Effects of Aspirin on metabolic alterations in Type 2 Diabetic Rats

Layla Ishaq Hussain Rahmatalla Amiri

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EFFECTS OF ASPIRIN ON METABOLIC ALTERATIONS IN TYPE 2 DIABETIC RATS

Layla Ishaq Hussain Rahmatalla Amiri

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Biochemistry and Molecular Biology)

Under the Supervision of Professor Haider Raza

April 2016
Declaration of Original Work

I, Layla Ishaq Hussain Rahmatalla Amiri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Effects of Aspirin on Metabolic Alterations in Type 2 Diabetic Rats”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Haider Raza in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Copy 2 of 10
Abstract

**Background:** Increased oxidative stress, inflammatory responses and mitochondrial dysfunction have been implicated in diabetes, obesity (diabesity) and cardiovascular diseases. Type 2 diabetes is the most common metabolic disorder, characterized by insulin resistance and pancreatic islet β-cell failure. The most common complications associated with type 2 diabetes are hyperinsulinemia, hyperglycemia, hyperlipidemia/dyslipidemia, increased inflammatory and reduced insulin response. Aspirin (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with the prevention of diabetes, obesity and related cardiovascular disorders. Aspirin has been used in many clinical and experimental trials for the prevention of diabetes and associated complications. **Aims and hypothesis:** Since inflammation, oxidative stress and mitochondrial dysfunction are hallmarks of many degenerative diseases, including diabetes, and aspirin and other NSAIDs have been used in clinical trials for the prevention of diabetes and related complications, we hypothesize that ASA might be involved in altering the molecular and metabolic targets affected in type 2 diabetes. Thus our main aim was to elucidate the mechanism of aspirin action in the pathophysiology and progression of disease using an experimental rat model for type 2 diabetes. **Methods:** In this study, five month old Goto-Kakizaki (GK) rats, which showed signs of hyperglycemia were used. Two subgroups of GK and Wistar control rats were injected intraperitoneally with 100 mg aspirin/kg/body weight/day for 5 weeks. Animals were sacrificed and blood and tissues were collected after performing glucose tolerance (2 h post 2g IP glucose ingestion) tests in experimental and control groups. **Results:** Aspirin caused a decrease in hyperglycemia which was accompanied by a significant improvement in glucose tolerance after ASA treatment in GK rats compared to the nondiabetic Wistar rats. Also, the ASA treated GK rats exhibited a significant decrease in insulinemia. ASA treatment caused a marked reduction in the pro-inflammatory prostaglandin, PGE2, which was significantly higher in GK rats. Aspirin treatment also improved energy metabolism in peripheral tissues. On the other hand, no significant organ toxicity was observed after ASA treatment at this dose and time period. However, the total cholesterol and lipoprotein levels were significantly
increased in GK rats, which decreased after ASA treatment. Immunofluorescence staining for insulin/glucagon secreting pancreatic cells showed improved β-cell structural and functional integrity in ASA-treated rats which was also confirmed by SDS-PAGE and Western blot analysis. **Conclusion:** The improved glucose tolerance in ASA-treated GK rats may be associated with increased insulin responses due to the anti-inflammatory properties of ASA and improvements in ROS/RNS – dependent (oxidative stress related) alterations in mitochondrial functions which facilitated insulin signaling and energy utilization in target tissues. These results may have implications in determining the therapeutic use of ASA in insulin-resistant type 2 diabetes. **Significance:** Since the UAE is one of the fastest growing countries where type 2 diabetes is prevalent, our study could help in identifying the metabolic targets affected in diabetes and after treatment with aspirin. The study might be significant in designing therapeutic strategies and in the management of this chronic disease.

**Keywords:** Type 2 diabetes mellitus (T2DM), insulin resistance, insulin signaling, metabolic alterations, aspirin (ASA), oxidative stress, energy metabolism.
تأثيرات الأسبرين على التغيرات الاستقلالية في النوع الثاني لداء السكري

الملخص

تسببت زيادة الاكسدة، والاستجابات الالتهابية و ضعف الميتوكوندريا في مرض السكري، والسمكة (diabesity) و أمراض القلب والأوعية الدموية السكري من النوع 2 هو اضطراب التمثيل الغذائي الأكثر شيوعا، والتي تتميز بمقاومة الأنسولين و فشل خلايا بيتا جزيرة في البنكرياس. المضاعفات الأكثر شيوعا المرتبطة بداء السكري من النوع 2 و فرط ارتفاع السكر في الدم والدهون / اضطراب شحوم الدم، وزيادة التهابات وانخفاض استجابة الأنسولين. الأسبرين (ASA) وغيرها من المعايير غير المترتبة المحتملة للالتهابات (المسكنات) ارتبطت مع الوقاية من مرض السكري، والسمكة واضطرابات القلب والأوعية الدموية ذات الصلة. وقد استخدم الأسبرين في العديد من التجارب السريرية والتجريبية للوقاية من مرض السكري والمضاعفات المصاحبة

الأهداف و الارياء: مُتَّوَّلًا لإظهار التأثيرات الإجهاد التأكسدي و ضعف الميتوكوندريا في مرض السكري، وحماية الأنسولين من التدمير بواسطة الأسبرين والمضادات المضادة للالتهابات، وجمع المشتقات، والأجسام المضادة لالتهابات، PGE2، الميتوكوندريا، وحدة الأنسولين. وهذا كان هدفنا الرئيسي لتوضيح أي تأثيرات من الأسبرين في الفيزيولوجيا السريرية وتطوير المرض باستخدام نموذج الحشرات فيلم السكري من النوع 2

الطريقة: في هذه الدراسة، تم استخدام الفئران عمر خمسة أشهر (GK)، والتي أظهرت علامات على ارتفاع السكر في الدم (الجلوكوز صحفي في الدم 80-95 ملغم/دسمتمتر مربع وديريلتر فائران وبستر سليمة). تم فحص مجموعة عينتين من GK وجرذان وديسيلتر سليمة البريتون مع 100 ملغم الأسبرين / كغم / وزن الجسم / يوم، لمدة 5 أسابيع. تمت التضحية بالحيوانات. جميع الفئران والأنسولين بعد انتهاء الجلوكرز (GIP2) اختبارات في المجموعة المضمنة للبكترية GIP2 في الجلوكرز التجريبي GIP2 بالفترة الزمنية. ومع ذلك، فقد ارتبط إجمالي الكولسترول والبروتين الدهن

النتائج: تسبب الأسبرين انخفاض في ارتفاع السكر في الدم، والذي كان يراقبه تحسن كبير في تحمل الغلوكرز بعد العلاج GK بالمقارنة مع الفئران ASA. أيضا، أظهرت الأسبرين ارتفاع انسولين في الفئران ASA GK ASA PGE2، الذي كان أعلى بشكل ملحوظ في الفئران ASA PGE2 في هذه الجرعة والفترات الزمنية. ومع ذلك، فقد ارتبط إجمالي الكولسترول والبروتين الدهن ASA في هذه الجرعة والفترات الزمنية مع الفئران ASA. أظهر المنايع تطبيق للأنسولين
الخلاصة: تحسن سكر الدم في الفئران ASA يترافق مع زيادة استجابات الأنسولين غكي. وقد أكده مايتفا SDSDS PAGE. يظهر هذا أيضًا في SDS-PAGE، ولذا يتم استخدامه في الفئران المعالجة بواسطة ASA.

لا يمكن أن يساعد دراسة أيضًا في تحديد أهداف الأيض التي تتأثر بمرض السكري بعد العلاج مع الأسبرين. قد تكون هذه الدراسة هامة في تصميم الاستراتيجيات العلاجية وفي إدارة هذا المرض المزمن.

المفاهيم الرئيسية: D2DM (T2DM)، مقاومة الأنسولين، إشارات الأنسولين، تعديلات التمثيل الغذائي، الأسبرين (ASA)
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Dubai Health Authority, for giving me the sponsorship and fulltime study leave so I can improve my professional skill and do my Master’s degree.
Dedication

To my beloved parents, husband and family
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<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA</td>
<td>Aspirin</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>GK</td>
<td>Goto-Kakizaki</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>ROS/RNS</td>
<td>Reactive oxygen species/reactive nitrogen species</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Hb1ac</td>
<td>Glycosylated hemoglobin</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
</tr>
<tr>
<td>MENA</td>
<td>Middle East and North Africa</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adenocorticotropin hormone</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>IMCL</td>
<td>Intramyocellular lipids</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxy-nitrite radical</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>RAGEs</td>
<td>AGEs receptors</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Overview

Diabetes mellitus (DM), the most widespread complex metabolic disorder among the world's population, is affecting millions of people globally. The prevalence is increasing rapidly. A recent study by Guariguata et al. representing 130 countries reported in 2013 that 382 million people were diabetic and this number was expected to rise to 592 million by 2035 (1). Furthermore, there were a number of undiagnosed cases of diabetes mellitus and impaired glucose tolerance, which could further increase leading to higher numbers of people suffering from diabetes-associated vascular and metabolic complications (2). Similarly, the prevalence of diagnosed and undiagnosed diabetes, generally associated with obesity, is increasing in the Middle-East region. Almost one in 20 people suffer from diabetes in the United Arab Emirates and this number is among the highest worldwide (3).

Apart from hyperglycemia, dyslipidemia and glycosylated hemoglobin (Hb1ac), there are a number of biomarkers being used to elucidate the chronic complications of diabetes. The main pathophysiological complications of diabetes are considered to be associated with hyperglycemia, hyperlipidemia, and insulin resistance and may include cardiomyopathy, retinopathy, nephropathy, urinary tract infections and other metabolic complications (4).

The diagnosis and classification of diabetes has evolved over time and has been revised due to challenges in the etiological, diagnostic and pathophysiological complications (5). The classification system of type 1, type 2 are mainly based on
etiological factors still presents challenges due to the presence of additional causative factors, therefore a number of subgroups under the different types of diabetes have also been suggested (5-7).

American Diabetes Association has come up with a new classification system for diabetes based on a β-cell-centric classification scheme that excludes inherent and unnecessary confusion (8). Although still popular as type 1 (previously, insulin-dependent diabetes mellitus) or type 2 (insulin-independent diabetes mellitus) there is an urgent need to review the current classification system towards the consensus on a more useful and unified system.

1.2 Type 1 diabetes

Type 1 diabetes (T1DM) accounts for 5–10% of the diabetic population. It results from a cellular mediated autoimmune destruction of the β-cells of the pancreas that leads to absolute insulin deficiency. The pathogenesis of T1DM is known to include recruitment of CD4+ helper and CD8+ killer T lymphocytes and infiltration of macrophages, which lead to inflammatory cytokine-dependent islet destruction.

(9). Antibodies against insulin secreting cells, including those against insulin, glutamic acid decarboxylase (GAD) and against the whole islet cell involved in the development of T1DM can be detected at birth or in infancy (10). Markers of this immune destruction of the cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to tyrosine phosphatase IA2. These autoantibodies are present in 85-90 % of the patients. The disease seems to rapidly progress in infants and children, whereas in adults, it takes a longer time to progress (11). Children who are overweight (higher
BMI) are more prone to develop the disease (12-14). T1DM is characterized by total insulin absence, or very low insulin levels resulting in hyperglycemia and ketoacidosis (15).

The symptoms generally associated with the disease include frequent urination (polyuria) due to the osmotic effect of excess glucose in the urine, drinking plenty of water (polydipsia), muscle cramps due to electrolyte disturbance, blurred vision and weight loss. Excessive fluid loss leads to dehydration (16).

1.3 Type 2 diabetes

Until a few years ago type 2 diabetes (non-insulin dependent) was considered a less complicated type of disease. This idea has changed since type 2 diabetes mellitus (T2DM) shortened life expectancy of patients; and is also a leading cause of premature death due to its complications which include cardiovascular disease, blindness, amputations, and renal insufficiency. The major causes of T2DMM and the most understood are insufficient β-cells response to insulin resistance and decrease in its peripheral action. Genetic and environmental factors are the major causes of T2DM.

The prevalence of T2DM has been increasing steadily all over the world. As a result of this trend, it has become an epidemic in some countries due to the increased number of people affected. T2DM increases with ageing, population, smoking, and high calorie diet intake, thereby adding to the already existing burden for healthcare providers, especially in under developed countries (1, 3, 17).

Pollution and high levels of environmental toxins are also related to the development of T2DM. Recently, some studies found a weak but positive
association of bisphenol (found in some plastic types) concentration in the urine and T2DM (18).

<table>
<thead>
<tr>
<th>Type</th>
<th>Characteristic</th>
<th>Clinical comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type 1</strong></td>
<td>Autoimmune, previously called juvenile or insulin-dependent diabetes mellitus</td>
<td>Potential association with other autoimmune diseases</td>
</tr>
<tr>
<td><strong>Type 2</strong></td>
<td>Polygenic and affected by environment</td>
<td>Increasing incidence associated with higher life span and western cultural habits</td>
</tr>
<tr>
<td><strong>Gestational</strong></td>
<td>Rapid clinical progress</td>
<td>Can continue after pregnancy</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td>Side effect of medications or pancreas dysfunction (e.g: steroids, and alcoholism)</td>
<td>Causative disease or medication may also influence ocular and lacrimal function</td>
</tr>
<tr>
<td><strong>Genetic</strong></td>
<td>Genetic defects in insulin secretion or action</td>
<td>Potential ocular associated malformations</td>
</tr>
</tbody>
</table>

Table 1: Classification and observations on types of diabetes mellitus (19)
According to the International Diabetes Federation (IDF) statistics, it was estimated that 366 million people were diabetic in 2011; this number increased to 387 million in 2014. DM led to almost 4.6 million deaths in 2011 (20). Estimates by the IDF indicate that the number of diabetics is expected to rise and reach up 592 million by 2035 (1). The prevalence of diabetes is increasing in the general population every year. This increase is attributed mainly to sedentary life style, increased intake of high calorie diet, central obesity (large abdomen), and a high body mass index (BMI: weight (kg)/height (m)^2). Almost 80% of people with DM live in low- and middle-income countries (21). The incidence of T2DM varies noticeably from one geographical region to another, as a result of environmental and lifestyle risk factors. Though prevalence of T2DM in Africa has been low, studies have shown a drastic increase in prevalence in both rural and urban areas, and affecting both gender similarly (20).

In the last few years, there has been a drastic rise in T2DM among all age groups. The UAE has been a member of the IDF since 2000; it comes under the Middle East and North Africa (MENA) region. The MENA region has the second highest prevalence (9.7%) of diabetes in the regions (figure 1). The UAE has the one of the largest number of diabetics in the world; around 19% of the UAE population is diabetic (22). There were 803,900 cases of diabetes in UAE in 2014. These numbers are expected to increase each year (20).

These figures indicate that the region has high risk factors for diabetes, mainly due to the rising levels of obesity and physical inactivity.

Sedentary lifestyle and increased consumption of unhealthy diet are the main contributors to the increased levels of obesity in the UAE. High-risk people need to
be alert about their physical activities and diet. This can decrease the chances of developing more complications. Better understanding of the early symptoms of diabetes help people to be prepared to manage the disease and its complications (20).

