneration of microvessels.

Table 1.2 | Active Forms of Oxygen

<p>| | | |</p>
<table>
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<tbody>
<tr>
<td>( \text{O}_2^- )</td>
<td>superoxide ion</td>
<td>( \text{RO}^- )</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>hydrogen peroxide</td>
<td>( \text{OONO}^- )</td>
</tr>
<tr>
<td>( \text{OH}^- )</td>
<td>hydroxyl radical</td>
<td>( \text{RO}^- )</td>
</tr>
<tr>
<td>( \text{HOO}^- )</td>
<td>hydroperoxyl radical</td>
<td>( \text{ROOH} )</td>
</tr>
<tr>
<td>( \text{O}_2^1 )</td>
<td>singlet oxygen</td>
<td></td>
</tr>
</tbody>
</table>

More specifically, ROS including superoxide (\( \text{O}_2^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and peroxynitrite have been implicated in endothelial dysfunction. Though the formation of free radicals in biological systems is not restricted to oxygen derivatives, oxygenated free radicals constitute the majority of biological free radicals. Molecular oxygen (\( \text{O}_2 \)), present in all aerobic organisms is a common terminal electron acceptor in biochemical processes a property that can render it toxic to cells. To review some of the principle active forms of oxygen:
**Superoxide Anion (O$_2$•⁻)**

The oxygen atom contains two unpaired electrons in its external orbit, reduction of one electron facilitated by a variety of oxidases can lead to the formation of the superoxide anion (O$_2$•⁻). Intracellular sources that can generate (O$_2$•⁻) include enzymes of the mitochondrial respiratory chain (Freeman 1982), NADPH or NADH oxidase present in endothelial cells, cytochrome P$_{450}$ in liver and adrenal glands (Estabrook 1976) and xanthine oxidase to name a few.

**Hydrogen Peroxide (H$_2$O$_2$)**

As a relatively unstable free radical O$_2$•⁻ can spontaneously convert to molecular oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$). Addition of another electron to O$_2$•⁻ results in the formation of the peroxide ion (O$_2$⁻²) which at physiological pH is rapidly converted to H$_2$O$_2$. O$_2$•⁻ in aqueous solution can also result in the formation of H$_2$O$_2$ via intermediary generation of the hydroperoxyl radical (HOO•) (Southorn 1988; Warner 1992):

\[
O_2^{•⁻} + H^+ \rightarrow HOO^• \\
HOO^{•} + O_2^{•⁻} + H^+ \rightarrow H_2O_2 + O_2
\]

By definition H$_2$O$_2$ is not a free radical, however it is an oxidant with a longer half-life than O$_2$•⁻ and thus able to influence various signaling events.

Other sources of H$_2$O$_2$ include certain oxidases present in peroxysomes while the antioxidant enzyme superoxide dismutase (SOD) present in three isoforms (extracellular ecSOD, cytoplasmic Cu/ZnSOD, and mitochondrial MnSOD) accelerates the decay of O$_2$•⁻ to form H$_2$O$_2$ and O$_2$.

**Hydroxyl Radical (OH⁺)**

The hydroxyl radical (OH⁺) is a highly reactive oxygen free radical. Its formation can occur in aqueous solution where O$_2$•⁻ and H$_2$O$_2$ may combine to yield OH⁺ in the presence of
a ferric ion catalyst (Fe$^{+3}$) (Haber Weiss reaction), ferrous ion Fe$^{+2}$ (Fenton reaction) or other metal ions including Ti$^{+3}$, Cu$^+$, Co$^{+2}$ (Halliwell 1984; Southorn 1988).

\[
\text{Fenton reaction: } H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + OH^- + OH^.
\]
\[
\text{Haber Weiss reaction: } H_2O_2 + O_2^- \rightarrow OH^- + OH^- + O_2
\]

### 1.3.2 Biological Sources of ROS

The **mitochondrial respiratory chain** (in particular complex III and IV) can serve as source for ROS. Mitochondria can produce considerable amounts of ROS as electrons from the respiratory chain can reduce oxygen to form O$_2^-$ however, due to high levels of MnSOD in the mitochondria O$_2^-$ levels may be regulated.

Cyclooxygenases and lipoxygenases found in **plasma membranes** catalyze the formation of prostaglandins and leukotrienes respectively, and in the process generate radicals such as OH$^-$.

**NADPH** is thought to be a major source of ROS within the vascular wall (Hwang 2003). NADPH oxidases are multicomponent plasma-membrane associated enzymes which catalyze the formation of O$_2^-$ with NADPH as the electron donor:

\[
2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + H^+
\]

Monooxygenases such as **cytochrome P$_{450}$** present in the endoplasmic reticulum can also generate O$_2^-$, H$_2$O$_2$, OH$^-$ and ROO$^.$ via hydroxylation and demethylation reactions.

**Xanthine oxidase** (XO), a biochemically modified form of xanthine dehydrogenase with greatest activity in the liver and intestine is also found in endothelial cells and catalyzes the oxidation of hypoxanthine and xanthine to uric acid resulting in the subsequent formation of O$_2^-$ and H$_2$O$_2$. 


1.3.3 Cellular Antioxidant Systems

Since reactive oxygen species generated in an uncontrolled manner can cause severe damage to cellular components, cells have in place several enzymatic and non-enzymatic mechanisms to restore the delicate balance between cellular oxidant and anti-oxidant systems.

Enzymatic Antioxidants

Glutathione Peroxidase (GPx)

Glutathione peroxidase is a selenium-containing enzyme that catalyzes the breakdown of lipid hydroperoxides to their corresponding alcohols and \( \text{H}_2\text{O}_2 \) to water. Glutathione peroxidase 1 is the most abundant isozyme present in mammals and possesses a high affinity for \( \text{H}_2\text{O}_2 \).

Catalase (CAT)

Like GPx, catalases catalyze the reduction of \( \text{H}_2\text{O}_2 \) to oxygen and water. This heme-containing enzyme is most prominently found in peroxisomes and requires manganese or iron as a co-factor (Chelikani 2004).

Superoxide Dismutase (SOD)

Superoxide dismutases are metalloproteins that catalyze the breakdown of the superoxide anion yielding hydrogen peroxide and molecular oxygen (Bannister 1987). There are three main SOD isozymes which vary in cellular location and metal ion cofactor specificity. Copper/Zinc SOD is found in the cytosol while mitochondrial SOD contains Manganese. A third form of SOD is also present in extracellular fluids.

Non-enzymatic Antioxidants

Besides enzymatic antioxidants, many dietary components also possess antioxidant capability.
Introduction

**Ascorbic Acid (Vitamin C)**

Vitamin C is a monosaccharide reducing agent that can reduce free radicals such as peroxyl radicals, superoxide, and singlet oxygen (Meister 1994). It cannot be synthesized in humans and therefore is obtained primarily via dietary sources.

**α-Tocopherol (Vitamin E)**

Vitamin E is a lipid-soluble antioxidant and therefore able to protect cell membranes from lipid peroxidation via the elimination of free radical intermediates (Herrera 2001). Vitamin E can react with radicals such as superoxide, hydroxyl and singlet oxygen rendering them inactive via the donation of a Hydrogen atom (Tappel 1962).

**1.3.4 Oxidative stress and Hyperglycemia**

Oxidative stress in the pathophysiology of diabetes is a well-established phenomenon. Indeed, increases in glucose concentrations of the blood and tissues have been correlated with increases in the formation of ROS (Ha 1999; Brownlee 2001). The brain is especially susceptible to ROS due to its high demand of oxygen, high content of transition metals such as Fe$^{2+}$, and poor anti-oxidant defense systems (Leutner 2001). Furthermore the anti-oxidant enzyme, catalase, is expressed in relatively low levels in neurons of the brain (de Haan 1998), in turn damage to neuronal cell membranes can lead to further ROS generation due to excitotoxicity resultant of excessive glutamate accumulation in the extracellular milieu (Saransaari 1999; Atlante 2001). Hyperglycemia in a rodent model has been linked to an increase in oxidative stress of brain mitochondria (Santos 2001) as well an increase in lipid peroxidation (Makar 1995).
HYPOTHESIS & OBJECTIVES
HYPOTHESIS

A substantial amount of literature warrants a vascular basis for the pathophysiological effects of diabetes on the CNS. Among a host of diabetes-related changes in the CNS, detrimental effects of the disease have been associated with altered barrier and transport function of the cerebral microvasculature. Furthermore, altered or elevated prostaglandin production is among the many factors implicated in the pathogenesis of diabetic vascular disease. While oxidative stress and the initiation of the arachidonic acid cascade via the activation of COX enzymes can appear as two separate pathogenic mechanisms, a common link between these presumably distinct processes is the oxidation of arachidonic acid and subsequent formation of bioactive prostaglandins such as 15d-PGJ₂. More specifically, increased prostaglandin production has been demonstrated to occur in patients suffering from DKA.

Derived from previous implications in a range of pathological events, including the induction of oxidative stress, apoptosis (particularly on endothelial cell cultures) and inflammation, and with basal concentrations of its parent compound PGD₂ exceeding those of other prostaglandins in the brain, it was postulated in the present thesis that:

*The abundant generation of 15d-PGJ₂ is warranted in an experimental model of type I diabetes with ensuing ketoacidosis and this cyclopentanone in turn acts as an important mediator of microvascular degeneration in the brain.*
OBJECTIVES

Streptozotocin (STZ)-induced diabetes model:

To establish an experimental model of type I diabetes via IP administration of a high dosage of STZ.

To ensure the occurrence of DKA by providing no insulin treatment to the hyperglycemic mice and comparing blood chemistry (glucose, ketones, pH, bicarbonate, & electrolytes) between STZ-injected mice versus saline-injected controls.

To characterize the levels of 15d-PGJ2 production in the brains of STZ-injected mice versus saline-injected controls relative to the levels of other prostaglandins.

To ascertain the efficacy of selenium tetrachloride (SeCl4) as an inhibitor of 15d-PGJ2 formation following continuous administration of the compound into the lateral ventricles.

To examine vascular density in the brains of STZ-injected mice and assess any changes before and after the administration of SeCl4.

15-deoxy-Δ12,14-PGJ2:

To characterize the effects of 15d-PGJ2 (1) in vivo via intracerebroventricular administration into healthy adult mice, (2) ex vivo via treatments of brain explants and aortic rings, (3) in vitro via treatments of brain microvascular endothelial cells relative to other endothelial cell cultures, smooth muscle cells and astrocytes.

