

Combination of Metformin and Inorganic Nitrate: Effects on Metabolic Disease and Lactic Acidosis



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Nitric oxide (NO) is a diatomic gaseous molecule generated in our bodies by enzymes known as NO synthases. More recently another pathway for generation of NO has been delineated in which dietary or endogenous inorganic nitrate and nitrite are metabolized to form NO. Dietary supplementation with nitrate has proven beneficial for a number of disorders e.g. by improving metabolic function and lowering blood pressure, making it an attractive novel drug candidate in metabolic and cardiovascular disease. Besides utilizing direct therapeutic effects of nitrate itself, another attractive option might be to combine nitrate with another drug in order to increase the overall effect or reduce the side effects of the other drug.

The aim of the proposed thesis was to determine whether simultaneous acute administration of a low dose of metformin and nitrate can act synergistically to improve metabolic functions and decrease blood glucose level in a metabolic syndrome genetic mouse model (A2BKO mice) and also if this combination can prevent lactic acidosis produced by metformin in rats.

The results showed that both nitrate and metformin alone had a beneficial effect on glucose tolerance but there was no clear synergistic effect to decrease blood glucose levels. We speculate that the lack of synergy may be due to their same mechanism of action in targeting NADPH oxidase and AMP-activated protein kinase. However, the glucose disposal was better in metformin and nitrate combination group compared to metformin group. Moreover, in separate experiments we found that acute administration of nitrate had no impact on prevention of lactic acidosis induced by high dose of metformin in rats. However, future chronic studies are necessary in order to ameliorate our knowledge of the proposed combination mechanism.

Keywords: Nitrate-nitrite-NO pathway, Nitrate, A2BKO mice, Metformin

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ABBREVIATIONS

Abcc8	ATP binding cassette subfamily c member 8
A2B	adenosine type 2b receptor
A2BKO	adenosine type 2b receptor knockout mice
AMPK	adenosine monophosphate-activated protein kinase
AT1	angiotensin II type 1
ATP	Adenosine Triphosphate
AUC	area under the curve
BAT	brown adipose tissue
BMI	body mass index
C/EBP β	ccaat/enhancer-binding protein beta
CVD	cardiovascular disease
DKA	diabetic ketoacidosis
DPP-4	dipeptidyl peptidase-4
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FFA	free fatty acids
GI	gastrointestinal
GLP-1	glucagon-like peptide-1
HDL	high-density lipoprotein
HFD	high fat diet
HHNS	hyperosmolar hyperglycemic non-ketotic syndrome
HNF4A	hepatocyte nuclear factor 4 alpha
IARC	international agency for research on cancer
IDF	international diabetes federation
iNOS	inducible nitric oxide synthase
IRS-2	insulin receptor 2
KATP	ATP-dependent potassium channel
KCNJ11	potassium inwardly-rectifying channel, subfamily j, member

MALA	metformin associated lactic acidosis
NADPH	nicotinamide adenine dinucleotide phosphate
NIDDM	non-insulin-dependent diabetes mellitus
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OGTT	oral glucose tolerance test
Pi	inorganic phosphate
PKA	protein kinase a
PKC	protein kinase c
PKG	protein kinase g
PPAR- γ	peroxisome proliferator-activated receptor gamma
SAR	serious adverse reaction
SUR1	sulfonylurea receptor 1
TCF7L2	transcription factor 7 like 2
T2DM	type 2 diabetes
TZD	thiazolidinedione
VLDL	very low-density lipoprotein
WAT	white adipose tissue
WHO	world health organization
XOR	xanthine oxidoreductase

1. Introduction

The metabolic syndrome has become a serious public challenge in recent years. It was first delineated in 1923 by Kylin as a triad of hypertension (high blood pressure), hyperglycaemia (high blood glucose) and hyperuricaemia (high blood uric acid) [1].

In 1947 Vague revealed that distribution of body fat has a great impact on the development of metabolic disease [2] and in 1988 Reaven revealed that the so called syndrome X contributes to insulin resistance, excess amount of insulin and glucose in blood, decreased HDL cholesterol, high level of VLDL triglycerides and hypertension [3]. The metabolic syndrome contributes to type 2 diabetes (T2D) and cardiovascular disease. The risk factors of metabolic disease are as follows, abdominal obesity, elevated blood pressure and high level of fasting plasma glucose, low amount of high-density lipoprotein (HDL) and high serum triglycerides [4].