Figure 1: International Diabetes Federation; world regional diabetes classification. (21)

1.4 Metabolic changes in Type 1 diabetes

T1DM is considered a more simple type of diabetes when compared to T2DM. Despite high blood glucose levels, the cells seem to lack glucose as its uptake is insufficient due to insulin deficiency. As a result, the liver keeps synthesizing glucose (gluconeogenesis) and ketone bodies (ketogenesis). High glucagon levels have adverse action in DM. The increased glucagon/insulin ratio lead to decreased levels of fructose 2,6 bisphosphate that is vital for activating glycolysis and inhibiting gluconeogenesis. Failure of glycolysis and excessive
gluconeogenesis exacerbate hyperglycemia. Glucose is secreted in the urine accompanied by water; this explains the thirst and excessive urination in acute diabetes (23).

Insulin deficiency affects other hormonal secretions such as decreased leptin from the adipose tissue and increased glucagon secretion. Recent studies have shown that leptin deficiency in diabetic rats lead to increased pituitary gland production of adenocorticotropic hormone (ACTH). This hormone stimulates the production of corticosterone from the adrenal gland, leading to increased lipolysis and production of free fatty acids (FFA) and glycerol from triglycerides. The FFA and glycerol produced reach the liver via the blood and get converted to glucose and ketone bodies. This leads to hyperglycemia and ketoacidosis (15). B oxidation produces acetyl CoA which in this case is unable to enter the TCA cycle due to insufficient oxaloacetate (from pyruvate as an end-product of glycolysis). The energy supply is changed to fat instead of carbohydrates. The high concentration of ketone bodies affects the kidneys’ ability to maintain the acid-base balance.

Uncontrolled diabetes can lead to coma due to low blood pH levels and dehydration. Ketosis is not seen in T2DM, as the insulin is sufficiently active to prevent lipolysis in the liver and adipose tissue (23).

1.5 Metabolic changes in Type 2 diabetes

T2DM is the most common endocrine metabolic disorder and is characterized by insulin resistance and pancreatic islet β-cell failure. Obesity, inflammation, oxidative stress, mitochondrial dysfunction have been implicated as etiological and pathophysiological risk factors for T2DM. Cardiomyopathy and other cardiovascular complications associated with increased inflammatory and oxidative stress responses
are the major causes of accelerated atherosclerosis in obesity and diabetes. Insulin resistance is an early feature in T2DM development (24-27).

Obesity is considered as one of the main reasons for developing insulin resistance in T2DM. Inappropriate diet-related obesity and visceral adiposity are the main risk factors for the development of T2DM. Many studies have shown that nutrient-induced β-cell dysfunction could be related to indirect initiation of low grade inflammation (28). Hypertriacylglyceridemia and hypercholesterolemia due to increased circulating lipoproteins, may lead to high levels of triglycerides in blood and tissues that exceed the storage capability of adipocytes, leading to fat deposition in the organs. The liver and muscles are the main sites for fat deposition. Fat accumulation leads to the development of insulin resistance. The rate of fatty acid β-oxidation increases in response to high fat concentration. The mitochondria reach a limit where they are unable to process the extra fatty acids by β-oxidation. Consequently, fat is deposited in the cytoplasm and is accompanied by increased levels of diacylglycerol and ceramide. Diacylglycerol activates protein kinase C which regulates insulin receptor substrate (IRS) mediated insulin signaling leading to reduced insulin sensitivity. Ceramide inhibits glucose uptake and glycogen synthesis (23).

In T2DM, mitochondrial dysfunction in the tissues, particularly in the pancreas, due to proinflammatory responses and disturbance in redox homeostasis signaling have also been reported which may lead to increased oxidative stress (26). Studies have also suggested that prolonged exposure to insulin, as seen in resistant T2DM suppresses mitochondrial biogenesis and function and this may lead to impairment of insulin sensitivity (29-30).
In chronic uncontrolled T2DM, insulin resistance induces stress on the β-cells in the pancreas to produce more proinsulin. Proinsulin is stored in the ER, and excessive amounts can cause ER stress. The ER stress lead to a signal induction that eventually protects the cell from damage. It removes the extra proteins, and inhibits general protein synthesis. If this fails to relieve the ER stress, apoptosis is activated leading to β cell death (23).

1.6 Insulin structure

Insulin was discovered in 1921 by Banting and Best (31). It is a small protein with a molecular weight of 6000 Daltons. It is made from two chains bound together by disulfide bonds. Insulin's amino acids are highly conserved in vertebrates; structurally same insulin is active in different mammals. Most insulin used to treat diabetics is extracted from pig’s pancreas (32).

1.6.1 Biosynthesis of insulin

Insulin is synthesized in large quantities in the β-cells of the pancreas. Insulin mRNA is first translated as preproinsulin which is a single chain insulin precursor, which is then converted to proinsulin when the peptide group is removed after being moved to the ER (Figure 2).

Proinsulin is made of three parts: an amino terminal A that consists of 21 amino acids, a carboxy terminal B chain which consists of 30 amino acids, and a C-peptide in the middle. In the ER, many specific endopeptidases cut off the C peptide to form the mature form of insulin leaving the A chain and B chain connected together with a disulphide bond between A7-B7 and A20-B19. Mature insulin and free C peptide are packaged in the Golgi and stored in secretory granules in the cytoplasm (33).
Insulin production is stimulated by high blood glucose level, some neural stimuli, and high levels of fatty amino acids. Glucose metabolism is needed for insulin release to be stimulated, this activates a sequence of events including generation of ATP, closure of K+ channels in the β-cell membrane and increased Ca$^{2+}$ entry into the cell. Subsequently, the β-cells excrete insulin by exocytosis and it diffuses into the blood (34). C peptides are also secreted, but their functions are not known. Insulin released into the blood circulation is recognized by insulin receptors in target cells.

1.7 Insulin receptor and cell signaling

Insulin receptors (IR) are heterotetrameric glycoproteins on the cell surface. They consist of two α extracellular subunits that attach to insulin, and two β subunits that have tyrosine kinase specific protein activity, with a disulphide bond to link them. When an insulin molecule attaches to the receptor extracellularly, the tyrosine kinase is activated leading to the receptor's autophosphorylation and other intracellular proteins as well (insulin substrates -1, -2, -3, and -4). Multiple downstream signaling pathways are activated after the phosphorylation (Figure 3).
The insulin receptor has different proximal substrates. These include IRS-1, IRS-2, IRS-3, IRS-4. IRS-1 and IRS-2 are the most expressed substrates, whereas IRS-4 is only expressed in the brain, kidney and thymus. IRS-3 is expressed in the adipose tissue of rodents (35).

The insulin signaling pathway starts when an insulin molecule binds to the insulin receptor (IR) activating tyrosine kinase activity (Figure 3). The activated IR phosphorylates and recruits different substrates such as IRS-1 and IRS-2. The tyrosine phosphorylated IRS then provides binding sites for many signaling molecules. Among them, PI3K has a vital role in insulin function, mainly by the activation of the Akt/PKB and the PKCζ cascades. Activated Akt then activates glycogen synthesis through inhibition of GSK-3; protein synthesis through mTOR and downstream elements; and cell survival through inhibition of many pro-apoptotic agents, such as (Bad, FoxO transcription factors, GSK-3, and MST1). Akt phosphorylates and inhibits FoxO transcription factors that control metabolism and autophagy. AMPK also directly regulates FoxO3 and activates transcriptional activity. Insulin signaling plays a vital role in growth and mitogenic effects that are mediated by the Akt cascade as well as by activation of the Ras/MAPK pathway. Insulin stimulates glucose uptake in muscles and adipocytes through the translocation of GLUT4 vesicles to the plasma membrane. Furthermore, insulin signaling prevents gluconeogenesis in the liver, by interfering with CREB/CBP/mTORC2 binding. Insulin signaling activates fatty acid and cholesterol synthesis by regulating SREBP transcription factors (36).
Hyperinsulinemia is a major characteristic of the metabolic syndrome. It is the over production of insulin from pancreatic β-cells, and is considered a primary contributor to T2DM development and cardiovascular dysfunction (37).

Understanding the mechanisms of insulin action and resistance is a vital step in the management of metabolic syndrome as well as development of therapeutic interventions to prevent or treat T2DM.

1.8 Insulin and glucagon hormonal responses in diabetes

Insulin and glucagon are considered the most important two hormones in diabetes. Insulin is released in case of elevated blood glucose levels to stimulate glucose uptake by the liver and muscle to lower circulating glucose level. Glucagon is secreted in case of decreased blood glucose levels, it increases glucose production from the liver to maintain normal blood glucose levels. Most studies focus on insulin, whereas glucagon has been interpreted as a consequence, rather than a cause of diabetes. But several studies have concluded that there is an association between elevated glucagon secretion and T2DM.
Weiping Han and co-workers at the A*STAR Singapore Bioimaging Consortium concluded from a study done in 2010 that dysregulated glucagon secretion, or lack of glucagon secretion inhibition with high blood glucose levels, can be a reason to develop T2D. The study design included a high-fat diet given to normal mice and to genetically modified ‘knockout’ mice with impaired glucose tolerance, low insulin secretion and very less glucagon secretion. High-fat diet is known to reduce insulin sensitivity in mice. It was predicted that the knockout mice (known to have impaired insulin secretion) will develop T2D sooner than the control mice. Surprisingly, the control mice developed high glucose levels, whereas the knockout mice did not. This finding is evidence that reduced insulin secretion and sensitivity may not be the only factors to the development of T2D, but also dysregulated glucagon secretion. That is why diabetes should be considered as a bi-hormonal disease and more studies should focus on glucagon regulation (38-39).

1.9 Insulin resistance in diabetes

One of the main characteristics of T2D is insulin resistance, when cells are unresponsive to insulin signaling. In the late stages of insulin resistance, the pancreatic β cells fail to produce sufficient amounts of insulin. Which eventually affects insulin signaling. Skeletal muscles play an important role in insulin resistance, being the major organ for glucose storage obtained from food (40). Alterations in lipid metabolism and mitochondrial dysfunction in skeletal muscle have been connected to the etiology of T2D but the underlying relationships with insulin resistance development remain to be clarified (41-42). Increased levels of lipid oxidation are seen in the early stages of insulin resistance; however increased plasma levels of non-esterified fatty acids (NEFA) inhibit glucose uptake and
glycogen synthesis in skeletal muscles (43). Additionally, it has been found that there is an increase in intramyocellular lipids (IMCL) (44).

Insulin resistance, also known as insulin resistance syndrome is a major causative factor of metabolic syndrome. There has been an alarming increase in metabolic syndrome. The main reasons for this syndrome are obesity, hyperglycemia, hyperlipidemia, and hypertension. Various animal studies have demonstrated the role of insulin and its signaling cascade in controlling cell growth, metabolism and survival through the activation of mitogen activated protein kinase (MAPKs) and phosphotidylinositide 3 kinase (PI3K). PI3K activation is associated with insulin receptor substrate 1, 2 (IRS1, 2) and eventually Akt and FOXO 1 phosphorylation. This cascade plays a vital role in nutrient homeostasis control and organ survival. Hyperinsulinemia, metabolic inflammation, and over nutrition lead to inactivation of Akt and activation of FOXO 1, by suppressing IRS1, 2 in different organs leading to metabolic syndrome. A good therapeutic intervention for diabetes and its complications could be through targeting IRS, Akt, FOXO 1 signaling cascade (4).

Patients with T1DM suffer from insulin deficiency due to failure of pancreatic β-cells to produce insulin. The therapeutic intervention is insulin supplement to control hyperglycemia. The scenario is different in T2DM, where pancreatic β-cells are producing sufficient insulin, which is not utilized properly. T2DM is non-insulin dependent, although insulin therapy can help reduce the hyperglycemia, but weight gain and cardiovascular risks are exacerbated.
Insulin sensitivity (black line), acute insulin response (AIR, blue line), and blood glucose (red line). Impaired insulin sensitivity arises before evident changes in glucose; the insensitivity is compensated by an increased insulin secretion (AIR) during normoglycemia (blue shading). However, overtime AIR declines leading to impaired fasting glucose (gray shading) and development of T2DM (45).

1.10 Chronic metabolic complications of diabetes

As mentioned above, diabetic complications which arise due to insulin insufficiency (type 1) or insulin resistance (type 2) may develop into metabolic stress associated with energy metabolism, mitochondrial dysfunction and oxidative stress. These alterations may further complicate the pathophysiology of disease due to progressive accumulation of advanced glycation end products (AGE) leading to neuropathic, nephropathic and retinopathic symptoms complications. Some of these metabolic etiological and pathophysiological factors are discussed below.

1.10.1 Oxidative stress in diabetes

Many experiments and clinical trials have shown that oxidative stress has a major role in the development of diabetes mellitus. Excessive reactive oxygen
species (ROS) are produced in diabetes. There are different cellular sources of ROS generation: peroxisomes, plasma membrane proteins, such as NADPH oxidase (NOX), cytosolic enzymatic reactions, and mitochondria. Mitochondria produce around 90% of total cellular ROS (46). Around 1% to 5% of the mitochondrial oxygen consumption is converted into superoxide anions and other ROS (47). Mitochondrial dysfunction leads to increased ROS production, increasing the potential for chromosomal abnormalities (48). Increased ROS production has been implicated in abnormal glucose oxidation, non-enzymatic glycation of proteins, and oxidative degradation of glycated proteins. High levels of ROS production and low levels of cellular anti-oxidant defense may damage cellular organelles and enzymes; it also increases lipid peroxidation leading to insulin resistance. The most important and first ROS generated by either mitochondria or by NOX is superoxide (O$^{2-}$).

Superoxide anion radicals get dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H$_2$O$_2$), which is eventually degraded under normal conditions by catalase or glutathione peroxidase enzyme systems. Excess H$_2$O$_2$ which is not metabolized by these antioxidant enzymes may also be non-enzymatically metabolized by Fe$^{2+}$ or other divalent ions to produce hydroxyl radicals. Superoxide anions react with nitric oxide producing reactive peroxy-nitrite radicals (ONOO-).

Hyperglycemia, as seen in diabetes, increases lipid peroxidation of low density lipoprotein (LDL) using a superoxide dependent pathway producing free radicals. Hyperglycemia increases the levels of glucose that interact with proteins, producing Amadori products followed by advanced glycation end products (AGEs). The AGEs receptors (RAGEs) inactivate some enzymes by altering their structures leading to malfunctioning. RAGEs also promote free radical formation, and inhibit
the anti-proliferative effects of nitric oxide. As AGEs increase oxidative stress, they promote the up-regulation of various NF-kβ target genes. NF-kβ increases nitric oxide production, which is an islet β cell damage mediator (49-50).

Glucose plays a major role in activating the sorbitol (polyol) pathway that leads to diabetes complications, such as eye lens cataract and neuropathy. Increased sorbitol dehydrogenase activity is closely related to altered NAD+ levels that lead to protein modification by non-enzymatic glycosylation of lens protein. However, the mechanisms are still not fully understood (51-52).