To elucidate possible downstream mechanisms of 15d-PGJ2-induced responses based on preceding studies.
2. MATERIALS & METHODS
2.1. Chemicals

The following materials were purchased: 15d-PGJ$_2$ (Cayman Chemical, Ann Arbor MI); antibodies to factor VIII, smooth muscle-specific actin and GFAP (Dako, Capinteria, CA); Streptozotocin, Allopurinol, Rotenone, Catalase, NAC, MTT, FITC/TRITC-conjugated lectin *Griffonia simplicifolia*, GW9662, AH6809, BW245c (Sigma-Aldrich, St.Louis MO); Apocynin (Calbiochem); Hoechst 33342, Propidium Iodide (Molecular Probes, OR); Selenium tetrachloride (Pfaltz Bauer Inc. Waterbury CT); DMEM, EGM-2, SmBM, ABM, EBM-2 (Clonetics Cambrex Bio Science, Walkersville MD); Matrigel (BD Biosciences, San Jose CA); 15d-PGJ$_2$ ELISA kit (Assay Designs Inc.); Isoflurane (Abbott, Montréal, Québec).

2.2. Animals

A total of 65 adult male C57BL/6J mice (figure 2.1) weighing approximately 25g were purchased from Charles River Canada (Montreal, PQ, Canada) for these studies. Upon arrival to the animal facility mice were group-housed in a temperature (22°C) and humidity controlled pathogen-free environment with no more than 4 animals per cage. Food (standard laboratory chow) and water were provided ad libitum. A light/dark cycle of 12/12 h was maintained with lights on at 6:00 AM. Prior to the commencement of all experimental proceedings the animals were allowed to acclimatize to the novel environment for at least 3-4 days. Procedures regarding the care and use of animals were carried out in accordance with the regulations of the Canadian Council of Animal Care, while all protocols involving animals were approved by the Sainte-Justine Hospital’s Animal Care Committee, Montréal (Appendix I).

1- to 3-day-old Yorkshire piglets (Fermes Me´nard, L’Ange-Gardien, QC, Canada) were also used in these studies for the acquisition of primary cell cultures. The animals were anesthetized with halothane (2%) and euthanized with intracardiac pentobarbital (120 mg/kg).
in accordance with regulations of the Canadian Council of Animal Care Committee and approval of the Sainte-Justine Hospital’s Animal Care Committee.

2.3. Induction of Diabetes

Animals were divided into two treatment groups: Group I (treatment group) received a single intra-peritoneal (IP) injection of a high-dose of STZ dissolved in saline while Group II (control group) received a single IP injection of vehicle (saline). Animals were weighted to ensure that each would receive an optimal high-dosage of STZ established at 200mg/kg in a volume of 100μL. Prior to injection, animals were anesthetized in an isoflurane drop jar and upon slowed breathing 100μL of saline / STZ was administered within 5 minutes of preparation. The awakened animal was replaced in the cage and allowed free access to food and water throughout experiments.

![Schematic Representation of the Streptozotocin-induced Diabetes Model.](image)

2.4. Biochemical Analysis of Blood Samples

Blood Glucose

Blood glucose and blood ketone measurements were made with a portable glucose and ketone meter (Accucheck compact, Roche) on blood drawn from the tip of the tail. This assessment was performed prior to each IP injection of saline/STZ to ensure normality, on days 1 through 7 post-injection (figure 2.2) and prior to each surgical procedure.
Blood Chemistry

Blood was drawn from the animal via the terminal (acute) method of cardiac puncture. Following anesthesia with isoflurane, the mouse was placed in dorsal recumbency. A 22 gauge needle with syringe was then introduced at angle approximately 10-30 degrees away from the body where the xiphoid process and the last rib meet. With the needle inserted slightly to the left of the midline into the heart a volume of approximately 2mL of blood was collected for analysis of blood pH, sodium, potassium, chloride, bicarbonate, ketones (β-OH) and glucose. This assessment of complete blood biochemistry was performed as a separate experiment to establish the characteristics of each treatment group while blood ketone and blood glucose levels were consistently measured as described previously.

2.5. Levels of Prostaglandin Production

2.5.1 15d-PGJ2 Enzyme Immunoassay

Once the treatment groups were established animals from each group were anesthetized and euthanized via intracardiac perfusion with 5mL of saline. Brains were excised from the skull and suspended in ice-cold tris buffer (pH 7.4) of the following composition: 5mMTris · HCl, 1.1mM ASA, and 1mM EDTA in distilled water. Any tissue samples not immediately used for analysis were frozen in liquid nitrogen and at stored at -80°. Whole brain tissue samples were homogenized twice at 30,000 rpm for 30 seconds following which the homogenate was centrifuged at 1,000g for 20 min at 4°C to remove undisrupted cells and nuclei. A small volume of the supernatant was collected and proteins were measured in aliquots by the dye-binding method, Bradford analysis (Bradford 1976). The remaining supernatant was then re-centrifuged at 14,000 rpm for 45 min at 4°C to remove membranes and enhance the extraction of prostanoids on octadecylsilyl silica columns. The supernatant was dissolved in 8.5mL of 100% ethanol and acidified to pH 3.0 with 300μL glacial acetic acid following which the volume was completed to 10mL with tris buffer. The 10mL samples were applied to the octadecylsilyl silica columns which were pre-activated with methanol and distilled water. Following addition of the samples the columns were washed with 10mL of 15% aqueous ethanol followed by 10mL of hexane. Prostanoids were eluted in 10mL ethyl acetate and evaporated under vacuum to dryness. 15d-PGJ2 levels in these samples were measured by ELISA assay (enzyme-linked
Materials & Methods

Immunosorbent assay (Assay Designs Inc.) as per manufacturer’s instructions. The levels of 15d-PGJ2 were quantified and normalized to the protein content (Bradford analysis) of the brain tissue. Each sample was representative of one brain (n = 7 per group) while the ELISA assay was run in duplicates.

2.5.2 Radioimmunoassay of TXB2, PGE2, PGF1α

The levels of prostaglandin E2 (PGE2), 6-keto-prostaglandin F1α (6-keto-PGF1α, stable metabolite of PGI2), and thromboxane B2 (TXB2, stable metabolite of thromboxane A2) in brain tissue was measured by radioimmunoassay. [3H]PGE2, [3H]6-keto-PGF1α, and [3H]TXB2 were used to measure PGE2, 6-keto-PGF1α, and TXB2, respectively. This assay is based upon the competition by PGE2/6-keto-PGF1α/TXB2 in the test sample with labeled PGE2/6-keto-PGF1α/TXB2 for anti-PGE2/6-keto-PGF1α/TXB2 antibody binding sites. Measurements were performed on homogenized brain tissue samples from each treatment group (STZ vs saline). As described in the previous section, prostaglandins were extracted on octadecylsilyl silica columns, eluted in ethyl acetate and subsequently evaporated to dryness under a vacuum. The dried samples were then re-suspended in 200μL radioimmunoassay (RIA) buffer (10mM phosphate buffer, pH 7.0, containing 10% sodium azide, and 10% γ-globulin) and mixed with the appropriate amount of labeled tracer and reconstituted antiserum. Samples were assayed in duplicate. The mixture was incubated overnight at 4°C following which assay tubes were placed in an ice bath, and 375μL of cold charcoal-dextran suspension was added. The tubes were centrifuged at 1000rpm for 10 min at 4°C; the supernatants were decanted into scintillation vials and radioactivity was determined by scintillation spectrometry where 10mL of Ready-Solv scintillation cocktail (Beckman) was added and the emitted radioactive energy was counted in a β-scintillation counter (Beckman). Percent binding was compared against a standard curve, and the amount of PGE2/6-keto-PGF1α/TXB2 in the sample was calculated. In each case the amount of PGE2/6-keto-PGF1α/TXB2 produced was normalized and all data is expressed as a percentage of control.

2.6. Analysis of Vaso-Obliteration

Vascular Density in the Diabetic and Non-Diabetic Brain

Mice were sacrificed on day 7 post-injection with STZ/saline. Isolated brains were excised from the skull and immediately fixed in 4% formalin and transferred to 30% sucrose
Materials & Methods

(in PBS) overnight. Brains were washed three times with PBS, frozen in a tube containing isopentane immersed in liquid nitrogen and stored at -80°C or at least 24 hours prior to use. Brain sections embedded in optimal cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA) of 10µm were cut with a cryostat (Microm International, HM500 O) and mounted on Superfrost Plus slides. Sections were then permeabilized with methanol for 10 minutes (–20°C) and incubated with FITC-conjugated lectin *Griffonia simplicifolia* (endothelial cell marker) (1:100) in phosphate-buffered saline overnight at room temperature. Sections were then washed with PBS and visualized by epifluorescent microscopy. Regions of interest were photographed (DMC Ie, Polaroid), subsequent to which vasculature was quantified using Image-Pro Plus 4.5 software. Quantification of vessel density was averaged on 10 to 15 sections per animal (approximately 5 images per section) and varied by ≤5%. This approach to measure vascular density has been abundantly used on another neural tissue, namely the retina (Alon 1995; Brooks 2001; Sennlaub 2001; Beauchamp 2004; Kermorvant-Duchemin 2005). The microvessel density in the cortical area of STZ-injected mice was compared with that in age and weight-matched saline-injected mice (vehicle), which was assigned a value of 100%. Vascular density was measured as capillary length per surface area (mm/mm²).

2.7. Intracerebroventricular delivery of 15d-PGJ₂

2.7.1 Stereotaxic Injections

Four to five week old C57BL6/J males weighing approximately 25g were housed individually in cages for at least 5 days prior to surgical interventions to allow for acclimatization. Each mouse was subjected to an intracerebroventricular (ICV) injection of vehicle (artificial cerebrospinal fluid, CSF) or freshly prepared 15d-PGJ₂ in artificial CSF under sterile conditions. Prior to surgical procedure, mice were anesthetized in an isoflurane chamber and mounted onto a stereotaxic frame (David Kopf Instruments, Tunjunga, California). Throughout surgical procedures the animals remained under anesthesia and were placed on a heating pad to avoid any drop in body temperature as a result of the anesthesia. A small incision was made with a scalpel in the skin above the skull in the anterior-to-posterior direction, beginning between the eyes and terminating near the cerebellum. The skin was then spread apart and the surface of the skull was cleaned with a disinfected cotton-swab.
Using a microscope the Bregma was located following which a 26s gauge Hamilton syringe (Hamilton, Reno, Nevada) was fitted into the stereotaxic arm and positioned above the Bregma. The stereotaxic coordinates were then zeroed above Bregma and a small hole was created in the cranium at the coordinates of interest via circular motions of a hand-held Dumont 5-45 tweezer (Fine Science Tool, Vancouver, British Columbia). This was done with careful precision so as to avoid any damage to the brain tissue. The stereotaxic coordinates for injection into the left lateral ventricle (figure 2.3) were chosen ahead of time based on previous literature (Paxinos 2001; Ahmad 2006) pertaining to ICV injections in C57BL6/J mice and were as follows: 0.5mm posterior to anterior, lateral 1.0mm from bregma, and ventral 2.5 mm relative to dura. Using the 26s gauge Hamilton syringe in a total volume of 5μL, 100 μg/kg bodyweight 15d-PGJ₂ (García-Bueno 2005) or aCSF was administered at a rate of 1μL/minute (n = 4 per group). Following ICV delivery, the needle was left in place for an additional 20 minutes to prevent reflux subsequent to which it was slowly withdrawn in several steps over a 5 minute period. Upon completion of all surgical procedures mice were returned to their cages following full recovery from anesthesia. Food intake and body weight were monitored until sacrifice.

**Figure 2.3** Schematic of 15d-PGJ₂ administration. LV indicates lateral ventricles, 3V indicates third ventricles, 4V indicates fourth ventricles. Adapted from: (The Jackson Laboratory 2003).

2.7.2 Brain Tissue Preparation

Animals were sacrificed at 24 hours post-injection when they were euthanized via intra-cardiac perfusion with saline under anesthesia with isoflurane. Brains were immediately excised, post-fixed in 4% formalin for 2 days and transferred to 30% sucrose (in PBS)
overnight. Brain sections were prepared with the use of the cryostat, mounted on Superfrost Plus slides and vascular density was assessed as previously described in section 2.6.

2.7.3 Validation of Injection site

Prior to the initiation of the stereotaxic injections with vehicle or 15d-PGJ2, preliminary experiments were conducted where ICV injections of 10% trypan blue were administered. The brain was excised, fixed in 4% formalin followed by 30% sucrose and prepared for cryostat sectioning. Successful injection was defined by the presence of trypan blue staining in the lateral ventricles.

2.8. Pharmacological Inhibition of PGD2 Synthase

Previous studies investigating the role of PGD2 in regulating sleep-wake activities have demonstrated the inhibition of brain lipocalin-type PGD synthase in vitro (Islam 1994) as well as in vivo (Matsumura 1991; Lee 2002) with the use quadrivalent selenocompounds such as SeCl4. Since no known pharmacological inhibitor of 15d-PGJ2 has been identified to date SeCl4 was used to inhibit L-PGDS and subsequently the formation of 15d-PGJ2. The reversible effects of SeCl4 necessitated continuous administration of the compound which was achieved via the use of the ALZET Brain Infusion Kit and Micro-osmotic pump (Durect Corporation, Cupertino CA). Brain infusion kits with 3mm-5mm cannular depth and osmotic pumps with a flow rate of 0.5μL per hour for a maximum of 7 days were selected for the current protocol. The study by Matsumura et al. (1991) determined the efficacy of the SeCl4 administration to be optimal at a rate of 400pmol/0.2μL per minute, based on this data and the specifications of the ALZET osmotic pumps (flow rate of 0.5μL/h) approximately 0.9mg of SeCl4 was dissolved per mL aCSF given that each pump could contain a total volume of 100μL.

2.8.1. Brain Infusion Assembly

Briefly, stereotaxic coordinates for intracerebroventricular drug delivery were determined as previously described (section 2.7.1) and accordingly ideal cannular length was established to 2mm for optimal drug delivery into the lateral ventricle. The depth of the cannula provided in the kit typically penetrates approximately 5mm below the surface of the
Materials & Methods

skull therefore spacers (provided in the kit) were used to alter this depth to restrict the penetration to 2mm below the surface of the skull. Once spacers were attached to the cannula and the length was verified, catheter tubing was used to attach the cannula to the flow moderator of the ALZET pump. The distance between the location of the pump implantation site and the location of the cannula was determined such that catheter length was 25% longer than this distance to allow free movement of the animal’s head and neck. Following this assembly, the brain infusion construct (figure 2.4) was filled with either vehicle (aCSF) or SeCl₄ (prepared in aCSF) and the filled osmotic pump was fit to its flow moderator such that the brain infusion assembly and osmotic pump were all completely filled and free of air bubbles. To minimize the chance of clotting and to ensure the proper functioning of drug delivery and flow rate the assembled pumps were primed in sterile saline at 37°C one day prior to implantation.

![ALZET Micro-osmotic Pump and Brain Infusion Assembly](image)

**Figure 2.4 | ALZET Micro-osmotic Pump and Brain Infusion Assembly.**
(Adapted from: Durect corporation)

2.8.2. Implantation of Brain Infusion Assembly

Animals were prepped for surgery and site of cannula placement for ICV drug delivery was determined using the stereotaxic apparatus as described previously in section 2.7.1. Following identification of the implantation site a small hole was made in the skull with the use of a micromotor stereotaxic drill (Model 1471 David Kopf Instruments). The cannula was then fitted into the hole and cemented onto the cranium with the pump placed in the midscalpular region on the back. Upon completion of the surgical procedures the animals
were returned to their cages and maintained on a heating pad until recovery from the anesthesia. Food and water intake was monitored until sacrifice.

For the measurements pertaining to whole brain content of 15d-PGJ₂ osmotic pumps containing aCSF or SeCl₄ were implanted on day 5 post-injection of STZ/saline yielding a total of four treatment groups (non-diabetic-aCSF, non-diabetic-SeCl₄, diabetic-aCSF & diabetic-SeCl₄). Animals were sacrificed at 48hours. For the experiments pertaining to the assessment of vascular density, osmotic pumps were implanted 24hours prior to the administration of STZ/saline. Animals in all four treatment groups were sacrificed 7 days post-surgery following which the brain was excised and primed for immunohistochemical staining to reveal vascular density as previously described in section 2.6.

2.9. Brain Explants and Quantification of Vascular Density

Brain explants from healthy adult mice were cultured *in vitro* based on a modification of a retinal explant protocol (Quiniou 2006). Following anesthesia with isoflurane animals were euthanized via intracardiac perfusion with 5mL saline. Excised brains were then sectioned in ice-cold culture medium using a vibratome. 150 μM thick sections were delicately placed in six-well dishes on top of a free-floating membrane (Nuclepore polycarbonate Track Etch, pore size 0.03 μm; Whatman, Brentford, UK). Brain explants were cultured for 2 days in EBM-2 without FBS containing vehicle (control), or 15d-PGJ₂ (10μmol/L). *Ex vivo* monitoring of vascular degeneration was visualized by live-staining the endothelium using FITC-conjugated lectin *Griffonia simplicifolia*. Following 24hours of treatment vascular density was quantified using a software program (ImagePro Plus 4.1) and as previously described (Mannick 1999).

2.10. Aortic Ring Angiogenesis Assay

The aortic ring angiogenesis assay was performed as previously described (Masson 2002; Kermorvant-Duchemin 2005; Mwaikambo 2006). Thoracic aortas were extracted from adult C57BL6/J mice sacrificed by CO₂ asphyxiation. Explanted aortas were immediately transferred to a culture dish containing ice-cold EGM-2. With the aid of a microscope the peri-aortic fibroadipose tissue was carefully removed with fine micro-dissecting forceps and scissors as carefully as possibly so as to avoid inflicting damage to the aortic wall. One millimeter–long aortic rings (10-12 per aorta) were sectioned and rinsed extensively in six to
eight consecutive washes of EGM-2. The rings were then embedded individually in 48-well culture plates previously coated with 45 µL of synthetic basement membrane (Matrigel; BD Biosciences) per well. An additional 45 µL of Matrigel was placed over each ring. Following 30 minutes of time allotted to allow setting, 500 µL EGM-2 was added to each well and the cultures were incubated at 37°C in a humidified environment for five days. The culture medium was changed on day 3 and the test compounds added: vehicle, 5µM & 10µM 15d-PGJ2. Photographs of the aortic rings, 24 hours prior to (day 3) and post addition of the test compounds (day 4) were taken at 4x magnification with an inverted microscope (Eclipse TE300; Nikon); all images were digitized under the same conditions (light, contrast, magnification). The angiogenic response was determined by measuring the area of neovessel formation by the use of an image-analysis software program (Image Pro). On each image the aortic ring and neovessel formation areas were determined by tracing polygonal figures around the respective areas. The total area (mm²) covered by the neovessel formation (minus the area of the aortic ring per se), its average width and the number of microvessels observed at the external limit of the neovessel formation area were noted.

2.11. Cell Culture

2.11.1 Brain Microvascular Endothelial Cell Cultures

For primary cultures of brain microvascular endothelial cells, newborn piglets were anesthetized with 2% halothane and sacrificed by intracardiac injection of pentobarbital (120 mg/kg). The brains were removed and immediately kept in ice-cold Krebs buffer (120 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 27 mM NaHCO₃, 1 mM KH₂PO₄, 0.01 mM sodium edetate, and 10 mM glucose). Subsequently the brains were harvested and dissected into small cubes (2-3mm³) in Hanks Balanced Salt Solution (HBSS pH 7.4) containing 2.8mM KCl, 0.2mM KH₂PO₄, 68mM NaCl, 0.16mM Na₂HPO₄, 2.8mM glucose, 100mM HEPES, and 0.01mM Phenol Red. Brain tissue was homogenized gently (5-6 strokes) in ice-cold phosphate-buffered saline containing 20% Ficoll-400 using a glass homogenizer with loose fitting glass pestle. The homogenate was centrifuged at 20,000 ×g for 20 min at 4°C. The pellet containing the microvessels was washed 3 or 4 times with 20 volumes of ice-cold phosphate-buffered saline to eliminate Ficoll. The resultant microvessel preparations were assessed for purity by light microscopy and γ-glutamyl transpeptidase
activity (Goldstein 1975; Li 1995). Microvessels were re-suspended in selective endothelial growth medium containing 5% fetal bovine serum (FBS), gentamycin (10 units/mL), penicillin (50 units/mL), and streptomycin (50mg/mL), seeded in 75cm² flasks and placed in a humidified atmosphere at 37°C until a confluent monolayer of cells was observed. Confluent endothelial cells were trypsinized, centrifuged, reseeded in culture flasks, and subcultured. Cell viability was verified by trypan blue exclusion and was >90%. Endothelial cells were identified by their cobblestone morphology at confluence, positive reactivity to factor VIII antibody, and negative reactivity to smooth muscle-specific actin and glial fibrillary acidic protein antibodies. Immunostaining for factor VIII, smooth muscle-specific actin, and glial fibrillary acidic protein was performed by fixing cells on cover slips with acetone for 10s and subsequently rehydrating in PBS for 20 min. The cells were then washed for 15 min in PBS containing 0.2% BSA, 5% goat serum, and 0.2% Triton X-100. Fixed cells were incubated for 60 min with factor VIII, smooth muscle actin, or glial fibrillary acidic protein antibody (1:50) diluted in PBS containing 10% FCS and 5% goat serum with 0.1% Triton X-100. After five washes in PBS, the secondary FITC-conjugated goat anti-rabbit antibody (1:100) was applied under the same conditions, and cells were washed again in PBS and water. Cover slips were then mounted in Immunomount and examined under an epifluorescent microscope (Leitz Diaplan).