1.10.2 Redox homeostasis in diabetes

Coupled oxidation reduction (redox) reactions, are vital to survival. ROS are by-products of metabolism. Natural antioxidants include vitamins (A, C, and E), glutathione, and enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (53). They work in harmony to remove ROS and control its adverse effects in the body. There are countable evidence for increased levels of oxidative stress in T2DM. The expression of antioxidants (SOD1, SOD 2, and catalases) in pancreatic β-cells is low compared to other tissues. Chronic diabetes decreases the γ-glutamylcysteine ligase, the rate limiting enzyme for glutathione synthesis. T2DM is associated with elevated markers of chronic oxidative stress, as β cells maintain a low level of antioxidant gene expression with a high level of glucose to inhibit the rate limiting enzyme for glutathione (54). Prolonged hyperglycemia and hyperlipidemia are considered the main causes of increased oxidative stress, mitochondrial dysfunction, and fibrosis with diabetes complications.
1.10.3 Mitochondrial dysfunction

Cellular energy is produced by the mitochondria through inner membrane bound respiratory chain electron transport enzymes and oxidative phosphorylation (OXPHOS) of ADP. It also produces (ROS). The mitochondrion is the main organelle controlling programmed cell death which is caused by energetic deficiency and ROS damage associated oxidative stress. Most of the cellular ROS (O$_2^-$, H$_2$O$_2$ and OH$^-$) are toxic byproducts of mitochondrial OXPHOS. Increased ROS production due to abnormal oxygen metabolism in diabetes may result in reduced bioenergetics and mitochondrial dysfunction which ultimately may lead to insulin resistance and other complications of cell survival and death (apoptosis/mitophagy). The mitochondrial inner membrane respiratory complex enzymes include five multipolypeptide enzyme complexes. Complexes I, II, III and IV make up the electron transport chain (ETC) and Complex V is adenosine triphosphbate (ATP) synthase.

Metabolism of carbohydrates and fats generate NADH and FADH2 which donate electrons to the ETC. Complex I oxidizes the NADH$^+$ (NADH: ubiquinone oxidoreductase, or NADH dehydrogenase). Electrons are transported to ubiquinine (CoQ$_{10}$) that gets reduced to ubisemiquinone (CoQ$_{10}$H). Electrons are then transported to Complex II from succinate (succinate oxidoreductase, succinate dehydrogenase (SDH)) that reduces CoQ$_{10}$ to CoQ$_{10}$H. The electrons are then ferried to Complex III (ubiquinol: ferrocytochrome c oxidoreductase) to move them to cytochrome c. Electrons are then passed on to complex IV (ferrocytochrome c: oxygen oxidoreductase), resulting in the formation of H$_2$O (Figure 5). The energy produced is used for proton pumping from the mitochondrial matrix through the
mitochondrial inner membrane, to the intermembrane space via complexes I, III, and IV.

Abnormal mitochondrial respiratory complexes structure and/or function may lead to the leakage of electrons from ETC. Single electron reduction of oxygen in the mitochondria may produce ROS. Increased ROS production may further increase oxidative modification and inhibition of respiratory complexes which might trigger the production of more ROS, i.e., ROS-induced ROS production. Some of the consequences of dysfunctional respiration have been studied. A point mutation in the mitochondrial DNA (mtDNA) gene MTATP6 reduces mitochondrial respiratory activity and helps cancer promotion by inhibiting apoptosis (55) (56). Growing evidence suggests that mitochondrial dysfunction due to oxidative damage is a major cause of aging, degenerative diseases like cancer, and metabolic syndrome, such as obesity and T2DM (42, 56).

Insulin resistance in skeletal muscle plays a vital role in the pathogenesis of metabolic syndrome and T2DM. Recent studies reported that insulin resistance is associated with impaired skeletal muscle oxidation capacity and reduced mitochondrial number and function (57). Protecting the mitochondria from oxidative damage in order to improve mitochondrial function in skeletal muscle is a strategy to prevent and treat diseases associated with mitochondrial dysfunction (58). Rosiglitazone improves the suppression of adipose mitochondrial biogenesis in db/db and high fat diet-fed mice (59). Pioglitazone works by reducing hyperglycemia, hyperlipidemia, and hyperinsulinemia in male fatty rats (60), it also improves mitochondrial function and stimulates mitochondrial biogenesis in human adipocyte/tissue in vitro (61) or human neuron-like cells (62). Metformin has a role
in delaying the expression of diabetes and vascular dysfunction; it also decreases mitochondrial oxidative stress in Goto-Kakizaki (GK) rats (63). However, trials have shown that any pharmacological therapy for T2DM, such as thiazolidinedione, insulin, metformin, and other oral hypoglycemic agents or combination therapy with or without insulin is associated with an increased risk of heart failure and body weight gain. Therefore, effective treatments without obvious side effects are needed for preventing and treating diabetes and other metabolic syndromes (64).

1.11 Mitochondrial structural and functional integrity in diabetes

T2DM is 2-3 times more probable in individuals with diabetic mothers, and risk doubles if the father is diabetic. DM is significantly linked with mitochondrial system, mainly mtDNA mutations. Researchers found a 10.4 kb deletion in mtDNA light strand origin of replication (OL) in maternally inherited IDDM. They also found the A3243G mutation that is associated with mitochondrial encephalopathy in T2DM. A3243G is transmitted through a carrier mother to all her children and by 80 years of age, almost all of them will develop DM (66).

Figure 5: Mitochondrial respiratory chain (65)
Changes in mtDNA in T2DM are both qualitative and quantitative, a 35\% decrease of mtDNA in peripheral leukocytes in T2DM patients compared to the control group. Reduction was also found in presymptomatic patients who developed the disease two years later. A 50\% decrease in skeletal muscle mtDNA was found in T2DM patients but four genes encoded by mtDNA (COI, COIII, ND4 and 12s rRNA) had 1.5-2.0 fold increased expression. Similar decreases have been found in GK T2DM rats. Such decrease leads to impaired insulin secretion (66-67).

Glucose stimulated insulin secretion (GSIS) is strongly linked to mitochondrial functional integrity. Insulin release is inhibited by (ETC) function inhibitors like: rotenone, antimycin A, sodium azide, and cyanide. In pancreatic β cell islets, the high capacity, low affinity receptors GluT1, and GluT2 helps the glucose equilibrium across the plasma membrane (67).

Sufficient extra and intracellular glucose equilibrium is maintained by GLUT2 high capacity that is 100 times double the maximal rate of glycolysis. Cytosolic kinase (an enzyme that controls the rate of flux through glycolysis and pyruvate generation) phosphorylates glucose to glucose 6-phosphate. Pyruvate generated from glycolysis enters the mitochondrial TCA cycle. A high level of glucose derived carbon enters the β cells and gets converted to mitochondrial CO₂. Mitochondrial pyruvate is metabolized by pyruvate dehydrogenase, generating acetyl-CoA or pyruvate carboxylase to form oxaloacetate to ensure anaplerosis to the TCA cycle. Anaplerosis can be crucial in pyruvate/malate shuttle activation causing an increase in cytosolic NADPH. TCA cycle generates reducing equivalents in the form of NADH and FADH2 that are transformed to mitochondrial ETC with Adenine Triphosphate (ATP) production. Systolic ATP/ADP ratio increase as the
ATP enters through adenine nucleotide translocase (ANT), this results in depolarization of the plasma membrane as the ATP sensitive K⁺ channels close. β-cell UCP-2 up regulation leads to increased ROS production, decreased ATP, ATP-K⁺ sensitive channels closure and eventually impaired insulin secretion. In mice models the knockout of UCP-2 activity leads to improved β cell ATP production and during glucose stimulation, insulin gets secreted. Plasma depolarization leads to Ca²⁺ sensitive channels opening and a sharp increase in systolic Ca²⁺, the main event for insulin secretion by exocytosis of secretory granules (56).

Animal models demonstrated evidence to the intact mitochondrial ETC function for insulin release. Mitochondrial DNA is needed for glucose-induced stimulus secretion coupling in pancreatic β cells (MIN6) of mice. Insulin is affected by the glucose stimulated Ca²⁺ inhibition which is a result of mitochondrial MIN6 depletion (56).

1.12 Advanced glycation end-product (AGE) and diabetes

Hyperglycemia, whether extracellular or intracellular have damaging effects on different organs. Extracellular hyperglycemia leads to the formation of AGEs while intracellular hyperglycemia increases mitochondrial activity, protein kinase C (PKC), and NADPH oxidase, promoting oxidative stress complications and inhibition of insulin signaling pathway.

Many researchers still consider hyperglycemia as the main factor for developing diabetic complications. Hyperglycemia contributes to the formation of AGEs. AGEs are formed early in embryonic life, but at a slower rate when compared to diabetics. This increase is due to high levels of glucose. AGEs are a heterogenous group of molecules, made from the non-enzymatic reaction between
reducing sugars and free amino groups originating from proteins, lipids, and nucleic acids. The first product of the previous interaction is the Schiff base, which rearranges itself to make the amadori products (e.g. haemoglobin A1c ). AGEs covalently bind to proteins, leading to altered protein structures and functions. AGEs also interact with their cell surface binding receptors (RAGEs). AGEs are endocytosed and either degraded or lead to the activation of pro-oxidant and inflammatory responses (68).

AGEs play a role in developing micro and macro angiopathies. High levels of AGEs are present in diabetics’ retinal vessels. The higher the level of AGEs, the more severe the retinopathy. Aminoguanidine is used to inhibit AGEs formation in diabetic patients, and help to reduce its complications.

AGEs are found in large quantities in the peripheral nerves of diabetics. The use of AGE inhibitors showed improved nerve conduction velocity and neuronal blood flow.

AGEs are also found in high concentrations in diabetics’ nephrons, which lead to glomerulosclerosis and interstitial fibrosis due to thickened glomerular basement membrane and mesangial expansion.

Animal and human studies have shown that AGEs contribute in the development of cardiovascular diseases. They alter normal blood vessel and smooth muscle structure making them atherogenic. AGEs also change the LDL cholesterol structure and make it prone to oxidation, leading to its deposition in the blood vessels wall (68).
1.13 Glucokinase mutations in diabetes

Glucokinase, a member of the hexokinase enzymes, is located on chromosome 7p 15.3-15. It consists of 12 exons that span 45,168 bp and encode for a 465-amino acid protein, its molecular weight is 52,191 Da. It is expressed in the pancreas, liver, brain, and endocrine cells. Tissue specific promoters helps in differential regulation and transcription of different transcripts resulting in three exon 1 (a,b,c) that differ in size. The upstream promoter is active in the pancreas and brain, whereas the downstream promoter is active only in the liver. It works as a monomer; it helps to phosphorylate glucose on carbon 6 with Mg-ATP to form glucose-6-phosphate (G6P). It is a glucose sensor in pancreatic β-cells. Glucokinase is a key regulatory enzyme for insulin secretion. There are many mutations in glucokinase gene (GCK) (620 mutations found) and most are in the protein area called allosteric activator site. Glucokinase mutations cause reduced insulin secretion, and lower glucose uptake by the liver leading to type 2 diabetes mellitus (69-70).

1.14 Diseases associated with diabetes complications

Long term diabetes increases the chances of developing many complications in different organs. These complications contribute to increased diabetes morbidity and mortality rate. The development of different complications is related to diabetes duration and glycemic control. The organs that develop diabetic complications show insulin independent glucose uptake. Aldose reductase induces nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) to activate the pentose phosphate pathway, which leads to a protein kinase C–induced increase in glomerular prostaglandin production and loss of mesangial cell contractility, which can be a
cause of hyper-filtration and glomerular dysfunction in diabetes (71-72). Impaired insulin and growth factor signaling, hyperlipidemia and hypertension are major factors for the development of diabetic complications. The most common diabetic complications are macrovascular disease, nephropathy, retinopathy and neuropathy diseases. (71) (Figure 6).

Figure 6: Major complications of diabetes mellitus (73)

1.14.1 Diabetic Retinopathy

Diabetic retinopathy (DR) is a microvascular complication which might harm the peripheral retina, the macula, or both. It is considered a major cause of vision disability and blindness in diabetics. The severity of DR ranges from non-proliferative and pre-proliferative to the more serious proliferative DR, where abnormal new vessels are formed. Neglecting vision problems in diabetics can
eventually lead to partial or complete blindness through hemorrhage or retinal detachment (74). Prolonged diabetics are more susceptible to DR. A recent study in patients with both T1DM and T2DM, showed that after 30 years of diabetes, most of the patients developed some degree of DR, and more than half developed proliferative DR. T1DM and people taking insulin had the highest prevalence of DR (75). Another study concluded that retinopathy can occur as a late stage complication in T1DM, while in T2DM 20% of patients are diagnosed with retinopathy at the time of diabetes diagnosis (76).

Loss of pericytes is an early marker for DR. Pericytes are elongated contractile cells that surround endothelial cells of small vessels, they play a role in maintenance of capillary tone (ie, dilatation and constriction), and protection against ROS damage. Loss of pericytes in DR would affect normal capillary constriction as well as new capillary generation (77).

**1.14.2 Diabetic neuropathy**

Diabetic neuropathy is due to damage in peripheral nerves. There are two types of peripheral neuropathies: focal mononeuropathies and sensorimotor polyneuropathies. One of the most common examples is feet nerve damage, due to poor blood flow that increases the chances of various foot complications. This can cause tingling, numbness, burning or pain which begins at the tips of the toes or fingers and slowly spreads upward. Neglecting the signs and untreated cuts and blisters can lead to the development of serious infections; they heal slowly and may ultimately require toe, foot or leg amputation (78).

Researchers found that diabetic mice fed high cholesterol diet, developed severe hypertriglyceridemia and advanced atherosclerosis. The previous finding
suggests that lesion progression is exacerbated in obese diabetics who mostly have T1DM (78).

1.14.3 Diabetic nephropathy

Diabetic nephropathy (DN) is caused by damage to small blood vessels in the kidneys leading to decreased kidney efficiency or failure. Diabetics are more prone to develop kidney disease. Retaining near normal levels of blood glucose and blood pressure can significantly reduce the risk of kidney disease (79).

The first sign of DN is usually microalbuminuria, which progresses to overt and more severe renal dysfunction and ultimately to renal failure and is the leading cause of end-stage renal disease (ESRD) (80).

Around 25% of people with T2DM have microalbuminuria or a more advanced stage of DN. Patients with DN also show thickening of glomerular basement membranes and glomerular hyperfiltration, which leads to mesangial (centre of the renal glomerulus) extracellular matrix extension and promotes increased urinary albumin excretion and development of glomerular and tubular sclerosis and renal failure. The risk factors for DN include hyperglycemia, duration of diabetes, age of onset, tobacco use, dyslipidemia, hypertension, and obesity (75).

1.14.4 Macrovascular complications

DM is considered as a major risk factor for developing cardiovascular events. The Framingham Heart Study described diabetes as one of the causative factors associated with probable ratios for coronary heart disease of 1.5 and 1.8 for men and women, and risks for stroke of 1.4 and 1.7 for men and women, respectively (81).
Diabetics have a 2- to 4-fold increased possibility of developing cardiovascular events compared to non-diabetics (82).

Heart disease in DM develops as a result of negative modulation at different levels leading to vascular (angiopathy), myocardial (diabetic cardiomyopathy), and intracardial nervous (autonomic neuropathy) dysfunction. Coronary artery disease (CAD), which eventually leads to occlusion of arteries that supply the heart, has been reported to be directly responsible for much of the condition of CVD in patients with DM. Atherosclerotic CAD is more frequent and more severe in diabetics when compared to non-diabetics (83).