2.11.2 Other Cell Types

Retinal microvascular endothelial cells (HRMEC), coronary artery smooth muscle cells (SMC), human umbilical vein endothelial cells (HUVEC) & human neuronal astrocytes were obtained from Cell Systems and Cambrex (USA).

2.11.3 Cell Culture and Treatments

Confluent cells of passages 6 to 12 were used for experiments. Cells were growth arrested by reseeding in DMEM (BMECs)/SmBM (SMCs)/EGM-2 (HUVECs) or ABM (Astrocytes) without fetal calf serum for 24h and subsequently incubated with $10^{-9}$M, $10^{-8}$M, $10^{-7}$M, $10^{-6}$M, 5x$10^{-6}$M and $10^{-5}$M 15d-PGJ₂ for 24h in 2% FBS media. In other experiments, cells were pre-treated with established concentrations of the following
inhibitors prior to the addition of 10μM 15d-PGJ2: xanthine oxidase inhibitor and free radical scavenger Allopurinol (1mM), glutathione precursor N-acetyl-cysteine (NAC, 2mM), catalase (500 units), NADPH oxidase inhibitor Apocynin (1mM) and mitochondrial complex I inhibitor Rotenone (1μM), irreversible PPAR-γ antagonist GW9662 (10μM & 50μM), non-selective DP1 and DP2 receptor antagonist AH6809 (10μM). Following the addition of test compounds, cell viability was assessed as described in the following section.

2.11.4 Cell Growth / Viability (MTT assay)

Cell viability with 15d-PGJ2 treatment was estimated by the tetrazolium based colorimetric viability assay via the reduction of 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma). The soluble yellow MTT salt is reduced to an insoluble blue formazan product by the mitochondrial dehydrogenases in living cells. The amount of formazan produced is proportional to the amount of living cells present (Denizot 1986). MTT (0.5mg/mL in PBS, pH 7.2) was incubated with cells for approximately 3 hours at 37°C. To avoid interference of the MTT assay by the various treatments described above, the culture medium was aspirated and cells were rinsed with fresh medium prior to incubation with 50μL MTT solution. Following incubation period the culture medium was once again aspirated and the purple formazan product was solubilized with acidified (40mM HCl) isopropanol solvent. 100μL of this solution was then transferred to a 96-well plate and the optical density was measured with a spectrophotometer at 560 nm, with 690 nm as a reference read-out. Cell viability was expressed as a percentage of optical density relative to control.

2.12. Determination of Apoptosis

DNA ladder assay

BMECs were seeded at 70% confluence. After 24 h, apoptosis was induced by replacing complete media with reduced FBS media (2% DMEM) supplemented with 15d-PGJ2. A final concentration of 10 μM 15d-PGJ2 was used. Both adherent and floating cells were harvested at 24 post-treatment, placed in a 15 ml tube and pelleted at 1500 rpm for 15 min. Pellets were resuspended in 1 ml final volume of 1 M Tris-HCl pH 8, 0.5 M EDTA pH 8 and 10% SDS containing 100 μg/ml of Proteinase K (Fermentas, Burlington, Canada)
and samples were incubated at 37°C with shaking overnight. The day after, samples were chilled on ice for 10 min. A 400 μl volume of 5 M NaCl was added and tubes were inverted several times to mix. Samples were centrifuged for 15 min at 3400 rpm. The supernatant was transferred to a new tube and DNA was precipitated by adding 2 volumes of 100% ethanol. The pellet was resuspended in 20 μL of water and incubated at 37°C for 30 min with RNase A at a final concentration of 20 μg/mL. Electrophoresis was performed in 1.6% agarose gel in TBE buffer, after which DNA was visualized by ethidium bromide staining. DNA fragmentation analysis was assessed by resolving 10–20 μg of DNA for each sample (Bossolasco 2006).

**Apoptotic Nuclear Morphology (Hoescht / PI staining)**

Apoptotic nuclear morphology was determined via a Hoescht stain based on a modification of the protocol described by (Bossolasco 2006). Approximately 30,000 BMECs were seeded on microscope coverslip glass (no. 12, Fisherbrand, Fisher Scientific, Leicestershire, UK) in 24-well plates (Falcon, Becton Dickinson, Palo Alto, CA, USA). Cells were allowed to attach for 24 h prior to treatment with 15d-PGJ2. Following 24h treatment with 10 μM 15d-PGJ2, characterization of the type of cell death (necrosis or apoptosis) was studied by using a membrane-permeable DNA-binding dye, Hoechst 33342, (McGahon 1995; Moore 1998). Cells were loaded for 15 min at 37°C with (0.1 μg/mL) propidium iodide (PI) and Hoechst 33342 (5 μg/mL) (H33342 trihydrochloride trihydrate) and visualized under a fluorescent microscope (excitation wavelength 365 nm, emission wavelength 465 nm, 40 x 0.60 magnification, NIKON eclipse TE300, Tokyo, Japan) with an immersion objective placed directly above the culture medium using red and ultraviolet filters. PI-positive cells (necrotic cells) and cells with fragmented or condensed nuclei and intact membranes (apoptotic cells) were determined in five fields per well.

**Flow Cytometry Detection of Annexin V Binding**

Loss of phospholipid asymmetry at the plasma membrane resulting in exposure of phosphatidylserine residues on the outer leaflet is often used as a marker of apoptosis, but cell surface exposure of phosphatidylserine groups detected by annexin V binding can also be associated with necrosis, (Lecoeur 2001). Annexin V binding was determined on cells incubated with 15d-PGJ2 (10^-5 M) for 18 and 24 hours. Concomitant PI staining, clearly
reflective of necrosis, was also studied. Cells were collected with trypsin, washed twice with PBS buffer (pH 7.4), and stained with the annexin-V conjugated to fluorescein (FITC annexin V), according to the manufacturer’s instructions. Intensity of fluorescence was monitored (annexin V: excitation 488 nm, emission 527 nm; incorporation of PI: excitation 488 nm, emission 599 nm) with a flow cytometer (BD Biosciences, Lincoln Park, NJ) and data were analyzed using the accompanying software (Cell Quest; BD Biosciences). Since PI is impermeant to live cells and apoptotic cells but stains dead cells, live cells would exhibit little or no fluorescence; apoptotic cells would exhibit green fluorescence as a result of FITC-conjugated annexin V binding, while dead cells would exhibit red-green fluorescence.

2.13. ROS production (DCFH-DA assay)

The formation of intracellular ROS following treatment of BMECs with 15d-PGJ\(_2\) was assessed using the peroxide-sensitive probe 2′-7′-dichlorofluorescin diacetate (DCFH-DA) (Hempel 1999). This probe diffuses through the cell membrane and is hydrolyzed to dichlorofluorescein (DCFH), following cleavage of the diacetate group by intracellular esterases. In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent 2′-7′-dichlorofluorescein (DCF). BMECs were seeded at 70% confluence in 48-well cell culture plates and incubated with the fluorescent probe with 7\(\mu\)M DCFH-DA (dissolved in PBS) for 15 minutes at 37\(^\circ\). Cells were then treated with 10\(\mu\)M 15d-PGJ\(_2\) for 5 mins. The accumulation of the oxidized fluorescent derivative DCF in the cells is indicated by an increment in fluorescence at 525nm when the sample is excited at 488 nm by a Microplate Fluorescence Reader. Background fluorescence was corrected by the inclusion of parallel blanks.

2.14. Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA). Student t-test (two-tailed) was used to assess the difference between two groups. One-way ANOVA was used to assess differences among groups (more than three). When appropriate, two-way ANOVA and Bonferroni post-hoc tests were used to assess differences among groups with two independent variables. All significance levels were set at p < 0.05.
3. RESULTS
### 3.1. Blood Chemistry Indicative of Diabetes and Acidosis

Biochemical analysis of blood samples from diabetic and non-diabetic mice 7-days post-injection of STZ/saline revealed significant elevations in blood glucose (44.5 ± 2.3 mM), β-OH-butyrate (1.00 ± 0.2 mM) and osmolality (table 3.1) in diabetic animals indicating sufficient levels of hyperglycemia and acidosis for further experimentation. STZ-injected mice appeared to be lethargic while several wet patches were observed in their cages presumably as a result of osmotic diuresis.

### 3.2. Significant Elevations in 15d-PGJ₂ Correspond to Concomitant Reductions in Cortical Vessel Density

The baseline production of PGD₂ from the whole mouse brain has been calculated to be approximately 2nM (Qu 2006) higher concentrations may however occur during pathophysiological conditions. With elevated levels of this parent compound being present in the brain the formation of 15d-PGJ₂ may too be warranted in a pathophysiological state such as that occurring in diabetic ketoacidosis. It was first examined whether endogenous levels of prostaglandin production were altered in the STZ-induced diabetes model. STZ-injected mice suffering from hyperglycemia and acidosis displayed elevated levels of TXB₂, PGE₂, PGF₁α production in the brain relative to control animals though these increases did not appear to be significant. However, contrary to the other PGs, elevations in 15d-PGJ₂ appeared to be significant (figure 3.1a) and occurred as early as day-five post-injection of STZ (figure 3.1b). Pharmacological inhibition of 15d-PGJ₂ synthesis was achieved following ICV administration of the tetravalent selenium compound SeCl₄ which has previously been reported to be a reversible inhibitor of PGD synthase in the brain (Matsumura 1991; Islam 1994), with no observable effects on other enzymes in the arachidonate cascade. Based on the time point experiment demonstrating elevations in 15d-PGJ₂ over the course of 7 days (figure 3.1b) and given the reversible effects of SeCl₄, continuous administration was initiated on day 5 post-induction of diabetes. Efficacy of the inhibitor, in the diabetes model, was ascertained when brain 15d-PGJ₂ levels reverted to near basal amounts relative to control following 48 hours of continuous SeCl₄ administration (figure 3.1d).
Diabetic vessels have been found to be susceptible to perturbations in vessel structure and function in organs such as the kidneys as well as the eyes where an ocular manifestation of the disease results from microvascular retinal changes (Klein 1996). It thus became crucial to examine if any vaso-obliteration could be observed in the diabetic brain subsequent to development of diabetes and ketoacidosis. Cortical brain vascular network was revealed via immunohistochemical staining of frozen cryostat sections with FITC-conjugated lectin *Griffonia simplicifolia* (figure 3.1c). A significant reduction in brain vessel density of was observed in the diabetic animals relative to control, with more pronounced effects in the cortex by day 7 post-injection of STZ. On average there was a 40% reduction in vessel density (figure 3.1c), measured as capillary length per surface area (mm/mm²), p < 0.05, n = 5 per group. To substantiate the hypothesis that elevations in the brain levels of 15d-PGJ₂ in diabetes may increase the incidence of microvessel damage owing to previously reported cytotoxic properties of 15d-PGJ₂ (Bishop-Bailey 1999; Rohn 2001; Kondo 2002; Bernardo 2003; Smith 2003; Yagami 2003; Xiang 2007), vascular density was studied in the STZ-model following pharmacological inhibition of 15d-PGJ₄ with SeCl₄. When SeCl₄ administration was initiated prior to the induction of diabetes a partial rescue in vascular density was observed (Figure 3.1e). Though this effect remained partial the difference in vessel density between the SeCl₄ treated and non-treated diabetic animals remained highly significant p < 0.001 with latter group exhibiting marked decrease in vessel density.

3.3. 15d-PGJ₂ Inhibits Angiogenesis ex vivo and Induces Vaso-obliteration in vivo & ex vivo

Having observed a significant increases in the production of 15d-PGJ₂ and the amount of vaso-obliteration in the diabetic brain in vivo, it was imperative to further elucidate the role of 15d-PGJ₂ itself in the modulation of vascular degenerative responses. The subsequent crucial steps involved studying these effects in healthy animals following ICV administration of a supraphysiological dose in vivo, and via treatments of cultured brain explants and aortic rings ex vivo with the cyclopentanone.

*In vivo*

With the previous reports identifying the formation of PGD₂ within the CSF, a pathophysiological dose of 15d-PGJ₂ (100 μg/kg) was administered into the left lateral ventricles of healthy adult mice. This in turn could facilitate a better understanding of the role
Results

of 15d-PGJ$_2$ in isolation from other factors within the pathological setting. ICV administration of 15d-PGJ$_2$ resulted in significant vaso-obliteration in the brain of healthy adult mice within 24 hours (figure 3.2a) as revealed via immunohistochemical staining with TRITC-conjugated lectin *Griffonia simplicifolia*. These observations in effect highlight the involvement of 15d-PGJ$_2$ in the elicitation of acute vascular degenerative responses.

**Ex vivo**

The observations *in vivo* were corroborated *ex vivo* when cultured brain explants from healthy adult C57BL6/J mice stained with FITC-conjugated lectin *Griffonia simplicifolia* revealed significant amounts of vaso-obliteration when incubated with 10μM 15d-PGJ$_2$. A marked ~50% reduction in vessel density relative to control could be observed as early as 24 hours (figure 3.2c). *Ex vivo* effects of 15d-PGJ$_2$ were further corroborated with the use of an aortic ring angiogenesis assay which allows for a three-dimensional assessment of vessel sprouting. Angiogenesis refers to the formation of new capillaries from pre-existing vessels and is often constitutive of a complex cascade of events involving the activation of endothelial cells (ECs). ECs in turn, proteolytically degrade the extracellular matrix and progressively migrate toward an angiogenic stimulus with subsequent proliferation and alignment to form a new capillary network (Risau 1997). The aortic ring angiogenesis assay thus served to further ascertain the effects of 15d-PGJ$_2$ on vasculature as well as to substantiate previously reported data implicating the anti-angiogenic potential of 15d-PGJ$_2$ (Xin 1999; Murata 2000; Huang 2004; Quesada 2005). Aortic rings cultured with 5μM and 10μM 15d-PGJ$_2$ exhibited significant reductions in vessel outgrowth p < 0.01 and p < 0.001, respectively (figure 3.2d).

**3.4. 15d-PGJ$_2$ is a Potent Inducer of Neuromicrovascular Endothelial Cell Death**

Endothelial cells are involved in many aspects vascular biology and remain an important component of the microvasculature and their demise is often associated with compromised regional circulation (Peson 1981; Nishigaya 1991). Furthermore, endothelial cells appear to be more susceptible than other cells of the microvasculature to oxidative stress and inflammation (Beauchamp 2001; Brault 2003). The loss of endothelial cells is pivotal factor in the vaso-obliterative phase and therefore *in vitro* studies were performed to assess the effects of 15d-PGJ$_2$ on endothelial cell cultures including, BMECs, HRMECs, and HUVECs. Cytotoxic effects were also studied on astrocyte cultures given that they interact
with endothelial cells at the BBB and SMCs which also remain an important component of the vasculature.

Since 15d-PGJ2 is often referred to as the endogenous ligand for PPAR-γ (Forman 1995; Kliwer 1995; Lehmann 1995; Nosjean 2002; Ide 2003; Scher 2005) and has also been shown to be a selective agonist of the PGD2 receptor, DP2 (CRTH2) (Monneret 2002; Monneret 2003; Powell 2003) its effects on cell viability were compared with PGD2, BW245c (PGD2 analog), and Ciglitazone (PPAR-γ agonist). 15d-PGJ2 revealed itself to be the only potent inducer of cell death (figure 3.3a) such that a significant reduction in BMEC viability was detected at 24hours (EC50 = 4.6μM). This effect appeared to be time (figure 3.3b) and concentration-dependent (figure 3.3c) while PGD2, BW245c and Ciglitazone showed no significant reductions in cell viability in comparison. Cell survival markedly began to decrease following 8h through 24h exposure to 15d-PGJ2. Similar effects with 15d-PGJ2 treatment were observed in HUVECs, HRMECs, Astrocytes and SMCs (figure 3.3d). The most pronounced effects however remained in the BMECs where cell viability was reduced to approximately 10% relative to control following 24hours of exposure.

3.5. Nature of Cerebrovascular Endothelial Cell Death Induced by 15d-PGJ2

To determine whether the mechanism of 15d-PGJ2-induced cell death was attributable to apoptosis, flow cytometric analysis was performed on cells exposed to 10μM 15d-PGJ2 for 24hours. A significant increase in the percentage of apoptotic cells was observed, p < 0.0001 (figure 3.3e), following 15d-PGJ2 treatment detected as the number of cells showing green fluorescence (Annexin positive cells – lower right quadrant) compared to live cells showing little or no fluorescence (lower left quadrant) and dead cells exhibiting red-green fluorescence (Annexin and PI positive cells - upper right quadrant). To further verify the apoptotic mechanism of cell death, changes in nuclear morphology were evaluated using a blue fluorescent cell permeable nucleic acid stain Hoechst 33342 which is sensitive to DNA conformation and the chromatin state of cells. As shown in figure 3.3f, the nucleus of normal control cells (vehicle) was big and round while 15d-PGJ2-treated cells exhibited condensed nuclear morphology, a typical feature of apoptosis. Finally, electrophoretic analysis of total cellular DNA isolated from BMECs treated with 10μM 15d-PGJ2 for 24hours revealed a pattern of inter-nucleosomal DNA cleavage specific for apoptosis (figure 3.3g).
3.6. Mechanism of 15d-PGJ$_2$-induced Cytotoxicity Involves ROS Production and Occurs Independently of the DP1, DP2 and PPAR$_\gamma$ receptors.

As mentioned previously 15d-PGJ$_2$ is often referred to as an endogenous ligand for the nuclear receptor PPAR-$\gamma$. In order to ascertain whether 15d-PGJ$_2$ may be mediating its cytotoxic effects via this nuclear receptor, BMECs were pre-incubated with an irreversible antagonist for PPAR-$\gamma$, GW9662 prior to addition of 15d-PGJ$_2$. The presence of this antagonist however did not appear to rescue cell viability (figure 3.4a). BMECs were also pre-incubated with a non-selective antagonist for the DP receptors (AH6809) since 15d-PGJ$_2$ has been described to possess weak agonistic activity on the DP2 receptor. Similarly the presence of AH6809 also did not appear to rescue cell viability (figure 3.4b).

Having previously established a pro-apoptotic effect of 15d-PGJ$_2$, it was thought that perhaps this observation may help to better understand the mechanism of 15d-PGJ$_2$-induced cytotoxicity. The possible involvement of oxidative stress was then evaluated given its previous implications in the pathogenesis of diabetes and the link between pro-apoptotic processes and the formation of ROS (Lennon 1991; Nencioni 2003). Furthermore, previous reports have postulated that 15d-PGJ$_2$ may induce intracellular ROS production (Kondo 2001; Shibata 2003). 15d-PGJ$_2$ treatment caused a marked increase in intracellular ROS levels as assessed by DCFH-DA oxidation (Figure 3.4c). ROS production was fairly rapid being already detectable at 30 min after the addition of 15d-PGJ$_2$. The involvement of ROS in 15d-PGJ$_2$-induced cell death was further evaluated in the presence of antioxidants. Catalase, NAC, apocynin, allopurinol and rotenone all effectively prevented the cell death induced by 15d-PGJ$_2$ with the most pronounced effects observed in the presence of NAC, catalase and allopurinol (figure 3.4d). Taken together the preceding data suggest that 15d-PGJ$_2$ induces BMEC cell death via PPAR-$\gamma$, DP1 and DP2-independent mechanisms involving the generation of ROS.
Table 3.1 | Blood biochemistry values from diabetic (STZ) and non-diabetic mice at 7-days post-injection.

Following anesthesia blood was obtained via intra-cardiac puncture for analysis of blood glucose, ketones, electrolytes, and osmolality. Effective osmolality was calculated as follows ([Na⁺ mmol/L x 2] + Glucose mmol/L). All groups represent n = 4-5 per group (except control for glucose, n = 11) ***p< 0.0001, **p<0.001, *p<0.05, relative to control. Blood gases and pH were not reported as anesthesia required for intracardiac blood sampling resulted in artifactual changes in pH and CO₂ levels presumed to be resultant of altered respiratory rates.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mmol/L)</td>
<td>143.6 ± 0.68</td>
<td>143.0 ± 2.5</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5.14 ± 0.5</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>112.0 ± 0.7</td>
<td>108.0 ± 2.0</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>11.51 ± 1.0</td>
<td>44.5 ± 2.3 ***</td>
</tr>
<tr>
<td>Bicarbonate (mmol/L)</td>
<td>18.03 ± 1.7</td>
<td>16.46 ± 0.9</td>
</tr>
<tr>
<td>Effective osmolality</td>
<td>299.4 ± 2.5</td>
<td>330.5 ± 4.7 **</td>
</tr>
<tr>
<td>B-OH-butyrate (mmol/L)</td>
<td>0.56 ± 0.07</td>
<td>1.00 ± 0.2 *</td>
</tr>
</tbody>
</table>
Figure 3.1 | Increased brain prostaglandin production corresponds to elevations in 15d-PGJ\(_2\) with concomitant decreases in vascular density.