Both types of diabetes mellitus (type 1 and 2) have irreversible effects on small and large blood vessels. Oxidative stress and inflammation remarkably alter gene expression in the vasculature. Imbalance between pro-inflammatory and thrombogenic systems in diabetics are key contributors in developing the pathology of the disease. Eventually, the above mentioned few points lead to failure of vascular repair. Macrovascular complications leading to heart attacks and strokes are considered the main cause of death among diabetics (84).

CVD is very common in diabetes and particularly in T2DM, particularly in uncontrolled diabetes. These patients are 2-6 times more prone to develop CVD compared to people with other types of diabetes. CVD is responsible for half of the diabetes deaths. It includes angina, myocardial infarction and congestive heart failure (85).

1.15 Pharmacology and therapeutics in diabetes

DM progresses to tissue or vascular damage, leading to severe complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration (86). Thus, DM is involved in a wide range of heterogeneous diseases.
Medications are basically used to save lives and relieve symptoms. Secondary aims of treatment are to prevent long-term diabetic complications and, by eliminating various risk factors, to increase durability. T1DM treatment is mainly by insulin replacement, while diet control and active lifestyle are considered the basis for treatment and management. Insulin supplements are also vital in T2DM when blood glucose levels cannot be controlled by diet, weight loss, exercise and oral medications. Oral hypoglycemic agents like sulphonylureas, biguanides, alpha glucosidase inhibitors, meglitinide analogues, and thiazolidinediones help in controlling T2DM. The main role of hypoglycemic drugs is to control the underlying metabolic disorder, such as insulin resistance and inadequate insulin secretion. They are prescribed in combination with a low calorie diet and lifestyle changes. Diet and lifestyle help to reduce weight, improve glycemic control and reduce the risk of developing cardiovascular complications, which cause 70% to 80% of deaths among diabetics (87).

Oral administration of stevioside improves insulin sensitivity, and seems suitable as an adjuvant for diabetic patients and/or those that ingest large amounts of fructose (88).

Pharmacological treatments that increase brain and hypothalamic insulin sensitivity may provide new strategies for the prevention of dementia disorders, obesity, and T2DM (89).

The increase in the intracellular deposition of triglycerides (TG) in muscles, liver and pancreas in subjects prone to diabetes is well documented and demonstrated to attenuate glucose metabolism by interfering with insulin signaling and insulin secretion. The obesity often associated with T2DM is mainly central, resulting in the overload of abdominal adipocytes with TG and reducing fat depot capacity to protect
other tissues from utilizing a large proportion of dietary fat. To reduce the excessive fat outflow from the abdominal depots and prevent the ectopic fat deposition it is important to decrease the volume of central fat stores or increase the peripheral fat stores. This can be achieved by gastrointestinal bypass or gastroplasty, which decreases dietary fat absorption, or by direct means that include surgical removal of mesenteric fat. Indirect treatment consists of drastic lifestyle change comprising both diet and exercise and pharmacotherapy that reduces mesenteric fat mass and activity. The first step should be an attempt to effectively induce a lifestyle change. Next is pharmacotherapy including acarbose, metformin, PPAR gamma, or PPAR gamma alpha agonists, statins and orlistat. (90)

There are various reports suggesting the use of antioxidants in the treatment of diabetes. Some reports provide experimental and clinical evidence supporting antioxidant supplementation as a cardioprotective intervention in the setting of DM (91). Other conflicting reports conclude there is no established benefit for antioxidant use in the management of diabetic complications (92).

Different side effects have been associated with different hypoglycemic drugs. Weight gain and hypoglycaemia with sulfonylureas, gastrointestinal (GI) disturbances with metformin, weight gain, GI disturbances and liver injury with thiazolidinediones, GI malfunctions, weight gain and hypersensitivity reactions with meglitinides and flatulence, diarrhea and abdominal swelling with alpha-glucosidase inhibitors (87).
1.16 Therapeutic use of Non Steroidal anti inflammatory drugs (NSAIDs) and aspirin in diabetes

1.16.1 Type 2 diabetes as inflammatory disease

Insulin resistance is the primary cause of T2DM. Increased inflammatory response is a hallmark of obesity, diabetes and insulin resistance. Use of anti-inflammatory drugs may be an approach to increase insulin sensitivity and energy metabolism. The immune system components are different in patients with T2DM when compared to normal individuals. Changes are mainly found in adipose tissue, liver, pancreatic islets, and circulating leukocytes. Factors determining T2DM are divided into intrinsic and extrinsic factors. Intrinsic factors include mitochondrial dysfunction, oxidative stress, and lipid deposition. Extrinsic factors validity, cytokines, adipokines, serum fatty acids, and hypoxia, interfere with insulin signaling. Surprisingly, these pathways join the common inflammation pathway. It is also found that diabetics show changes in some leukocytes activation, higher rates of apoptosis and fibrosis. Extrinsic factors affect the nuclear factor – κB (NF-κB), kinase β (Ikkβ), and Jun kinase (JNK) inhibitor. As a result, insulin action gets inhibited by serine phosphorylation of insulin receptor substrates (IRS1, and IRS2). Similarly, multiple cytokine signaling suppressors are activated by cytokine proteins (SOCS) and adipokines, which lead to improper tyrosine kinase phosphorylation of IRS1, and IRS2, which finally degrade. Consequently, intracellular insulin signal transduction is inhibited. Pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and IL-12) and chemokines are also found in insulin resistant T2DM (93). The above variations support the idea that inflammation plays a role in the development of T2DM. Trials
using salicylate with diabetics showed lower blood glucose levels and reduced disease associated complications (94).

1.16.2 Utility of aspirin therapy in patients with type 2 diabetes

T2DM is strongly linked with cardiometabolic syndrome (CMS), as increased platelets aggregation and hypercoagulability are the main causes of CMS and to some extent causes T2DM. Platelets play an important role in atherosclerosis as they release a number of inflammatory mediators. Patients with increased levels of fasting blood glucose show high platelet dependent thrombosis. T2DM patients also show increased sensitivity of platelet purinergic receptors to ADP, resulting in higher levels of circulating platelet microaggregates that speed thrombus formation.

For many years aspirin (from NSAID family: non-steroidal anti-inflammatory drugs) was known by its vasodilator, anti-inflammatory, and anti-thrombotic functions, it interrupts the synthesis of prostaglandins. The synthesis happens through arachidonic acid oxidation that is derived from membrane phospholipids. After the oxidation, arachidonic acid is transformed by prostaglandin H synthase that is often called cyclooxygenase (COX). COX helps in the formation of thromboxanes and prostacyclin, they work as vascular mediators, have a vasodynamic function, and platelet activity. COX is found in two isoforms (COX1, and COX2). COX 2 is induced by cytokines and associated with inflammation. Aspirin inhibits both COX by irreversible serine 530 acetylation, which slows down arachidonic acid entrance to the active site. Aspirin and sodium salicylate are both known inhibitors of NF-κB and this may be the mechanism by which they exert their anti-inflammatory effects. A high dose of aspirin is found to reduce glucosuria in diabetes. High levels of free fatty acids lead to serine kinase cascade activation, which lead to higher levels of
TNFα which has a negative effect on insulin signaling. It is also found that TNFα activates serine phosphorylation of IRS 1 that prevents downstream metabolic signaling. One study found that aspirin can inhibit 4 out of 6 serine kinase involved in IRS1 phosphorylation, leading to improved insulin sensitivity by allowing maximum tyrosine phosphorylation and signaling by P13 kinase and protein kinase B pathway (95).

Different studies have shown that aspirin therapy is a secondary prevention approach for cardiovascular events; clinical guidelines recommend using low-dose aspirin (75-162 mg/day) for the primary prevention and secondary prevention of CVD in individuals with risk factors. Diabetics are considered as a high risk group, so they are good candidates for aspirin except for patients with contraindications (96) (97). The American Diabetes Association (ADA) recommends the use of aspirin as a primary prevention approach in patients with diabetes who are at increased cardiovascular risk, including patients with a family history of CVD, hypertension, smoking, dyslipidemia, or albuminuria and those who are above 45 years of age (98).

1.16.3 Aspirin side effects and aspirin resistance

Nevertheless, due to the action of aspirin on cyclooxygenase (COX), it is associated with upper GI complications including ulcers and bleeding. The severity of the side effects is less with low-dose aspirin compared with non-selective, NSAIDs. Co-administration of a gastro-protective agent like the proton pump inhibitors (PPIs) is useful for decreasing the chance of developing upper GI associated with use of low-dose aspirin. Treating existing H. pylori also helps to reduce the risk of these side effects, especially in those at high risk (96).
Aspirin resistance is the failure of aspirin to decrease platelet production of thromboxane A2. High degrees of aspirin resistance can be associated with increasing risk of cardiovascular events. Laboratory tests of platelet thromboxane A2 production or platelet function that is dependent on platelet thromboxane production can be used as a measure of aspirin resistance. Possible causes of aspirin resistance can be insufficient dose, drug interactions, genetic polymorphisms of COX-1 and other genes needed for thromboxane biosynthesis, upregulation of non-platelet sources of thromboxane biosynthesis, and increased platelet turnover. Tackling the reason of aspirin resistance can help in overcoming the problem. Recent research is focused on defining aspirin resistance, developing dependable tests for it, and measuring the risk of associated cardiovascular events (99).
1.17 Aim of the present work

Metabolic complications and atherosclerotic vascular disease is a major cause of premature morbidity and mortality in obesity and chronic T2DM. Increased oxidative stress, inflammatory responses and mitochondrial dysfunction have also been implicated in diabesity. Aspirin and other NSAIDs have been used in many clinical and experimental trials for the prevention of diabetes, cancer, obesity and atherosclerotic cardiovascular complications. We therefore, plan to investigate the effects of aspirin on molecular and metabolic targets altered in diabetes using experimental model of T2DM. The GK rat is a spontaneous non-obese animal experimental model of T2DM. This strain was developed by selective breeding of glucose intolerant Wistar rats over many generations. The GK rats exhibit decreased β-cell numbers and function which is accompanied by mild hyperglycemia, impaired glucose-induced insulin secretion, marked glucose intolerance, peripheral insulin resistance and chronic inflammation (100-102). Despite the mild hyperglycemia several manifestations of diabetes complications have been demonstrated in this model (103-105).

Growing evidence has linked T2DM with low-grade systemic inflammation. Thus, the GK rat represents a good animal model for studying human T2DMM pathophysiology and the effects of therapeutic options such as the use of aspirin or other NSAIDs. It is possible that existing low-grade systemic inflammation can be suppressed by experimental strategies aimed at blocking the production or action of proinflammatory signaling pathways, thereby treating T2DM.

Since the objective of the present study was to elucidate the mechanism of aspirin action in altering metabolic and molecular targets affected in diabetes, we intended to avoid gastric toxicity caused by oral administration of aspirin, and
therefore we have selected to administer a low dose aspirin (100 mg/Kg body weight) intraperitoneally for 5-weeks in young GK T2DM rats.

1.18 Hypothesis

Since inflammation, oxidative stress and mitochondrial dysfunction appears to be hallmarks of diabetes in general and T2DM in particular, we plan to elucidate the molecular and biochemical mechanisms of action of aspirin on the pathophysiology and metabolic complications of T2DM using insulin resistant hyperglycemic GK rats. Aspirin and other NSAIDs have been used in clinical and experimental trials for the prevention of diabetes, obesity and other complications. Our specific aims are to find wither ASA:

1. Improves pancreatic islet β-cell integrity and function.

2. Improves β-cell insulin secretory ability and regulate glucagon synthesis.

3. Improves insulin signaling.

4. Improves glucose uptake and energy metabolism in target tissues.

5. Improves mitochondrial function, oxidative stress and improve antioxidant redox homeostasis.

6. Alters drug metabolism and detoxification in the tissues in diabetic rats.

ASA might be involved in altering the molecular and metabolic targets affected in T2DM.
Chapter 2: Methods

2.1 Chemicals

Aspirin, bovine serum albumin (BSA), picric acid, paraformaldehyde, trisodium citrate, potassium phosphate, cytochrome c, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5'-dithio-bis(2-nitrobenzoic acid), 1-chloro 2,4-dinitrobenzene (CDNB), cumene hydroperoxide, dimethylnitrosamine (DMNA), erythromycin, glutathione reductase, NADH, NADPH, coenzyme Q2, antimycin A, dodecyl maltoside, sodium succinate, lucigenin and ATP Bioluminescent cell assay and Hexokinase colorimetric assay kits were purchased from Sigma-Aldrich Fine Chemicals (St.Louis, MO, USA). 2’, 7’-Dichlorofluorescein diacetate (DCFDA) was procured from Molecular Probes (Eugene, OR, USA). Polyclonal antibodies against insulin and glucagon were purchased from Dako, Denmark, while those against Akt, p-Akt, JNK, p-JNK and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against PPAR-γ was purchased from Abcam (Cambridge, UK) while that against 4-HNE was from Oxis Int. Inc. (Portland, OR, USA). FITC and TRITC conjugated secondary antibodies were purchased from Jackson Immuno-research Laboratories (West Groove, Pa, USA). Kits for insulin were purchased from EMD Millipore Corporation (Billerica, MA, USA), PGE2 from Arbor Assays (Michigan, USA), total nitrate/nitrite from R & D Systems, Inc., (Minneapolis, MN, USA), SOD and GDH from Abcam (Cambridge, UK), LPO kit from Oxis Int. Inc. (Portland, OR, USA). Reagents for SDS-PAGE and Western blot analyses were purchased from Gibco BRL (Grand Island, NY, USA) and Bio Rad Laboratories (Richmond, CA, USA).
2.2 Animals

Ten male GK rats (weighing 100-120 g) were procured from Taconic (Germantown, NY, USA) at five weeks of age. Ten male Wistar rats of similar age and weight were procured from the Animal House Facility of College of Medicine & Health Sciences, United Arab Emirates University (U.A.E) and were used as non-diabetic controls to evaluate the progression of diabetes in GK rats. All animals were maintained under standard laboratory conditions including a 12-hour light/dark cycle with free access to food, water and libitum. Approval for this project was obtained from the Animal Ethics Committee, College of Medicine & Health Sciences (Protocol Ref#A1-13); U.A.E and all the animals were used following the safe practice for animals in research guidelines as stipulated by NIH, USA. Body weights and blood glucose levels (One Touch UltraGlucose, LifeScan Inc, U.S.A) of the animals were regularly measured.

2.3 Animal treatment, tissue homogenisation and glucose tolerance test

After 4 months, the GK rats (avg. body wt. 319g) showed signs of hyperglycemia (fasting blood glucose 80-90 mg/dl) while the control Wistar rats (avg. body wt. 323g) had a fasting blood glucose level of 50-60 mg/dl. The animals were then divided into four subgroups, each containing 5 animals. Two subgroups of GK and Wistar control rats were injected intraperitoneally with 100 mg ASA/kg body weight/ day) for 5 weeks. This experimental dose and time points were selected based on the published reports in previous studies, using diabetic models including GK rats (106-112).
In order to avoid gastric toxicity caused by oral administration of aspirin, we administered aspirin intraperitoneally. Towards the end of the experiment, the animals were subjected to a glucose tolerance test. Briefly, after an overnight fast, animals were injected with glucose (2g glucose/kg body weight) intraperitoneally. Blood samples were collected from the tail vein at time 0 (prior to the glucose load), 60 and 120 minutes after the glucose challenge and glucose levels were measured.

At the end of 5 weeks, animals were sacrificed by decapitation and blood was collected from the jugular vein. Pancreas and the heart from the animals were quickly excised and a portion of these were fixed in Zamboni's solution and processed for immunofluorescence studies. The rest of the tissues were stored at -80 °C until further analysis. Portions of the tissues were homogenized (25 % w/v) in H-medium (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and used for isolating mitochondria, cytosol and microsome by differential centrifugation. Protein concentration in the serum and sub-cellular fractions was measured using Bio-Rad reagent as described before (113-115).

2.4 Serum analysis

Blood samples collected from the jugular vein after decapitation were transferred into a plain tube. Samples were centrifuged at 4000 rpm for 10-15 min and the serum was used to assay key organ function tests, cholesterol and lipoproteins, glucose levels and uric acid using the COBAS® INTEGRA 400 plus auto-analyzer (Roche, Basel, Switzerland). Insulin, PGE2, total NO and SOD assays
were also performed in the serum samples using appropriate kits as suggested in vendor’s protocols and as described before (116-117).

2.5 Insulin assay

Serum insulin level was measured using a non-radioactive quantitative insulin ELISA kit (Cat# EZRMI-13K). Briefly, serum samples were loaded onto microtiter plates that were pre-coated with monoclonal mouse anti-rat insulin antibodies to capture the insulin molecules, followed by addition of biotinylated polyclonal antibodies that bind to the captured insulin. The addition of horseradish peroxidase then binds to the immobilized biotinylated antibodies. The activity of horseradish peroxidase in the presence of 3,3’,5,5´ tetramethylbenzidine was measured at 450 nm. The increase in the absorbency was proportional to the amount of captured insulin in the sample. The concentration of insulin was calculated from the standard curve.

2.6 Detection of insulin/glucagon by immunofluorescence staining

Immunofluorescence (IF) is a technique that employs fluorescent-labeled antibodies to detect specific target antigens. It is commonly used in both scientific research and clinical laboratories. In our research we used indirect immunofluorescence which utilizes a two-step technique (118). Using a primary unlabeled antibody that binds to the protein of interest (insulin, or glucagon), followed by loading a fluorophore-labeled secondary antibody (directed against the Fc region of the primary antibody) to detect the first antibody.

Immunofluorescence staining was performed on pancreatic tissue sections, cut to 5 μm thickness. Sections were dewaxed by immersing in xylene (two changes) 5 minutes each at room temperature, followed by rehydration in descending
concentrations of ethanol (100%, 95%, 70%, and 50%) for 3 minutes each, washing with distilled water, followed by Antigen retrieval using citrate buffer (pH 6). The slides were then incubated for 1 min in the microwave using power 10, followed by 10 minutes incubation at power 1 and allowed to cool down to room temperature for 30 minutes. Slides were washed three times in PBS before incubating with blocking reagent at room temperature for 45 minutes. This was followed by the application of the first primary anti-insulin antibody (polyclonal guinea pig anti insulin diluted at 1:2000), which was incubated overnight at 4 °C followed by PBS wash (three changes, for 5 minutes each). First secondary antibody (anti-guinea pig FITC diluted 1:100) was added and incubated for one hour at room temperature. After a PBS wash (three changes, for 5 minutes each), the second primary antibody was added (pre-diluted polyclonal rabbit anti human glucagon), and incubated overnight at 4 °C, followed by Phosphate Buffered Saline (PBS) wash (three changes, for 5 minutes each). The second secondary antibody (Glucagon: anti-rabbit TRITC diluted 1:100) was added and incubated for one hour at room temperature followed by PBS wash (three changes, for 5 minutes each). Finally slides were washed with PBS (three changes, 5 minutes each) before mounting with CTTI-Fluore mounting media. Slides were examined using a fluorescence microscope. Sections were studied using AxioCam HRc digital camera with AxioVision 3.0 software (Carl Zeiss, Oberkochen, Germany) fixed with z-plane fluorescence. Contrast and brightness of sections were adjusted and images were merged using image J 1.48V

2.7 Prostaglandin E2 assay

Aspirin has been known for years for its vasodilator, anti-inflammatory, and anti-thrombotic functions. Its main function is to inhibit the synthesis of
prostaglandins. PG synthesis happens through arachidonic acid oxidation that is derived from membrane phospholipids. After oxidation, arachidonic acid becomes transformed by the action of prostaglandins H synthase which is better known as cyclooxygenase (COX) (119).

Prostaglandin (PG) E₂ is the most produced PG with different actions. PGE₂ is usually considered as an immunosuppressant as it inhibits T cell activation in vitro. However, in vivo its immunosuppressant action has been unclear. Recently, several researchers have found that PGE₂ facilitates expansion of the Th17 subgroup of T helper cells of both human and mouse through elevation of cAMP via PGE receptors, EP2 and EP4 (120).

PGE₂ was measured in the serum using the DetectX high sensitivity immunoassay kit (Cat # K018-HX1). Briefly, standards or diluted samples were added to a microtiter plate coated with an antibody that captures mouse IgG. A PGE₂ – peroxidase conjugate was added followed by a monoclonal antibody to PGE₂. Finally the substrate was added to react with the bound PGE₂ conjugate and the color generated was read at 450 nm using a microtiter plate reader. PGE₂ concentration was calculated from the standard curve.

2.8 Measurement of nitric oxide production

Nitric oxide (NO) is synthesized endogenously by an enzyme nitric oxide synthase (NOS) from the precursor L-arginine (121). NO is a gaseous free radical with a short half-life of a few seconds or less. Due to its instability, it is difficult to measure. Therefore, the levels of the more stable NO metabolites, nitrite (NO₂⁻) and nitrate (NO₃⁻), have been used to indirectly measure NO. Total NO was measured using the nitric oxide kit (R &D systems, Inc.) (Cat # ab65328). This assay estimates
nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by the colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is a two-step diazotization reaction where acidified $\text{NO}_2^-$ produces a nitrosating agent which reacts with sulfanilic acid producing a diazonium ion. This ion then couples to N-(1- naphthyl) ethylene diamine forming the chromophoric azo-derivative that absorbs light at 540 – 570 nm. The reaction mixture containing the reaction diluents, serum samples from treated and untreated groups, NADH, and diluted nitrate reductase was incubated for 30 mins at room temperature. The reaction was started by adding Griess reagents I and II, and after an additional incubation for 10 minutes at room temperature, the absorbance was read at 546 nm. NO concentration in the samples was calculated from the standard curve.

2.9 Measurement of Superoxide dismutase

ROS, like superoxide radicals, are involved in the pathogenesis of many diseases. Almost 3 to 10 % of the oxygen used by tissues is converted to its reactive intermediates that affect the functions of cells and tissues. Superoxide dismutase (SOD) is an important anti-oxidative (122) enzyme that catalyzes the conversion of single electron reduced species of molecular oxygen to hydrogen peroxide and oxygen ($\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$) (123).

SOD was measured using Abcam’s Superoxide activity assay kit (Colorimetric) (Cat # ab65354). It is a sensitive kit that uses a substrate, WST1 which produces a water soluble formazan dye on reduction with superoxide anion. Reduction rate is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. Thus, inhibition activity of SOD was detected using this method.
Briefly, serum samples and tissue homogenates were treated with WST in the presence of the enzyme working solution in the reaction buffer for 20 mins at 37 °C. The absorbance was read at 450 nm using a microplate reader and the percentage inhibition rate calculated.

2.10 Assay for Reactive oxygen species production

Oxidative stress has a major role in the development of DM. Oxidative stress in the tissues was evaluated by measuring superoxide production. Two different methods were used: the lucigenin-enhanced chemiluminescence method for NOX detection and the DCFH-DA method for ROS detection.

NAD(P)H-dependent lucigenin-enhanced chemiluminescence for detection of NOX was carried out using the method of Li Y et. al. (124). Briefly, around 50 - 100 µg of tissue homogenates were used with 0.1 M KPi buffer (pH 7.4), and 1mM lucigenin and 10 mM NADPH was added. Chemiluminescence was instantly measured using the Turner Designs TD-20/20 luminometer for an integration time of 60 s. Controls were used and blank reading was subtracted from the final values expressed.
ROS generation was also measured by the dichlorofluorescein method using a cell permeable probe DCFH-DA (2’, 7’-dichloro fluorescein diacetate) that measures peroxides. DCFH-DA is a stable compound that diffuses into cells and is hydrolyzed by intracellular esterases to produce DCFH which remains within the cells. DCFH is rapidly oxidized by peroxy radicals to the highly fluorescent compound, 2’, 7’-dichlorofluorescein (DCF). Briefly, mitochondrial samples were incubated with 5 µM of stable non-fluorescent DCFH-DA for 30 min at 37 °C in 0.1 M Kpi buffer, pH 7.4. The reaction was stopped using ice-cold 0.1 M Kpi buffer, containing 0.1 % Triton X-100 and was read in a spectrofluorimeter at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.11 Lipid peroxidation assay

Lipid peroxidation (LPO) is the oxidative degradation of lipids. Free radicals pull an electron from the lipids cell membrane, leading to cell damage. The LPO assay was carried out using the LPO-586™ assay kit.

The LPO-586™ assay is based on the reaction between a chromogenic reagent, N-methyl-2-phenylindole, and MDA and 4-hydroxyalkenals at 45 °C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of the reagent to produce a stable chromophore with maximal absorbance at 586 nm. Briefly, the standards, samples and reagent were mixed followed by the addition of 37 % HCl, incubated at 45 °C for 60mins, centrifuged and the clear supernatant read at 586 nm.

2.12 Measurement of total Glutathione sulfhydryl

Glutathione (GSH, γ-glutamylcysteinylglycine), the primary non-protein sulfhydryl in the cells of most aerobic organisms is the body's major antioxidant (125). It is a tripeptide, that is formed from three amino acids; glutamic acid,
cysteine, and glycine (126). It plays a major role in cellular metabolism. For example, in the detoxification of certain arenes, or as a cofactor in the biosynthesis of leukotriene and prostaglandin, it is a major antioxidant, that protects cells from free radicals. Moreover, it is an essential electron donor to glutathione peroxidase in the reduction of hydroperoxides (126). Glutathione is found in the reduced sulfhydryl (GSH) and oxidized disulfide (GSSG) states. In healthy cells and tissues, it is retained in the reduced state (GSH) by the action of glutathione reductase (127).

GSH levels were measured as protein-free sulfhydryl content using Ellman’s reagent (128) with few modifications from the method of Tietze (127). The assay depends on the enzymatic recycling method, via using glutathione reductase for GSH quantification. The general thiol reagent, 5-5’-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with the sulfhydryl group of the GSH and produces a yellow colored 5-thionitrobenzoic acid (TNB) and GS-TNB (between GSH and TNB). This is then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (β-NADPH), to produce more TNB, thus recycling the GSH. The TNB production rate is directly related to this recycling reaction, which is directly proportional to the concentration of GSH in the sample. TNB absorbance measurement at 412 nm gives an accurate estimation of GSH in the sample. The glutathione reductase reduces all oxidized GSH (GSSG) present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB to GSH. Therefore the assay measures the total glutathione in the sample. Briefly, the reaction mixture (total volume of 1 ml) contained 143 mM NaPi/6.3mM EDTA, pH 7.5, DTNB, 20 mM NADPH and 30-40-µg cytosolic/mitochondrial protein which was precipitated with 10 % 5-sulfosalicylic acid.
The reaction was initiated by the addition of glutathione reductase for a period of 2 mins at 30 seconds interval. GSH amounts in the sample were estimated by running standards of known concentration under the same conditions.

2.13 Measurement of Glutathione s-transferases activity

Glutathione S-transferases (GST), a phase II detoxification enzyme exists in different isoforms. It plays an important role in the protection against various toxicants by catalyzing the conjugation of glutathione with the reactive electrophilic metabolites and xenobiotics (129). Glutathione S-transferases (GSTs) can reduce lipid hydroperoxides through their Se-independent glutathione peroxidase activity and these enzymes have the ability to detoxify lipid peroxidation end products such as 4-hydroxynonenal (4-HNE). Earlier studies together with results of recent studies in our laboratory suggests that lipid peroxidation products, particularly hydroperoxides and 4-HNE, are involved in the mechanisms of stress-mediated signaling, and can be controlled by the alpha class GSTs through the regulation of the intracellular concentrations of 4-HNE (130).

The cytosolic and mitochondrial GST activities were measured using 1 chloro-2, 4-dinitrobenzene (CDNB) according to the method of Habig et al. (131), with few modifications. Briefly, the reaction mixture contained 0.3 M potassium phosphate buffer, pH 6.5, 1.0 mM GSH, 1.0 mM CDNB, and 40-50 µg mitochondria or cytosol. The reaction was carried out at 25°C, and the change in absorbance at 340nm was monitored for 2 mins using a spectrophotometer (model DU-70, Beckman). All initial rates were corrected for the background non-enzymatic reaction. One unit of activity is the formation of 1 µmol product min⁻¹ at 25°C (extinction coefficient at 340 nm = 9.6 mM⁻¹ cm⁻¹ for CDNB).
2.14 Glutathione peroxidase activity (GSH-Px)

GSH-Px is a major antioxidant in the cellular system. It plays an important role in the detoxification of peroxides, thereby preventing cellular lipid peroxidation. GSH-Px uses glutathione to reduce hydrogen peroxide to water and lipid peroxides to alcohol. Glutathione peroxidase protects cells from oxidative damage by consuming free peroxide, in the presence of selenium as a cofactor. Considering the presence of non-selenium enzymes, cumene hydroperoxide was used as the substrate in order to measure the total GSH-Px (Se and non-Se enzymes) activity. This was done by NADPH oxidation using a coupled reaction system that contains glutathione, glutathione reductase, and cumene hydroperoxide. The reaction below demonstrates the action of GSH-Px that oxidizes 2 moles of reduced GSH to oxidized GSSG

\[
\text{GR}
\]

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}_2
\]

Glutathione Reductase (GR) reduces the oxidized glutathione to complete the cycle:

\[
\text{GR}
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The oxidation of NADPH to NADP\(^+\) leads to a decreased absorbance at 340 nm. The rate of decrease in absorbance is directly proportional to the glutathione peroxidase activity. The reaction mixture (total volume of 3 ml) contained 50 mM KH\(_2\)PO\(_4\)/5 mM EDTA buffer, pH 7.0, 28 mM NADPH, 3.75 mM NaN\(_3\), 0.5 mM GSH and 1 unit of GR that was incubated for 10 minutes at 25 °C before adding 50-100 µg of mitochondrial or cytosolic protein. The reaction was initiated by adding 0.2 mM cumene hydroperoxide, and measured using the spectrophotometer at 340
nm for 2-5 minutes. Results are expressed as nmol/min/mg protein, using the extinction coefficient of NADP (6.22 mM⁻¹ cm⁻¹).