(a) Brain production of 15d-PGJ\(_2\), TXB\(_2\), PGE\(_2\), PGF\(_{1\alpha}\) in diabetic vs. non-diabetic controls. Histogram represents the amount of 15d-PGJ\(_2\) TXB\(_2\), PGE\(_2\), PGF\(_{1\alpha}\) production as assessed via ELISA assay (15d-PGJ\(_2\)) or RIA assay (TXB\(_2\), PGE\(_2\), PGF\(_{1\alpha}\)) on tissue homogenates (7 days post-injection of vehicle /STZ). Values represent mean ± SEM, n = 4-6 per group, ***p< 0.001 expressed as a percentage of respective controls (2-way ANOVA). (b) Time course of 15d-PGJ\(_2\) elevation in the STZ model. Histogram represents mean ± SEM values of 15d-PGJ\(_2\) production from day 1 through day 6 post-STZ injection expressed as a percentage of control, n = 5 per group, ** p < 0.01, *** p < 0.001 (2-way ANOVA). (c) Vascular network was revealed in brain sections from STZ-injected diabetic mice and saline-injected control mice (vehicle) with FITC-conjugated lectin (Griffonia simplicifolia), scale bar represents 100\(\mu\)m. Values in histogram represent mean ± SEM of vessel density in cortical region relative to that of vehicle-injected mice, n = 5 mice per group, *p < 0.05 expressed as a percentage of control (t-test). (d) Effect of SeCl\(_4\) administration on 15d-PGJ\(_2\) production. Values are expressed as pg/mg of protein, n = 4 – 8 per group, *** p < 0.001 (2-way ANOVA) (e) Effect of SeCl\(_4\) on vascular density. Histogram represents mean ± SEM of vessel density in cortical region expressed as percent control relative to aCSF administered non-diabetic animals, n = 4 per group, *** p < 0.001.
Figure 3.1

a.)

PG levels (% control)

0 50 100 150 200 250
TXB₂  PGF₁α  PGE₂  15d-PGJ₂

b.)

PGJ₂ levels (% control)

0 50 100 150 200
Day 1  Day 2  Day 3  Day 4  Day 5  Day 6

Vehicle  STZ

***

Vehicle  STZ

***

Vehicle  STZ

**

Vehicle  STZ

†

Vehicle  STZ

0 25 50 75 100 125
Vascular Density (% control)

Vehicle  STZ

0 100 200 300 400 500 600 700 800 900
15d-PGJ₂ (pg/mg protein)

Vehicle  STZ

***

Vehicle  STZ

***

Vehicle  STZ

†

Vehicle  STZ
Figure 3.2 | 15d-PGJ2 inhibits angiogenesis \textit{ex vivo} and induces vaso-obliteration \textit{in vivo} and \textit{ex vivo}

(a) \textit{In vivo} effects of 15d-PGJ2 on brain vasculature. Mice were infused with a single ICV injection of aCSF (vehicle) or 15d-PGJ2 (100 µg/kg) in a final volume of 5µL. Cortical vessel density was revealed via TRITC-conjugated lectin (\textit{Griffonia simplicifolia}). Values in histogram represent mean ± SEM of vessel density from 10-15 brain sections per animal, *** p < 0.0001, n = 4 per group. (b) Validation of injection site. Trypan blue was injected in a volume of 5µL into the left lateral ventricle to visualize distribution in CSF and verify the accuracy of the stereotaxic coordinates. LV indicates lateral ventricle. (c) \textit{Ex vivo} effects of 15d-PGJ2 on neuromicrovascular density. Brain explants from C57BL6/J mice were cultured for 24h in the absence (vehicle) or presence of 10µM 15d-PGJ2. Neuromicrovascular endothelium was stained with FITC-conjugated lectin (\textit{Griffonia simplicifolia}). Values in histogram represent quantification of vessel density by computerized image analysis. Data are expressed as mean ± SEM, n = 3 animals per group, *** p < 0.0001 relative to control (vehicle). (d) Role of 15d-PGJ2 in angiogenesis \textit{ex vivo}. Photomicrographs of thoracic aortic explants cultured with 5µM (not shown), 10µM 15d-PGJ2 and vehicle were taken prior to and 24h post-addition of test compound. Histogram represents quantification of microvessel outgrowth by computerized image analysis of thoracic explants cultured with vehicle, 5µM or 10 µM 15d-PGJ2. Data are expressed as mean ± SEM, from three separate experiments n = 7 aortic rings per group from four animals, * p < 0.05, ** p < 0.01, scale bar represents 100µm.
Figure 3.2

a.)

b.)

LV

Vascular density (% control)

Vehicle 15d-PGJ₂

0 25 50 75 100 125

Vascular density (% control)

Vehicle 15d-PGJ₂

0 25 50 75 100 125

Vascular density (% control)

Vehicle 15d-PGJ₂

0 25 50 75 100 125

Vascular density (% control)

Vehicle 15d-PGJ₂

0 25 50 75 100 125

Vascular density (% control)

Vehicle 15d-PGJ₂
Figure 3.3 | 15d-PGJ\textsubscript{2} is the most potent inducer of neuromicrovascular endothelial cell death by apoptosis.

(a) Treatment of BMECs with 10μM PGD\textsubscript{2}, BW245c (DP receptor agonist), 15d-PGJ\textsubscript{2}, and Ciglitazone (PPAR\textgamma agonist) revealed 15d-PGJ\textsubscript{2} to be the most potent-inducer of cell death (EC\textsubscript{50} = 4.6μM). Cell death appeared to be time (b) and concentration-dependent (c) as assessed via MTT assay. (d) Similar concentration-dependent effects of 15d-PGJ\textsubscript{2} were observed in other cell types including HUVECs, HRMECs, SMCs and Astrocytes (d). Values are expressed as a percent of control and represent mean ± SEM of five to six experiments (c), and three separate experiments (d), each performed in triplicate. (e) Induction of chromatin condensation could be observed upon high magnification of the nuclei stained with Hoechst 33342 in BMECs treated with 10μM 15d-PGJ\textsubscript{2}. (f) Agarose gel electrophoresis exhibited DNA ladder formation in BMECs treated with 10μM 15d-PGJ\textsubscript{2}. Lane 1: DNA marker, Lane 2: control, Lane 3: 10μM 15d-PGJ\textsubscript{2}, Lane 4: DNA marker. (g) Fluorescence-activated-cell sorter (FACS) caliber flow cytometer analysis of 15d-PGJ\textsubscript{2}-induced cell death revealed an increase in the percentage of apoptotic cells (lower right quadrant) following 24 hour treatments with 15d-PGJ\textsubscript{2}. Data in histogram represent mean ± SEM, n = 3, *** p < 0.0001 relative to control (vehicle).
Figure 3.3

a.)

Concentration (log mol/L)

Cell Viability (% control)

b.)

Time [h]

Cell viability (% control)

c.)

Concentration (log mol/L)

Cell Viability (% control)

d.)

Concentration (log mol/L)

Cell Viability (% control)

e.)

% apoptotic cells

Control 15d-PGJ2

f.)

Vehicle 15d-PGJ2

g.)

Vehicle 15d-PGJ2

Control 15d-PGJ2

Annexin V

Annexin V

PI

6.31%

48.73%

Astro, EC50= 8.2 μM
HRMEC, EC50= 3.7 μM
HUVEC, EC50= 3.2 μM
SMC, EC50= 4.2 μM

Vehicle

15d-PGJ2
Figure 3.4 | 15d-PGJ2 cytotoxicity on neuromicrovascular endothelial cells involves ROS production and occurs independently of the PPAR-γ, DP1 and DP2 receptors.

(a) Cultured BMECs treated with 10µM 15d-PGJ2 in the presence or absence of the irreversible PPAR-γ antagonist GW9662 (10µM, 50µM) revealed a PPAR-γ-independent response with no rescue in cell viability following pre-treatment with GW9662 (10µM). Data are expressed as a percent of control and represent mean ± SEM of three separate experiments performed in triplicate. (b) Pre-treatment with 10µM AH6809 (non-selective DP antagonist) did not rescue cell viability while treatment with BW245c (DP1 receptor agonist) did not induce a decrease in cell viability, collectively revealing a DP receptor-independent response of 15d-PGJ2-induced cell death. Data are expressed as percent of control and represent mean ± SEM of three separate experiments. (c) BMECs were pre-loaded with the fluorescent ROS probe DCFDA for 15min then treated with 10µM 15d-PGJ2 for 5min. ROS production was assessed via DCFDA fluorescence intensity expressed as a percentage of control from three separate experiments performed in quadruplicate, mean ± SEM, *** p < 0.0001. (d) Survival of BMECs treated with 15d-PGJ2 (10 µM) in absence or presence of catalase (500 U), N-acetyl-cysteine (NAC, glutathione precursor, 2 mM), apocynin (1mM), allopurinol (xanthine oxidase inhibitor and free radical scavenger, 1 mM) or rotenone (mitochondrial complex 1 inhibitor, 1µM). Cell viability quantified by MTT assay showed that the presence of anti-oxidants partially rescued 15d-PGJ2-induced cell death. Data are expressed as percentage of control, mean ± SEM from three to five experiments performed in triplicate; * p < 0.05, ** p < 0.01, *** p < 0.001 compared to 15d-PGJ2-treated cells (one-way ANOVA). (e) Brain explants cultured for 24hours with catalase (500 U), NAC (2mM), apocynin (1 mM), allopurinol (1 mM) or rotenone (1µM) in the presence or absence of 10 µM 15d-PGJ2. Neuromicrovascular endothelium was stained with TRITC-conjugated lectin (Griffonia simplicifolia) (not shown) and vascular density assessed via computerized image analysis revealed that the presence of antioxidants partially rescued vessel degeneration induced by 15d-PGJ2. Values are expressed as percent of respective controls, mean ± SEM; * p < 0.05, ** p < 0.001 (one-way ANOVA).
Figure 3.4

(a) Cell Viability (% Control)

(b) Cell viability (% control)

(c) DCF (% of control)

(d) Cell Viability (% of Control)