2.15 Assay for cytochrome P450

The cytochrome P450 enzymes form the phase II detoxification enzymes, which exists in different isoforms. They play an important role in detoxification and protection against a wide variety of xenobiotics and various drugs. They are very important for metabolizing many endogenous compounds and for their detoxification (132). The CYP 2E1 isoenzyme helps in the reduction of oxygen to superoxide and peroxide which is important in lipid peroxidation (133). The activity of CYP 2E1 was measured as described by Czygan et al. (134). Briefly, the reaction mixture composed of assay buffer (50 mM KPi, pH 7.4 and 10 mM MgCl₂), 1 mM NADPH, 4 mM NDMA (N-nitrosodimethylamine N-demethylase), and 100-200 µg microsomal protein were incubated at 37 °C for 30 minutes. 25 % ZnSO₄ and saturated Ba (OH)₂ solution were added to stop the reaction. After centrifugation, an equal amount of NASH reagent (consisting of ammonium acetate, acetyl acetone and acetic acid) was added to the supernatant, heated at 60 °C for 30 minutes and then left to cool in ice water. CYP 2E1 activity was determined using a spectrophotometer set at 412 nm and calculated using a standard curve plot using different concentrations of formaldehyde run under the same conditions.

Another isoenzyme from the CYP 450 family is the CYP 3A4, which is the most abundant enzyme found in the liver and the intestine. Similar to the CYP 2E1, is vital in oxidizing xenobiotics, such as toxins or drugs. It plays an important role in the metabolism of approximately half the drugs in use today. Its catalytic activity variation is important in issues of bioavailability and drug-drug interactions (135).
The assay of CYP 3A4 activity was similar to that of CYP 2E1, except that erythromycin was used as the substrate for this isoenzyme.

2.16 Assay of mitochondrial respiratory complexes

Mitochondrial complexes are located in the mitochondrial inner membrane and make up the ETC system, which plays a major role in the production of ATP, the main energy intermediate in living organisms.

Mitochondrial complex I, also known as NADH:quinone oxidoreductase or NADH dehydrogenase is the first enzyme (Complex I) in the mitochondrial ETC. It works by transferring electrons from reduced Nicotinamide adenine dinucleotide (NADH) to coenzyme Q (CoQ). This complex also translocates protons, helping to provide the electrochemical potential involved in ATP production. Respiratory complex II oxidizes succinate to fumarate in the Krebs cycle and reduces ubiquinone in the electron transport chain (136). Complex III transfers the electrons from CoQH2 to reduce cytochrome c that is the substrate for complex IV. Complex IV, also known as cytochrome c oxidase transfers four electrons from four molecules of cytochrome c to molecular oxygen (O2) to produce two molecules of water (H2O). Together, it moves four protons across the membrane, producing a proton gradient to produce ATP.

Complex I specific activity was measured by following the decrease in absorption due to the oxidation of NADH at 340 nm (ε = 6.81 mM\(^{-1}\)cm\(^{-1}\)). 0.13 mM NADH, 65 mM ubiquinone and 2 µg/ml of antimycin were added to the assay mixture containing 50 mM Kpi, pH 7.2 and 5 mM MgCl\(_2\) to make a final volume of 1 ml. 10-20 µg mitochondria was added and the NADH: ubiquinone oxidoreductase activity was measured for 2 minutes, then rotenone (2 µg/ml) was added and the
activity was measured for an additional 2 minutes. Complex I activity is the rotenone sensitive NADH:ubiquinone oxidoreductase activity.

Complex II (succinate-ubiquinone oxidoreductase) and Complex III (ubiquinol-cytochrome c oxidoreductase) activities were measured by monitoring the reduction of cytochrome c at 550 nm ($\varepsilon = 19.1$ mM$^{-1}$cm$^{-1}$). 1M succinate was used as a substrate in the assay that comprised of 50 mM KPi buffer, pH7.2, 5 mM MgCl$_2$ and 2.5mM oxidized cytochrome c. Around 10-20 µg of mitochondrial protein was added and the Complexes II, III activities were measured for 2 mins at 550 nm. Rotenone (2 µg/ml) and 0.1 M sodium azide were added to block the activities of Complex I and Complex IV respectively. The activity was expressed as nmol/mg protein.

Complex IV activity was measured by monitoring the oxidation of reduced cytochrome c at 550 nm ($\varepsilon = 19.1$ mM$^{-1}$cm$^{-1}$). The assay was performed using 10 µg of mitochondrial protein at 550 nm using 50 µM cytochrome c (reduced) as the donor. Reduced cytochrome c was prepared fresh on the same day, using 0.1 M dithiothreitol (DTT) as a reducing agent. The assay mix contained 10 mM Tris-HCl pH 7.0, 120 mM KCl, and 250 mM sucrose in a final volume of 1 ml. 1 mM lauryl maltoside (LM) was added to make the external mitochondrial membrane permeable. The initial rate of cytochrome c reduction was used to calculate the activity. Complex IV activity is expressed as nmol/mg protein.
2.17 Measurement of cellular adenosine triphosphate levels

Adenosine triphosphate (ATP) has many essential roles in the cells. It is the main intermediate donor of energy needed for most of the energy-consuming anabolic and catabolic activities in the cell. ATP is present in all metabolically active cells. Thus it is used as a marker to evaluate cell viability. Its concentration rapidly declines when cells undergo necrosis or apoptosis. Total cellular ATP was measured by bioluminescence using the ATP-bioluminescent somatic cell assay kit (Sigma, Cat # FLASC). The assay is based on the following reaction:

\[
\text{ATP} + \text{D-luciferin} + \text{O}_2 \rightarrow \text{AMP} + \text{PPi} + \text{oxyluciferin} + \text{CO}_2 + \text{light}
\]

The intensity of the light emitted is proportional to the amount of ATP and is measured using a luminometer. The amount of ATP in the samples was expressed as LU/mg protein. Briefly, 100-200 µg of mitochondrial protein were lysed with somatic cell releasing reagent that leads the release of cellular ATP by altering membrane permeability. The free ATP reacts with Luciferin-Luciferase in the reaction mix and the light released was measured using a Turner Designs 20/20 luminometer. The emitted light was proportional to the ATP concentration in the sample.
2.18 Hexokinase assay

Hexokinase is an insulin sensitive enzyme and it is the first enzyme in the glycolytic pathway. It uses ATP to catalyse the phosphorylation of aldo- and keto-hexoses to hexose-6-phosphate. There are multiple isoenzymes of hexokinase (HK-I, II, III and IV), they differ in their kinetic and regulatory properties and distribution in the body. Studies have shown alterations in hexokinase in different diseases such as: x-linked muscular dystrophy, hemolytic anemia, and cancer (137).

Hexokinase activity was measured using a hexokinase colorimetric assay kit from Sigma –Aldrich (Cat # MAK091). The enzyme activity was determined by a coupled enzyme assay, in which glucose was converted to glucose-6-phosphate by hexokinase, which, in turn, was oxidized by glucose-6-phosphate dehydrogenase to produce NADH. This, in turn, reduces a colorless probe producing a colorimetric (450 nm) product proportional to the available hexokinase activity. The amount of NADH produced was determined from the standard curve. One unit of hexokinase enzyme is the amount that will produce 1.0 µmole of NADH per minute at pH 8.0 at room temperature.

2.19 Glutamate dehydrogenase assay

Glutamate dehydrogenase (GDH) is a mitochondrial enzyme, which plays a vital role in controlling insulin secretion. Nevertheless, it is not known if GDH expression levels in β-cells is rate limiting for the secretory response to glucose. GDH also plays an important role in controlling glutamine and glutamate oxidative metabolism (138).

Abcam’s glutamate dehydrogenase kit (Cat # ab 102527) was used to detect GDH in the mitochondrial samples. It is a sensitive method to detect the enzyme
activity, as low as 0.01 mU. In this assay, GDH in the sample consumes glutamate as a specific substrate and generates NADH, resulting in a proportional color development, which is read colorimetrically at 450 nm. Briefly, the mitochondrial samples and standards were incubated with the GDH developer and NADH for 30 mins to 2 h at 37 °C and the absorbance measured at 450 nm at 5 min intervals. The amount of NADH generated was determined from the standard curve. 1 unit is the amount of enzyme that will generate 1.0 umol of NADH/min at pH 7.8 and 37 °C.

2.20 SDS-PAGE and Western Blot analysis

50 µg protein from the pancreatic and heart total extract and 10-20 µg protein from serum samples from all the four groups were separated by 10 % SDS-PAGE. This separates the proteins according to their molecular size (139). The separated proteins were then electrophoretically transferred to a membrane (nitrocellulose paper) by Western Blotting (140). Membranes were blocked using 5 % non-fat milk in TBS-0.05 % Tween-20; it is an essential step to avoid any non-specific antibody binding. The membranes were immunoblotted with the antibodies to the proteins of interest, as described before (141) after which the membranes were treated with a secondary antibody for 1 hour at room temperature. Blots were then developed using ECL Plus Western Blotting Luminol Reagent kit(cat #), scanned and quantitated using the ImageJ 1.48 software. β-actin was used as the loading control.

2.21 Statistical analysis

Data analysis was performed using SPSS software, version 21. All results are expressed as means ± SEM of at least three to four determinations. Statistical significance of the data was assessed using analysis of variance followed by LSD’s post-hoc analysis. P values ≤0.05 were considered statistically significant.
Chapter 3: Results

3.1 Effect of ASA on body weight

Aspirin treatment marginally lowered the body weight in control and GK rats. The control rats which had an average weight of 325 g before treatment with ASA showed a weight of around 295 g after aspirin treatment. Similarly GK rats which showed an average weight of around 343 g were marginally reduced to around 326 g after ASA treatment. Although more planned studies are needed to further confirm this observation. None the less, our study may suggest an increased utilization of energy after aspirin treatment may be a cause of weight loss.

3.2 Effect of ASA on blood glucose and insulin levels

The fasting blood glucose level (Figure 7A) in GK rats was significantly higher compared to control rats, and was reduced modestly (17%) when treated with ASA from (14.7 mM to 12.5 mM). This could be due to more efficient glucose uptake and or utilization in ASA-treated GK rats. Similarly, blood insulin (Figure 7B) was significantly elevated (> 2-fold) in GK rats and ASA treatment significantly reduced the levels. No significant changes in blood glucose or insulin levels were observed in control non-diabetic rats treated with ASA. These results may suggest increased energy expenditure in ASA-treated diabetic rats which is presumably due to increased insulin sensitivity and responses in peripheral tissues.
3.3 Effect of aspirin on glucose tolerance

Improved glucose tolerance was observed at 60-120 mins after I.P. administration of 2g glucose/kg body weight in ASA treated rats. The diabetic GK rats showed a more significant improvement (20-30 %) compared to control rats (Figure 8). No significant alterations in glucose tolerance were observed in control
non-diabetic rats treated with ASA. These results suggest enhanced utilization of glucose by peripheral tissues after ASA treatment in the GK rats.

![Graph showing glucose tolerance test results](image)

**Figure 8**: Effect of aspirin treatment on glucose tolerance.

Glucose tolerance test in control Wistar (C) and GK rats treated with/without ASA (n=5). After an overnight fast, animals were injected with glucose (2g glucose/kg body weight) intraperitoneally. Blood samples were collected from the tail vein at time 0 (prior to the glucose load), 60 and 120 minutes after the glucose challenge and glucose levels tested as described in the Materials and Methods.

Results are expressed as mean ± S.E.M. from three independent experiments and

### 3.4 Effect of aspirin on serum biochemistry

No significant alterations in the level of total serum proteins; uric acid, ALT, AST, LDH and CK (*Figure 9A-F*) was observed after ASA treatment. This suggests that the ASA treatment had no significant toxicity in the tissues. However, a significant decrease in triglyceride level was observed in GK rats, with no appreciable effect after ASA treatment (*Figure 10C*). This may suggest increased uptake and utilization of lipids as an initial compensatory mechanism for the lack of
proper glucose utilization by peripheral tissues in GK rats. An increase in alkaline phosphatase and conjugated bilirubin was also observed in GK rats which were inhibited by ASA treatment (Figure 10D and 10E). This appears to be associated with mild cholestasis due to hypercholesterolemia seen in these rats.

Figure 9: Effect of aspirin treatment on tissue toxicity

Tissue toxicity was measured in control Wistar (C) and GK rats. Serum separated from the blood of the rats was subjected to analysis for total protein (A), uric acid (B), Alanine aminotransferase (ALT) (C), Aspartate aminotransferase (AST) (D), Lactate dehydrogenase (LDH) (E) and Creatine kinase (CK) (F) on the COBAS® INTEGRA 400 plus auto-analyzer. Results are expressed as mean ± S.E.M. from three independent experiments.
We measured cholesterol, lipoprotein, triglyceride, alanine phosphatase (ALP) and direct bilirubin levels. Serum separated from the blood of the rats was subjected to analysis for cholesterol (A), total lipoproteins (B), Triglycerides (C), Alkaline phosphatase (ALP) (D) and conjugated bilirubin levels (E) on the COBAS® INTEGRA 400 plus auto-analyzer. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. (* indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK).

Figure 10: Effect of aspirin treatment on blood lipid profile.
3.5 Effects of aspirin on prostaglandin E2 level

GK diabetic rats showed a significant (about 2-fold) increase in Prostaglandin E2 level indicating an increased inflammatory response (Figure 11). ASA treatment drastically reduced the level of PGE2 both in GK diabetic and control rats.

![Figure 11: Effect of aspirin treatment on Prostaglandin E2 levels.](image)

E2 was measured in control Wistar (C) and GK rats treated with/without ASA (n=5). Serum separated from the blood of the rats was assayed for prostaglandin E2 levels using an ELISA kit as described in the Materials and Methods. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. (** indicates p< 0.001 compared to control, ∆∆ indicates p< 0.001 compared to GK).

3.6 Effects of aspirin on reactive oxygen species and reactive nitrogen species

A significant decrease in serum NO level was observed in GK diabetic rats (Figure 12A). ASA treatment significantly increased the level of NO. A slight increase in NO production was also observed in ASA treated control rats. Serum
SOD level was significantly increased in ASA-treated GK diabetic rats but not in ASA-treated non-diabetic control rats (Figure 12B). The increased SOD could be due to the increased peroxide radicals, which in turn, would be converted to H₂O₂ and may be responsible for the increased insulin signaling. This supports our finding of increased glucose tolerance after ASA treatment since ASA is known to play an important role in increasing insulin sensitivity.

Figure 12: Effect of aspirin treatment on total nitric oxide and superoxide dismutase levels. Total NO and SOD were measured in control Wistar (C) and GK rats treated with/without ASA (n=5). Serum separated from the blood of the rats was assayed for total NO level (A) and SOD (B) activity using the respective kits as described in the Materials and Methods. Results are expressed as mean + S.E.M. from three independent experiments and asterisks indicate significant difference. (* indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK, ∆∆ indicates p< 0.001 compared to GK).
3.7 Effect of aspirin on pancreatic insulin/glucagon secretory functions

Immunoreactivity of insulin- (β) and glucagon- (alpha) secreting cells in the pancreatic islets was checked using paraffin sections of pancreas (Figure 13). A significant decrease in size and number of the hormone secreting cells was observed in GK rats while ASA treatment improved the structural integrity and size of pancreatic islets. Quantitative analysis confirmed increased pancreatic islet size (Figure 8A) in ASA treated cells which correlated with their ability to regulate insulin secretion confirmed by the increased percentage of insulin secreting cells (Figure 14B) after ASA treatment. In addition, ASA treatment also enhanced the insulin/glucagon ratio which was significantly reduced in GK diabetic rats (Figure 14C).

![Figure 13: Effect of aspirin treatment on β-cell insulin/glucagon level.](image)

Localization of insulin (green) and glucagon (red) in pancreatic islets was performed by a double-labeled immunofluorescence method and visualized with a Carl Zeiss fluorescent microscope as described in the Materials and Methods. Representative islets at X40 magnification selected from at least five sets of slides from each of the groups are shown. Bar represents 25 μm.
Figure 14: Effect of aspirin treatment on insulin/glucagon secretion

To assess the effect of aspirin treatment, the size of the islets (A) was measured using Axiovision® 3.0 Image Analysis System (Zeiss, Gottingen, Germany) attached to the Carl Zeiss fluorescent microscope. Insulin- or glucagon-positive cells were counted in visible sections at X40 magnification. The number of insulin-immunoreactive cells was divided by the total number of cells to give the percentage of insulin secreting cells (B). The ratio of insulin positive to glucagon positive cells was also calculated (C). A total of 10 random islets were analyzed from a total of 5 slides in each group. Results are expressed as mean ± S.E.M. and asterisks indicate significant difference. * indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK, ∆∆ indicates p< 0.001 compared to GK.
3.8 Effect of aspirin on the expression of insulin

Insulin expression in the serum and pancreas of GK and control rats was also studied by SDS-PAGE using a polyclonal antibody against insulin. An increased expression of serum insulin was observed in GK rats confirming the insulinemia in GK diabetic rats when compared to control non-diabetic rats (Figure 15A). ASA treatment reduced the amount of insulin in the serum supporting the results obtained by ELISA assay for serum insulin. These results suggest alterations in glucose stimulated insulin synthesis after ASA treatment. Insulin expression decreased significantly in the pancreas of GK diabetic rats. However, an increased expression was observed after ASA treatment suggesting the recovery of β cells to produce insulin, but the levels were still comparatively higher than that seen in control rats. This could be because though the ASA treatment had improved glucose tolerance, the low dose of ASA for this time period was not enough to bring the glucose levels back to normal and thus bring the insulin levels also to control levels.

![Figure 15: Effects of aspirin treatment on the expression of insulin in serum and pancreatic homogenates.](image)

The expression was measured in control Wistar (C) and GK rats treated with/without ASA (n=5). Pancreatic homogenates (50 µg) and serum samples (10 µg) were
electrophoretically transferred by 15 % SDS-PAGE and Western blot analysis to visualize the expression of insulin (A). B-actin was used as the loading control. The histograms in Fig. 9B shows densitometric analysis of the protein bands and are expressed as percentage protein compared to the control group. Asterisks indicate significant difference. (**) indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK). The figures shown are representative of 3-4 independent analyses.

3.9 Effects of aspirin on oxidative/nitrosative stress

A significant increase in mitochondrial ROS production was observed in the pancreas (almost 80 %) and in the heart (about 50 %) of GK rats which was reduced almost to control levels after ASA treatment (Figure 16A). Control Wistar rats treated with ASA showed negligible ROS formation. Membrane-bound NADPH oxidase, however, was increased only in the heart and not the pancreas of GK rats (Figure 16B). This could suggest differential sources of ROS production the pancreas and heart in GK rats.
Figure 16: Effects of aspirin treatment on mitochondrial ROS and membrane-bound NOX levels. ROS and NOX were measured in the pancreas and heart of control Wistar and GK rats. Mitochondrial ROS production (A) in the pancreas and heart of control Wistar and GK rats was measured using DCFDA as a probe. Total homogenates from the tissues of these animals were analyzed for membrane-bound NOX production using the lucigenin-enhanced chemiluminescence method as described in the Materials and Methods. Asterisks indicate significant difference. * indicates p < 0.05 compared to control, ** indicates p < 0.001 compared to control, ∆ indicates p < 0.05 compared to GK, ∆∆ indicates p < 0.001 compared to GK. The figures shown are representative of 3-4 independent analyses.

A significant increase in pancreatic SOD and decrease in heart SOD levels was observed (Figure 17). ASA treatment again brought back the values close to the control. No difference was observed in the control rats treated with ASA. This explains the increased insulin signaling by ASA treatment due to the low levels of NO/H$_2$O$_2$. 
3.10 Effects of aspirin on lipid peroxidation

Microsomal LPO was significantly increased (about 60-70 %) in both the pancreas and heart of GK rats (Figure 18). ASA treatment markedly reduced the levels in both, though it was still much above the control levels. This could be due to the increased peroxide formation suggestive of increased oxidative stress in diabetic rat tissues.
Figure 18: Effects of aspirin treatment on lipid peroxidation in the pancreas and heart

LPO was measured in control Wistar and GK rats. NADPH-dependent LPO was measured in the pancreas and heart of control Wistar and GK rats using the LPO kit from Oxis Int. as described in the Materials and Methods. Asterisks indicate significant difference. * indicates p< 0.05 compared to control. ** indicates p< 0.001 compared to control, ∆∆ indicates p< 0.001 compared to GK. The figures shown are representative of 3-4 independent analyses.

3.11 Effects of aspirin on GSH-dependent redox metabolism

A significant increase (about 30 %) and non-significant in the cytosolic protein-free GSH were observed in the pancreas and heart respectively (Figure 19A). There was no effect on the mitochondrial GSH in the pancreas though the heart showed a slight decline in the GK animals (Figure 19B).

GSH-CDNB conjugating activity by GST enzyme in the cytosolic fraction showed very mild alterations both in the pancreas and heart (Figure 19C). However, the mitochondrial fraction of the pancreas showed a significant decrease in GST activity in the GK rats and ASA treatment increased the activity (Figure 19D).
could be due to the increased conjugation of the GST enzyme due to the increased mitochondrial ROS production in the pancreas. Comparatively, only a mild increase in the activity was observed in the heart tissue, though ASA treatment seemed to increase the activity significantly. These results suggest the differential response in maintaining the GSH pool and its conjugating activity in the pancreas and heart of diabetic GK animals.

Increased GSH-Px activity in the pancreas was observed both in the cytosolic as well as the mitochondrial fractions of GK rats as well as after treatment with ASA (Figure 19E, F). The heart, however, showed no significant alterations in the cytosol whereas mild alterations were observed in the mitochondria. This may suggest that the pancreas is under more oxidative stress conditions when compared to the heart and that there seems to be more protection in the heart from increased peroxide formation.
GSH-Px was measured in the pancreas and heart of control Wistar and GK rats treated with ASA. GSH concentration in the pancreas and heart of control Wistar and GK rats was measured in the cytosol (A) and mitochondria (B) by the enzymatic recycling method of Griffith as described in the Materials and Methods. GST activity, using CDNB as substrate, in the pancreas and heart of control Wistar and GK rats was measured in the cytosol (C) and mitochondria (D) as described in the Materials and Methods. GSH-Px activity in the pancreas and heart of control Wistar and GK rats was measured in the cytosol (E) and mitochondria (F) using cumene hydroperoxide as substrate as described in the Materials and Methods. Asterisks indicate significant difference * indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK, ∆∆ indicates p< 0.001 compared to GK. The figures shown are representative of 3-4 independent analyses.

Figure 19: Glutathione levels and metabolism.
3.12 Effects of aspirin on CYP activities

A significant increase (60 \%) in CYP 2E1 activity in the pancreas and a 35 \% increase in the heart were observed (Figure 20A). This increase in activity could be due to increased oxidative stress. ASA treatment brought the activity to control level in both the tissues. Similarly, CYP 3A4 activity was also significantly increased in both the pancreas and heart (Figure 20B). However, ASA treatment could not bring the activity back to control levels in both the tissues. In fact, the activity increased further after ASA treatment in the heart tissue. This could be because of differential isoenzyme expression in the heart and maybe because CYP 3A4 is involved in ASA metabolism.

Figure 20: CYP 450 levels.
CYP 450 was measured in the pancreas and heart of control Wistar and GK rats treated with aspirin. CYP 2E1 (A) and CYP 3A4 (B) activities in the liver and kidney microsomal fractions were measured using standard substrates as described in the Materials and Methods. Results are expressed as mean ± S.E.M. from three
independent experiments and asterisks indicate significant difference. * indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, Δ indicates p< 0.05 compared to GK, ΔΔ indicates p< 0.001 compared to GK. The figures shown are representative of 3-4 independent analyses.

3.13 Effects of aspirin on mitochondrial bioenergetics

Respiratory Complex I activity was markedly reduced in both pancreas and heart mitochondria of GK rats (Figure 21A). Treatment with ASA increased the activity to almost control levels. Similarly, Complex II/III activity also was significantly reduced in both the tissues of GK rats (Figure 21B). Treatment with ASA again significantly increased the activity to control levels in both the pancreas as well as the heart. However, not significant alteration in respiratory Complex IV activity was found in either pancreas or heart (Figure 21C). On the other hand, mitochondrial ATP levels were markedly reduced in the pancreas and heart of GK animals (Figure 21D) though ASA treatment brought the levels to be similar to control rats. This shows that ASA treatment has beneficial effects by increasing energy utilization and ATP production.
Figure 21: Effect of aspirin on the activities of mitochondrial complexes and ATP levels.

Measurement was done in the pancreas and heart of control Wistar and GK rats.
Mitochondrial respiratory enzyme Complexes I,II/III and IV (A,B,C) activities were measured in freshly prepared mitochondrial fractions from the pancreas and heart of control Wistar and GK rats using enzyme specific substrates as described in the Materials and Methods. The ATP content (D) in the rat tissues was determined using ATP Bioluminescent cell assay kit (Sigma, St Louis, MO). Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. * indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, Δ indicates p< 0.05 compared to GK, ΔΔ indicates p< 0.001 compared to GK).

3.14 Effects of aspirin on energy metabolizing cytosolic and mitochondrial enzymes

Glucose phosphorylation by hexokinase enzyme was significantly increased in both pancreas and heart of GK rats (Figure 22A). ASA treatment further increased the activity. On the other hand, activity of the mitochondrial enzyme, GDH was significantly reduced in both the tissues of GK rats (Figure 22B). However, ASA treatment improved the levels significantly. No change in activity was observed in the control rats treated with ASA. These results imply improved energy utilization and insulin responses in the pancreas and heart of GK diabetic animals after ASA treatment.
Figure 22: Effect of aspirin on hexokinase and glutamate dehydrogenase activities
Measurement was done in the pancreas and heart of control Wistar and GK rats. Hexokinase (A) and glutamate dehydrogenase (B) were measured in the pancreas and heart of control Wistar and GK rats using the respective kit protocols as described in the Materials and Methods. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. * indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, Δ indicates p< 0.05 compared to GK, ΔΔ indicates p< 0.001 compared to GK.

3.15 Effects of aspirin on the expression of key redox-sensitive marker proteins

The increase in tyrosine phosphorylation of downstream insulin signaling kinases promotes insulin signaling via the IRS1/2 signaling pathway. Increased phosphorylation of Akt protein was observed after ASA treatment (Figure 23A) in
the pancreas of control Wistar rats as well as GK rats. Increased phosphorylation was also observed in the heart of control rats after ASA treatment. Significant increase in phosphorylation of JNK protein was also observed in the pancreas and heart of both GK and control Wistar rats (Figure 23B). Increased activation of PPAR-γ was also observed after ASA treatment in both pancreas and heart tissues of GK rats (Figure 23C), though the increase was more significant in the heart tissues of GK rats. This suggests an improved fatty acid metabolism after ASA treatment, thus regulating lipid metabolism. This may also explain the reduced level of triglycerides in GK rats.

Increased carbonylation of proteins by 4-HNE conjugation was observed in the pancreas of GK rats (Figure 23D). ASA treatment markedly decreased the level of conjugation. The heart tissue however did not show any difference in protein carbonylation, again suggesting increased oxidative stress in the pancreas. The heart appears to be exhibiting extra level of redox-protection from the oxidative stress.
Figure 23: Expression of specific marker proteins.
Measurement was done in the pancreas and heart of control Wistar and GK rats treated with aspirin. Homogenates (50 µg protein) from the pancreas and heart of control Wistar and GK rats were subjected to 12% SDS-PAGE and Western blot analyses to visualize immunoreactivity of specific marker proteins: Akt, p-Akt (A), JNK, p-JNK (B) and PPAR-γ and HNE (C). Beta-actin was used as loading control. Histograms represent the ratio of the signals of phosphorylated to unphosphorylated proteins, in (A) and (B) whereas it represents the ratio of the protein to that of actin in (C) and are expressed as fold increase using the ratio of control proteins as 1.0. The figures shown are representative of 2-3 independent analyses. Molecular weights shown are in kDa.
Chapter 4: Discussion

DM is a progressive metabolic disorder attributed to rapid economic development and changes in life style that have led to reduced physical activity, increased intake of refined high calorie diet and a rise in obesity (1, 3). The increasing incidence of diabetes, particularly T2DM, worldwide, has cost billions of dollars in the treatment and management of this disease. T2DM is a multifactorial metabolic syndrome associated with progressive impaired insulin secretion, insulin response and resistance and characterized by compromised energy metabolism, hyperglycemia, hyperlipidemia, and cardiovascular abnormalities. However, the precise molecular mechanisms of disease progression and complications are still unclear. The GK rat is a spontaneous non-obese animal experimental model of T2DM. This strain was developed by selective breeding of glucose intolerant Wistar rats over many generations. The GK rats exhibit decreased β-cell numbers and function which is accompanied by mild hyperglycemia, impaired glucose-induced insulin secretion, marked glucose intolerance, peripheral insulin resistance and chronic inflammation (101-102, 142). Despite the mild hyperglycemia several manifestations of diabetes complications have been demonstrated in this model (143-145).

Growing evidence has linked T2DM with inflammation. Thus, the GK rat represents a good animal model for studying human T2DM pathophysiology and the effects of therapeutic options such as the use of ASA or other NSAIDs. It is possible that existing low-grade systemic inflammation can be suppressed by experimental strategies aimed at blocking the production or action of proinflammatory signaling pathways, thereby treating T2DM. There are several studies on the glycemic effects
of salicylates and ASA (acetylated salicylic acid) in the literature, but the mechanism of their antidiabetic effects is still unclear (106, 146). Recently, sodium salicylate and aspirin have been shown to inhibit activation of the transcription factor NF-κB (107) by preventing degradation of IKK-β, which regulates the inflammatory responses, and might therefore ameliorate insulin resistance and improve glucose tolerance in at least some patients with T2DM. However, the exact mechanism and effects of ASA on treatment of T2DM are still not fully understood.