(e) Vascular density % control
The observations in the present thesis suggest that 15d-PGJ$_2$ is abundantly generated in an experimental model of streptozotocin-induced diabetes with ensuing acidosis. This cyclopentanone in turn appears to be involved in eliciting vaso-obliterrative effects as demonstrated \textit{in vivo}, \textit{ex vivo} and \textit{in vitro}. While these effects appeared to occur independently of the DP1, DP2 and PPAR-$\gamma$ receptors they did involve the formation of reactive oxygen species (ROS). The generation of ROS and the involvement of oxidative stress in turn have been linked to pro-apoptotic processes suggesting a possible mechanism by which 15d-PGJ$_2$ may bring about endothelial cell death and consequential microvascular degeneration in the diabetic brain. Alternate mechanisms that may be involved include covalent inactivation of the NF$\kappa$B-mediated survival pathways and the transcriptional regulation of COX such that 15d-PGJ$_2$-induced inhibition of COX may lead to increased intracellular levels of arachidonic acid (AA). This accompanied by increased oxidative stress may lead to the production of oxidized lipids resulting in cell death. Dashed lines represent pathways verified in the present thesis. Solid lines represent postulated mechanisms.
Figure 3.5
DISCUSSION
The findings in the present thesis suggest a pathophysiological role for the cyclopentanone prostaglandin 15d-PGJ$_2$ such that concentrations \( \geq 1 \mu M \) detected in the STZ-induced diabetic brain \textit{in vivo} appeared to elicit cytotoxic effects on the vasculature \textit{ex vivo}, and more specifically on brain microvascular endothelial cells \textit{in vitro}. Microvascular degeneration is of significant pathophysiological relevance to patients suffering from diabetes-related cerebral edema. This condition is often associated with acute cerebromicrovascular perturbations occurring in states of diabetic ketoacidosis. Although the general physiological and anatomical concept of the cerebral microvasculature and the BBB is familiar to most, the involvement of the cerebrovascular endothelium in the pathogenesis of encephalopathies is often overlooked. Diabetic vessels possess a greater susceptibility to perturbations in vessel structure design as seen in organs such as the kidneys as well as the eyes where an ocular manifestation of the disease is underlined with microvascular complications. It can then be inferred that a substantial amount of literature warrants a vascular basis for the structural as well as functional alterations in the CNS within the pathogenesis of diabetes but the mechanisms underlying the neuropathology still remain elusive. Of particular interest is the observation that the blood-brain barrier, a structure often devoid of fenestrations under physiological conditions, can respond to increases in arachidonic acid products (Brault 2003; Kermorvant-Duchemin 2005; Gabryel 2006; Quiniou 2006). Acidosis, such as that occurring in DKA, can stimulate K$^+$ channel openings (Faraci 1994; Kontos 1996; Kinoshita 1997; Xu 2000) in endothelial cells, leading to increased activities of phospholipase A$_2$ (Hyslop 1993) thus activating the arachidonic acid cascade. Cerebrovascular arachidonic acid metabolism in turn plays an important role in the regulation of cerebral circulatory physiology as well as a cerebrovascular response injury.

A critical question that surfaces when considering the cyclopentanone prostaglandins is whether or not they play a role in normal physiology. In order to establish a physiological or pathophysiological role for these compounds it is essential to demonstrate their existence \textit{in vivo}. The present thesis is likely to be one of the first reports to demonstrate an abundant generation of 15d-PGJ$_2$ in the diabetic brain. The contribution of prostanoids to the pathogenic scheme has been recognized in the literature for a long time. Prostaglandins in general have been found to occur in bodily fluids in the picomolar to nanomolar range under physiological conditions (Fukushima 1990) however, concentrations may be greatly exacerbated reaching
micromolar amounts at sites of inflammation (Offenbacher 1986). Baseline production of PGD$_2$ in the mouse brain has been calculated to be approximately 2nM (Qu 2006) while an earlier study demonstrated the production of 15d-PGJ$_2$ in the medium of primary microglial cell cultures to be in the range of 10nM (Bernardo 2003). Additionally, the levels of PGD$_2$ in tissue homogenates of adult rats have been found to exist in the micromolar range and production levels could be strongly increased following the addition of exogenous arachidonic acid (Urade 1989). Taken together, these findings warrant the possibility that concentrations of 15d-PGJ$_2$ in the brain in vivo maybe orders higher due to the confined interstitial space as well as factors affecting arachidonic acid metabolism in the brain within the pathogenesis of diabetes itself. In a more recent study 15d-PGJ$_2$ itself was found to be increased to 600 pg/mg protein (Lin 2006) in the ischemic cortex. The present observations demonstrate that the levels of 15d-PGJ$_2$ production significantly exceeded those of other prostaglandins in the brains of STZ-induced diabetic animals reaching micromolar concentrations (~1-3μM). These results suggest that the toxicity of 15d-PGJ$_2$ at micromolar concentrations observed in vitro in the present thesis as well in several previously published reports may also occur in vivo. The concentrations reached in the local microenvironment are likely resultant of the rapid conversion of PGD$_2$ into its cyclopentanone derivatives (Fitzpatrick 1983), and were confirmed in vitro as sufficient to induce BMEC apoptosis.

While the adverse effects of 15d-PGJ$_2$ on endothelial cell viability appear to be an exception to its commonly known anti-viral and anti-tumoral effects (Honn 1985) the current observation of the cytocidal effects of 15d-PGJ$_2$ are in accordance with emerging findings highlighting its deleterious effects on various non-cancerous cell types including endothelial cells (Bishop-Bailey 1999), neurons (Rohn 2001; Kondo 2002; Smith 2003; Yagami 2003), microglia (Bernardo 2003), and more recently, oligodendrocyte precursor cells (Xiang 2007). Taken together these findings along with the current observations support a role for 15d-PGJ$_2$ in brain injury resultant of microvascular degeneration and propose it to be a possible underlying mediator in diabetes-related encephalopathies comprising of vascular injury. In vivo assessments of vascular degeneration within the diabetes model and that brought about as a result of 15d-PGJ$_2$ administration were primarily dependant on fluorescence imaging of brain sections following lectin *Griffonia simplicifolia* staining. Several criticisms have been put forth regarding this assessment such as the requirement of thresholding of the fluorescence intensity to delete background auto fluorescence. In the present thesis for all fluorescence
images used in final analysis thresholding was consistent among brain sections. Furthermore, image analysis was conducted in a blinded manner to avoid investigator bias.

Several studies investigating the effects of inflammatory prostaglandins have used inhibitors for COX such as Ibuprofen, however the use of such non-selective inhibitors suppress the formation of other COX-derived products making it difficult to interpret the role and possible interplay of the downstream prostaglandins. The search for a more specific inhibitor led to the discovery of the tetravalent selenium compound SeCl$_4$, previously demonstrated to be a reversible inhibitor of the L-PGDS (Matsumura 1991; Qu 2006). The toxicity of selenium compounds in several species of mammals has been documented previously (Ursini 1999) despite being an essential trace element. Furthermore, selenocysteine is present in the active site of glutathione peroxidase (Wingler 1999) and is component of other proteins such as selenoprotein P (Ursini 1999). However, experiments in the present thesis revealed no toxic effects of SeCl$_4$ in the diabetic or non-diabetic animals as determined by the display of normal behaviors in all treated groups similar to that observed prior to the administration of the compound. These observations would need to be interpreted with caution however, as selenium is extremely non-selective and toxic effects cannot be completely ruled out by behavioral observations. The findings in the present thesis in effect corroborate previous data pertaining to the efficacy of the compound as an inhibitor of PGD$_2$ as assessed by the significant reductions in brain 15d-PGJ$_2$ levels to near basal amounts in the diabetic group. However, with previous literature identifying a role for PGD$_2$ in the regulation of sleep-wake activities whereby SeCl$_4$ administration resulted in the inhibition of sleep, the present thesis does not propose SeCl$_4$ as a potential therapeutic to suppress the formation of 15d-PGJ$_2$ but rather highlights the precise role of this cyclopentanone in relation to other prostanoids in mediating vascular degenerative responses. The use of SeCl$_4$ to suppress the formation of 15d-PGJ$_2$ here represents a novel approach and is the first account to demonstrate the efficacy of this compound as an inhibitor of 15d-PGJ$_2$ in a pathological setting \textit{in vivo}. The observation that suppressing 15d-PGJ$_2$ formation appears to partially rescue vascular injury in turn affirms the partaking of this cyclopentanone in the detrimental process.

With the data proposing a novel pathological condition in which 15d-PGJ$_2$ is generated in substantial amounts, the attempt to further substantiate its biological activity confirmed its role as an important mediator in the elicitation of vascular degenerative responses likely at the
level of the blood-brain barrier. This was demonstrated by showing that 15d-PGJ\(_2\) induces neuromicrovascular endothelial cell (BMEC), smooth muscle cell and astrocytic demise \textit{in vitro} (figure 3.3d) and exerts, presumably species-independent cytotoxicity on brain microvessels in both \textit{ex vivo} and \textit{in vivo} models (figure 3.2). The biphasic effects of 15d-PGJ\(_2\) have been previously reported (Levonen 2001; Emi 2004) whereby the concentration of 15d-PGJ\(_2\) appears to dictate opposing biologic outcomes. Specifically, low concentrations (1\(\mu\)M) of 15dPGJ\(_2\) increase cellular proliferation, moderate concentrations induce cell cycle arrest and cellular differentiation, while (10\(\mu\)M) higher concentrations induce apoptosis. The reason for these paradoxical observations is yet to be determined, however the cytotoxic effects of 15d-PGJ\(_2\) at a concentration of 10\(\mu\)M used in the current study was confirmed to be attributable to apoptosis via an array apoptosis detection techniques.

With a majority of the reports focusing on \textit{in vitro} assessments of 15d-PGJ\(_2\)-induced responses, several mechanisms have been put forth since to explain its diversity of actions. The exact mechanism by which this cyclopentanone may exert its cytotoxic effects on brain microvessels however still remains to be elucidated. The cyclopentanone derivatives of PGD\(_2\) have a unique structural component that can account for receptor-independent modifications of their cellular targets. The structure of 15d-PGJ\(_2\) significantly differs from the synthetic class of drugs thiazolidinediones (TZDs) that also serve as ligands for the nuclear receptor PPAR-\(\gamma\). It is most likely that its nucleophilic ability can be attributed to the more potent apoptotic properties of 15d-PGJ\(_2\) compared to the TZD ciglitazone as well as PGD\(_2\) itself (figure 3.3a) and furthermore account for receptor-independent actions. PPAR-\(\gamma\) and PGD\(_2\) (DP1/DP2) receptor – independent induction of apoptosis by 15d-PGJ\(_2\) was further confirmed when the irreversible PPAR-\(\gamma\) antagonist GW9662 (figure 3.4a) and the non-selective DP receptor antagonist AH6809 (figure 3.4b) were unable to rescue cell death induced by the cyclopentanone.