Salicylates have also been shown to enhance metabolic rate and energy expenditure in GK rats (108). Recent studies on experimental rodent models of diabetes and clinical trials have demonstrated beneficial and deleterious effects of ASA on diabetes-associated complications and insulin sensitivity depending upon the dose of aspirin and experimental model used (109-112, 147).

Our recent studies in diabetic rats have demonstrated that diabetes-associated hyperglycemia caused tissue-specific increase in oxidative stress and mitochondrial dysfunction (114,116,117,148).

Low grade systemic inflammation is considered an integral part of T2DM and plays an important role in glycemic control (149). The GK rats are a useful model to evaluate the mechanism of effects of ASA on disease progression. The aim of the present study was to evaluate the potential role of aspirin and the biochemical mechanism involved in improving glucose tolerance and insulin sensitivity in type 2 mildly diabetic GK rats. The objective was to investigate the effect of ASA on 1) pancreatic β-cell structural integrity; 2) insulin secretion and insulinemia;3) insulin signaling; 4) energy metabolism and mitochondrial function;5) oxidative stress and
glutathione-dependent redox metabolism; 6) drug metabolizing and detoxification enzyme systems. We have demonstrated that low dose ASA (100 mg/Kg body wt. / I.P.) treatment for 5 weeks reduced hyperglycemia and hyperinsulinemia with improved glucose tolerance (utilization) by peripheral tissues without causing any appreciable toxicity in target tissues. Improved insulin secretory function in these rats was also observed. Our results have also suggested that insulin signaling was improved causing an increase in energy metabolism after ASA treatment. We also demonstrated an improvement in the drug metabolizing abilities in the pancreas and heart of ASA-treated GK rats as suggested by the alterations in the cytochrome P450 and GSH-conjugating drug metabolizing enzymes. However, the ASA treatment caused no significant alterations in the activities of ALT, AST, total proteins, uric acid, LDH or CK. A mild liver toxicity was, however, observed in rats treated with ASA. This may be due to increased cholestasis. This effect is mostly idiosyncratic and is independent of the dose administered (150).

Insulinemia, hypercholesterolemia and hyperlipoproteinemia, in GK rats may have caused insulin resistance resulting in hyperglycemia (149,151). Our results have shown a marked increase in total cholesterol level in GK rats which was significantly reduced after ASA treatment (Figure 9A). However, the cholesterol level remains higher in GK rats treated with ASA than that seen in the control Wistar rats. Although the individual lipoproteins were varying both in the control and GK diabetic rats, ASA treatment caused a significant reduction in the total lipoproteins (Figure 9B). Recent study by Wang et al. (151) also demonstrated that salicylate treatment, in addition to a decrease in blood insulin and blood glucose levels, reduces the levels of total cholesterol and lipoproteins. Serum triglyceride level was however
lower in GK rats compared to the control rats and ASA treatment had no significant
effect on the level of TG in GK rats. This may suggest increased uptake of lipids in
GK rats by peripheral tissues as a compensatory mechanism for obtaining energy
from fatty acid instead of glucose. ASA has been shown to inhibit lipogenesis in the
tissues and activate lipases to generate energy metabolizing fatty acids as a
compensatory mechanism. There are reports suggesting that ASA acts in synergy
with metformin in improving lipid metabolism and insulin sensitivity (152). ASA
treatment improves insulin sensitivity by increasing the whole body fatty acid
oxidation and by protecting the IRS from serine phosphorylation which inhibits
downstream insulin signaling (153). This effect of ASA is associated with its anti-
inflammatory action as well as direct activation of AMP-activated protein kinases
(154, 155).

Our results have also indicated a marked increase in serum PGE2 level in GK
rats which may have contributed to increased inflammation. It is possible that
treatment with anti-inflammatory agent may result in reducing the proinflammatory
signals and responses in type 2 diabetes. We have shown that ASA treatment
markedly reduced the production of PGE2 in control and GK rats. GK rats, on the
other hand showed markedly increased production of PGE2 (Figure 11). Increased
PGE2 and E-prostanoid receptors (EP2/3) negatively regulate insulin secretion and
signaling and has been linked to the pathophysiology of T2DM (156). Therefore,
inhibition of PGE2 by ASA may play a significant role in PGE2/ EP3 mediated
modulation in β-cell function and insulin signaling.
There are reports showing that ASA increases NO production in cells and tissues (155) and that NO plays an important role in increasing insulin sensitivity by reducing the sensitivity of cells towards prooxidants (155-157).

NO is a freely diffusible reactive nitrogen species (RNS). Local production of superoxide, such as within β-cells, protects the cells by scavenging NO. Low levels of H$_2$O$_2$ have also been shown to induce NO production and insulin sensitivity. ASA has been reported to protect cells from oxidative damages by stimulation of endothelial NO production (155). There are reports that NO synthesis is reduced in T2DM and hypertension and that insulin regulates NO production (156, 157). We observed that serum NO level was low in GK rats and ASA treatment increased NO production (Figure 12A), which may have stimulated glucose tolerance and increased insulin response. The regulation of NO metabolism in T2DM plays an important role in insulin dependent Akt signaling and therefore disturbances of NO generation are detrimental in insulin resistance (156). Our results show that ASA treatment appears to have beneficial effect on the NO production in GK rat tissues.

We observed that ASA treatment increased serum SOD activity (Figure 17). SOD converts toxic superoxides to H$_2$O$_2$ and thus may help in H$_2$O$_2$-dependent insulin signaling and increased glucose transport in target cells. Insulin signaling is reported to be positively and negatively affected by ROS production. At a low physiological level, H$_2$O$_2$ is involved in enhancing insulin signaling by activating insulin receptor-tyrosine kinase activity and engaging the IRS-1/PI3k/AKT-dependent downward signaling to increase basal glucose transport activity (158). At lower concentrations, H$_2$O$_2$ has been reported to mimic insulin signaling even in the absence or low levels of insulin and inhibits PTP1B activity to prolong insulin
receptor tyrosine kinase activity. In contrast, in the presence of insulin, H$_2$O$_2$ inhibits insulin signaling by activating deleterious serine/threonine kinases developing insulin resistance (159, 160). Persistent hyperglycemia and insulinemia, systemic and islet inflammation and increased ROS are all involved in developing insulin resistance and β cells structural and functional defects in T2DM (161, 163). Clinical and experimental trials have validated the beneficial effects of anti-inflammatory therapies including ASA treatment (149, 151, 162, 164). Emerging results suggest that both β cell mass and abnormal secretory functions are implicated in T2DM (165, 166). As shown in our immunofluorescence study (Figure 13), ASA treatment has improved both the structural and functional properties of β islets. This may be associated with the anti-inflammatory property of ASA which helps in regenerating functional β cells, glucose stimulated insulin secretion, insulin sensitivity, glucose transport and energy utilization in T2DM.

In diabetes, hyperglycemia and hyperlipidemia are associated with increased ROS production and oxidative stress (115, 167, 168, 169, 170). Excessive superoxide production, however, either by NOX or mitochondrial respiration may result in excessive formation of peroxynitrite a highly reactive nitrogen species, which may accelerate the deleterious metabolic complications in diabetes. Using both type 1 and type 2 diabetes rat models we have shown the excessive production of ROS, increased oxidative stress associated mitochondrial and metabolic stress in the tissues (114-117, 148). Our present study on GK diabetic rats has also exhibited an increased oxidative stress related metabolic changes and mitochondrial dysfunction in the pancreas and heart. ASA treatment however had some beneficial effects. As shown by SDS-PAGE analysis, the increased carbonylation by 4-HNE-
conjugation of oxidized proteins was observed in GK rat tissues. ASA treatment decreased the oxidative 4-HNE-conjugation of proteins. GK rats pancreatic β-cells have been shown to be oxidatively stressed and have high-HNE-modified proteins (161). ASA appears to be protecting the mitochondrial proteins from oxidative carbonylation thus preventing mitochondrial dysfunction which is linked to develop insulin resistance in the tissues (171).

Our SDS-PAGE analysis (Figure 23) also revealed that ASA treatment increased AKT and JNK phosphorylation which promotes insulin signaling via activation of IRS1/2 and insulin-dependent downstream signaling. ASA treatment has also been shown to inhibit serine phosphorylation of IRS as seen in inflammatory responses, thereby stimulating the tyrosine phosphorylation of IRS through AKT pathways resulting in increased insulin sensitivity. The effect is associated with the anti-inflammatory action of ASA (153, 154, 172, 173). Activation of PPAR-γ in the pancreas and heart tissues after ASA treatment also suggests an improved fatty acid metabolism in GK rats treated with ASA. PPAR-γ family of nuclear receptors play an important role in the regulation of lipid metabolism by stimulating the genes involved in the lipid uptake and adipocytes differentiation. PPAR-γ agonists of the thiazolidine (TZD) family are being extensively used for the treatment of T2DM due to their insulin sensitizing properties. Increased expression of this protein in the tissues of GK rats after ASA treatment suggests another mechanism of improving insulin responses by ASA treatment (174). Another mechanism proposed by TZD is that it inhibits mitochondrial respiratory complexes in mitochondria, like metformin, and pyruvate carrier resulting in the activation of AMPK which facilitates mitochondrial biogenesis and hence insulin responsive increase in energy utilization.
Our results on GK rats also support these observations and add a mechanistic explanation of ASA action in improving T2DM.

Mitochondrial oxygen /energy metabolism to generate ATP via oxidative phosphorylation is also accompanied by ROS production. Mitochondria generate moderate levels of ROS during normal oxygen metabolism in oxidative phosphorylation during the metabolism of glucose and fatty acids. The low level of ROS enhances insulin sensitivity upon redox regulation of insulin receptor (tyrosine kinase) and substrates (IRS family). However, chronic exposure to high levels of ROS produced in T2DM could alter the mitochondrial function and thereby cause insulin resistance. Our results on GK rats have shown a compromised mitochondrial respiratory function with altered enzyme activities of inner membrane respiratory complexes (Figure 21). These results confirm our previous observations on STZ-induced diabetes or on Zucker type 2 fatty rat tissues (114-117, 148). ASA treatment seems to have beneficial effects by altering the mitochondrial functions in favor of energy metabolism and improved insulin signaling as observed by increase glucose phosphorylation by hexokinase and by increasing the activity of glutamate dehydrogenase (GDH) which generates alpha-ketoglutarate, an intermediate of tricarboxylic acid cycle. Activation of GDH has been associated with increased insulin secretion and energy metabolism (177, 178).

We have also observed that ASA treatment not only affected the mitochondrial function and oxidative stress but also involved in regulating GSH-dependent antioxidant homeostasis as well as CYP-dependent metabolism in the pancreas and heart of GK rats. The CYP 2E1 activity was increased in GK diabetic
rats suggesting the ROS mediated oxidative and metabolic stress (*Figure 14A*) (114-117, 148). ASA treatment resulted in the reduction of CYP activity. Similarly, CYP 3A4 activity was also affected by ASA treatment suggesting the adaptation of metabolic stress in GK rats (*Figure 20B*). This may also suggest a differential isoenzyme specific metabolism of ASA in the pancreas and heart. GSH-antioxidant pool as well as GST enzyme which conjugates endogenous oxidants such as HNE and other lipid peroxides was also altered after ASA treatment. These results have confirmed our previous observation (114-117, 148, 179) of altered drug metabolism in diabetes. ASA appears to have some adaptation in improving the drug metabolizing abilities of the tissues.
Chapter 5: Conclusion

With more than 350 million estimated global cases, diabetes is a pandemic disease with increasing morbidity and mortality. In the absence of an appropriate prevention and poor management, the current predictions for diabetes prevalence could double by 2035. Approximately 90% of diabetics have T2DM with multiple etiological bases. While intervention by lifestyle management and dietary control is an acceptable strategy to control energy metabolism, it is difficult to adhere to lifestyle changes for a long period of time. Moreover, the efficacy of current anti-diabetic drugs is limited with unwanted side effects. Consequently there is a need and demand for the development of safer and more effective drugs in type 2 diabetes. Therefore, it is important to understand the mechanisms involved in the etiology and pathophysiology and progression of insulin resistant T2DM and to identify the key molecular targets for anti-diabetic therapeutics. Chronic hyperinsulinemia, glucotoxicity and lipotoxicity have been identified as the major causes of insulin resistance and diabetes-associated complications. The onset of diabetes may start with pancreatic β-cell structural and functional defects, insulin secretion and glucose stimulated insulin release, insulin circulation and insulin signaling. The progression of the disease due to excessive availability of glucose and fatty acids include insulin-insensitivity in target tissues. Additionally, mitochondrial dysfunction with reduced fatty acid and energy metabolism may result in increased ROS production and oxidative stress with compromised redox-homeostasis resulting in the deterioration of the structural and functional integrity of tissues. The most common features are progressive metabolic syndrome with diabetic-induced retinopathy, neuropathy and
nephropathy. Mitochondrial dysfunction is considered to be an important cause of insulin resistance with compromised energy utilization.

ASA has been around for a long time for the treatment of cardiovascular diseases. In 2007, the ADA and AHA jointly recommended that ASA (75 to 162 mg/day), to be used as a primary preventive drug in T2DM patients with increased cardiovascular risks (180). However, there are conflicting reports and disagreement regarding the use of ASA for primary prevention in diabetes (181). Moreover, measuring the response to ASA is often difficult as some patients may show ASA resistance. Furthermore, there are other potential factors which may affect ASA efficacy in patients which include: ASA dose, disease severity, genetics, obesity and inflammation, smoking and complications of the disease and interactions with other drugs.

The objective of the present study was to further investigate the mechanism of ASA action on young GK diabetic-insulin resistant rats using an accepted effective dose based on the literature search and injecting the drug intraperitoneally (I.P.) to avoid gastric toxicity caused by ASA. Our hypothesis was; 1) to elucidate the mechanism of ASA action on pancreatic β-cell structure and insulin secretion; 2) study the effect of ASA on insulinemia and hyperglycemia/dyslipidemia; 3) to study the effect of ASA on glucose tolerance; 4) to study the effect of ASA on insulin signaling; 5) to study the effect of ASA on energy metabolism; 6) to study the effect of ASA on mitochondrial functions and oxidative stress and 7) to study the effect of ASA on drug metabolism. Our results suggest that ASA has improved β-cells integrity and insulin secretory function. We have also shown that ASA treatment to GK rats has improved glucose tolerance, insulin signaling based on Akt/JNK
phosphorylations and energy metabolism with improved glucose and lipid metabolism in the pancreas and heart. ASA treatment also altered oxidative stress, mitochondrial function and redox homeostasis which may have consequences in developing insulin-sensitivity in diabetes.

Further studies are needed to study the mechanism of ASA action using different doses and routes of administration along with clinically approved anti-diabetic drugs. Also, we need to elucidate the effect of ASA on energy metabolism, mitochondrial function, oxidative stress, insulin signaling response in different tissues from young and chronic diabetic rats. Identification of molecular targets for downstream insulin signaling affecting the energy (glucose and fatty acids) metabolism in GK rats will highlight the efficacy of ASA alone or in combination with clinically approved anti-diabetic drugs in type 2 diabetic animal models. Our study might be helpful in designing strategies for the therapeutic management of insulin-resistant T2DM.
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