Apoptosis induced by various stimuli is frequently reported to be accompanied by ROS production (Martindale 2002). ROS levels when exceedingly high can overwhelm cellular oxidative defenses. Owing to the unstable nature of free radicals their high reactivity can result in cellular damage. Although 15d-PGJ\(_2\) has been reported to be a potential inducer of intracellular oxidative stress (Kondo 2001; Clay 2002) it is not clear whether this effect provides a sole cause for cellular demise induced by 15d-PGJ\(_2\) in brain microvessels. In the present study, 15d-PGJ\(_2\) stimulated ROS production in BMEC cells (figure 3.4c) and
antioxidants including catalase, NAC, allopurinol and rotenone appeared to at least in part protect the cell (figure 3.4d) as well as cultured brain explants (figure 3.4e) from demise. The production of ROS was significantly increased as early as 30 min after treatment (figure 3.4c) indicating that ROS production is an early event in the apoptotic signaling pathway. With what appeared to be a partial reversal of outcomes it is not to say that the detrimental effects of 15d-PGJ2 observed in BMECs and brain microvessels is entirely attributable to ROS, however ROS may in turn function as key signaling molecules that oxidize proteins resulting in protein degradation, or fragmentation ultimately reducing protein function (Byung 1994). They may also result in the oxidation of molecular mediators resulting in the induction of the apoptotic signaling cascade. Additionally ROS have been known to cause lipid peroxidation and DNA damage which can in turn contribute to cell destruction and ultimate induction of the apoptotic signaling cascade (Byung 1994). The ability of anti-oxidants to rescue 15d-PGJ2-induced apoptosis however suggests that ROS can be considered to be one of the major contributors for the initiation of the apoptotic death cascade.

Of particular interest is the observation that astrocytes appeared to be more resistant to the potent effects of 15d-PGJ2. Prostaglandins are rapidly metabolized in cells via glutathione transferase (GST)-mediated conjugation to glutathione (GSH) (Atsmon 1990; Bogaards 1997) then removed from the cell by the action of ATP-dependent efflux pumps (Paumi 2003). Variability between cell types in GSH and GST levels and efflux pump activity may explain the differential susceptibility, furthermore, astrocytes have been reported to possess (Bresgen 2006) better antioxidant defense mechanisms affirming the involvement of oxidative stress in 15d-PGJ2-induced responses. Accordingly, depletion of intracellular GSH (antioxidant) levels potentiates the effects of cyclopentenone PGs, while augmentation of cellular GSH content protects cells from these compounds (Atsmon 1990; Levonen 2004).

Alternate mechanisms that maybe be involved are depicted in figure 3.5. Cyclopentanone prostaglandins can modify cellular components necessary for defense, such as modification of proteins like NF-κB that can serve as key regulators of cellular anti-apoptotic defense mechanisms (Straus 2000; Cernuda-Morollon 2001). The exocyclic electrophilic carbonyl of 15d-PGJ2 covalently inactivates the IKK rendering NF-κB in its inactive form bound to I-κBα and I-κBβ (Castrillo 2000; Rossi 2000; Straus 2000; Cernuda-Morollon 2001). NF-κB plays an important role in the control of cell proliferation and survival (Karin 2002). 15d-PGJ2 is able to inhibit TNF-α- and tPA-induced activation of NF-κB in several cell types
Discussion

(Rovin 2001). Activation of NF-κB has been described as a key survival step through the expression of antiapoptotic genes of the Bcl-2 family and various members of the IAP family, which explains its capacity to inhibit caspase activation (Beg 1996; Wang 1998; Baldwin 2001). In the CNS, basal NF-κB activity in neurons is required for survival, while NF-κB inhibition can precipitate neuronal death and enhance neurodegeneration initiated by a variety of insults (Kaltschmidt 1999; Mattson 2001; Chiarugi 2002; Culmsee 2003). With 15d-PGJ2 previously demonstrated to be a potent inhibitor of NF-κB, this mechanism along with oxidative stress could also contribute to its cytotoxicity. Another possible mechanism involves the ability of 15d-PGJ2 to inhibit transcriptional activation of COX-2, and for that matter perhaps other arachidonic acid metabolizing enzymes (Xin 1999; Inoue 2000) this in turn may lead to increased intracellular levels of free arachidonic acid, an event also known to induce apoptosis (Surette 1996; Surette 1999; Cao 2000). Finally, in addition to its cytocidal effects, several lines of evidence demonstrate the anti-angiogenic potential of 15d-PGJ2 corroborated in the present thesis with the aortic ring angiogenesis assay (figure 3.2d). While 15d-PGJ2 has been shown to inhibit mitogen-induced EC proliferation and tube formation by ECs in an in vitro model of angiogenesis, the use of the aortic ring angiogenesis assay in the present study is the first report to use this assay to ascertain the anti-angiogenic potential of 15d-PGJ2. 15d-PGJ2 also inhibits VEGF-induced angiogenesis in a corneal assay (Sarayba 2005). Another study reports that though the downregulation of VEGF receptors may be involved this may not be a major mechanism and that 15d-PGJ2 strongly and PPAR-γ-independently inhibits activities of c-jun and c-myc, the transcription factors involved in regulation of cell cycle (Funovics 2006). This could in part explain 15d-PGJ2-induced responses in the present thesis though this remains to be investigated.

With multiple mechanisms put forth to explain the diversity of actions the relative importance of each has not been completely established. That being said the role of 15d-PGJ2 in the CNS is far from clear because in addition to toxic effects, neuroprotective actions of cyclopentanones have described. However the present study is one of the first attempts to characterize the role of 15d-PGJ2 in the CNS of diabetic animals. With the earlier findings demonstrating that cyclopentenone eicosanoids can perturb mitochondrial function and promote oxidative stress suggest that 15d-PGJ2 is more likely toxic than protective. Mitochondrial dysfunction is a well documented event in apoptosis (Cossarizza 1994; Zamzami 1995). A process known as permeability transition appears is responsible for the loss of the
mitochondrial membrane potential, leading to the opening of the permeability transition pore and release of solutes from the mitochondria. Of the proteins released are the apoptosis-inducing factor and cytochrome c which in turn leads to activation of caspases and subsequently apoptosis (Perkins 2000). It is likely that the source of ROS generation in the present study is the mitochondria however this remains to be established.

In conclusion, the findings in the present thesis in effect unveil a pathophysiological for the cylopentanone 15d-PGJ2 in an experimental in vivo model of diabetes. Though the cytocidal effects of 15d-PGJ2 have been extensively studied a majority of these reports have focused on an in vitro assessment. The present thesis therefore, signifies a novel role for 15d-PGJ2 in a pathological setting in vivo and provides a new perspective as to the upstream molecular mechanisms of oxidative stress on microvascular injury in the diabetic brain. While these findings are restricted to an experimental model of diabetes they can certainly provide new insights as to the underlying effects observed in diabetes-related encephalopathies and vascular injuries which can be extrapolated to human patients. These data situate 15d-PGJ2 as a pivotal mediator involved in eliciting vascular degenerative responses in the brain, however it is highly plausible that other factors may be involved in this process and necessitate further investigation into the downstream interplay between molecular mediators in the pathogenesis of diabetes-related encephalopathy.
CONCLUSIONS & FUTURE DIRECTIONS
Recent years have uncovered new and exciting findings on a variety of different functions of 15d-PGJ2 in the physiological and pathophysiological setting. This thesis has added some novel observations to the immense field on prostaglandins with an emphasis on the pathogenic scheme of 15d-PGJ2 in the CNS and a special focus on diabetes-related encephalopathies. It is clear that 15d-PGJ2 is an important factor in both CNS health and disease. The question as to whether 15d-PGJ2 is detrimental in diabetes-related CNS injuries is central to this thesis. Attempts to clarify this involvement were made \textit{in vivo} however given the focus of the present thesis on vasculature further studies are necessary to solidify this understanding. Within the STZ-model, experiments pertaining to capillary leakage via measuring trypan blue exclusion in the brain can strengthen the hypothesis that brain microvascular integrity is indeed diminished in diabetics. Furthermore, having ascertained that 15d-PGJ2 levels are elevated in the STZ model, an assessment of capillary leakage prior to and following SeCl4 administration could better elucidate the role of this cyclopentanone \textit{in vivo}.

While several attempts were made to demonstrate the harmful effects of 15d-PGJ2 on vasculature \textit{in vivo}, \textit{ex vivo} and \textit{in vitro}, the exact mechanism by which this detrimental process results in microvascular complications in diabetes-related encephalopathies remains to be elucidated. Downstream cellular mechanisms that necessitate further study include the involvement of NF-\kappaB. It is likely that 15d-PGJ2 elicits its effects via the inhibition of NF-\kappaB translocation to the nucleus. Since NF-\kappaB is a transcription factor, its inhibition may favor the formation of reactive oxygen species via decreased generation of antioxidants such as MnSOD (Manganese superoxide dismutase). The translocation of NF-\kappaB can be studied via confocal microscopy imaging of NF-\kappaB localization in the cytosolic versus nuclear regions of the cell or western blot analysis of NF-\kappaB content in whole cell versus nuclear extracts upon 15d-PGJ2 treatment. The levels of MnSOD in turn can be assessed via western blot analysis. Furthermore, the involvement of oxidative stress and the confirmation of an apoptotic death mechanism induced by 15d-PGJ2 suggests the possibility of the oxidation of a pro-apoptotic factors such as the caspases which in turn may initiate a specific death mechanism. Brain sections may in turn be evaluated for apoptosis (Tunnel and Caspase staining) to determine if the link between 15d-PGJ2 exists \textit{in vivo}. It may also be beneficial to identify the source of ROS generation.
i.e. whether or not it is mitochondrial since this structure has so often been associated with pro-apoptotic processes. Measurements of mitochondrial membrane potential (decreased membrane potential) as well as cytochrome c release may be interesting preliminary experiments to further uncover the death mechanism. Furthermore, while the in vitro studies on anti-oxidants elutes to the involvement of reactive oxygen species, the role of oxidant stress in vivo would benefit from a direct measure of oxidant stress markers.

Reverting to the role of 15d-PGJ₂, conditional tissue or cell-specific knockouts or knock-in models could be excellent tools to scrutinize the function of this cyclopentanone in vivo.


Appendix I

Certificate of Ethics