Based on the IDF definition, in order to be recognized with metabolic disease, individuals should have abdominal girth more than or equal to 94cm for men and more than or equal to 80cm for women, in addition to any two following risk factors: high blood pressure (diastolic blood pressure more than or equal to 85 mmHg or a systolic blood pressure more than or equal to 130 mmHg, or treatment of previously diagnosed hypertension; increased fasting plasma glucose (more than or equal to 100 mg/dL or have type 2 diabetes); high level of triglycerides (more than or equal to 150 mg/dL or treatment of previously diagnosed lipid abnormality); and reduced HDL cholesterol (for men: less than 40 mg/dL and for women: less than 50 mg/dL or specific treatment for this lipid abnormality) (<http://www.idf.org>). Moreover, insulin resistance in which body cells do not respond normally to insulin, obesity and inferior physical activity may contribute to development of metabolic syndrome. The metabolic syndrome increases the risk for Type 2 diabetes and cardiovascular diseases [5, 6].

1.1. Weight Gain and the Relationship to the Metabolic Syndrome

Obesity is recognized as a global epidemic [7] and is closely linked with an increased risk for the metabolic disease. Elevated amounts of fat in the body, particularly in the liver may result in insulin resistance and atherogenic dyslipidemia which may lead to high level of triglycerides and low level of HDL cholesterol in blood [8]. Most of the individuals diagnosed with metabolic disorder are obese (BMI of 30 kg/m² or above) or overweight (BMI of 25 kg/m² to 29.9 kg/m²) [9, 10]. The metabolic disorder was diagnosed in 4.6% of normal weight men, 59.6% of obese men and 22.4% of overweight men and also in 6.2% of normal weight women, 28.1% of overweight women and 50.0% of obese women [10]. Middle-aged men who gained weight since 20 years of their age are at higher risk of developing metabolic disease [11]. Moreover, weight gain during adulthood in women, is associated with higher risk of cardiovascular problems [12] and in general, individuals with weight gain during the last 10 to 15 years of their current age are more likely to develop metabolic disease in comparison with those with stable body weight [13].

1.2. Metabolic Syndrome and physical activity

Physical activity is body's movement which in consequence causes energy disbursement [14]. According to various studies, physical activity is closely linked with less risk of developing metabolic disease [15-19] and individuals with inferior physical activity can be at risk of developing metabolic disorder.

Frequent physical activity has a great positive impact on metabolic syndrome. It decreases body weight and fat accumulation, blood pressure and triglyceride levels. It also increases HDL cholesterol and improves sensitivity of insulin [18, 20, 21]. Rennie and colleagues [15] revealed that increasing physical activity contributes to decreased risk of developing metabolic syndrome in individuals independent of age, heavy alcohol ingestion and smoking.

1.3. Nitrate

Nitrate is a major component of our daily diet and is mostly found in green leafy vegetables. Moreover, nitrite and nitrate are used as preserving agents for meat products such as sausages, hot dogs and bacon. The proper daily intake amount of nitrate announced by the World Health Organisation is 0–3.7 mg/kg. Nitrate concentration in various food types is shown in Table 1 [22]. Nitrate was commonly believed as a harmful compound for our health until recent years. Based on the report from International Agency for Research on Cancer (IARC) in 2010, inorganic nitrate can trigger carcinogenesis. However, as described in more detail below it turns out to have various unexpected beneficial protective effects in animal models and in human. These include prevention from ischaemia reperfusion injury, lowering blood pressure, improved metabolic function and protection against gastric ulceration. According to recent epidemiological studies green leafy vegetables have shown to be protective against metabolic disease, cardiovascular problems and obesity [23, 24]. Moreover, it has been discussed recently that there is likely no link between inorganic nitrate consumption and gastric cancer [25-28].

Table 1. Nitrate concentration in various food types.

Nitrate content (mg/100 g)	Food type
< 20	Orange and banana Mushroom, onion, asparagus and pepper Processed meats such as ham, hot dogs, salami and bacon
<50	Carrot, cucumber, broccoli and cauliflower
<100	Cabbage and turnip
<250	Fennel and leek
>250	Beetroot, lettuce, spinach and celery

1.3.1. The nitrate-nitrite-NO pathway

There are two different pathways for NO formation in the body, the NOS-dependent pathway in which nitric oxide synthase (NOS) generates NO from L-arginine in the presence of oxygen molecule and the more recently discovered nitrate-nitrite-NO pathway that can be a replaced pathway for nitric oxide production particularly under oxidative stress and during tissue hypoxia [29].

In the classic pathway, NO is generated from L-arginine amino acid and NOS (nitric oxide synthases) in the presence of oxygen. NOS has three isoforms, calcium-dependent

forms which consist of endothelial nitric oxide synthases (eNOS) and neuronal nitric oxide synthases (nNOS) which generate small amounts of nitric oxide in response to elevated amounts of intracellular Ca^{2+} and a Ca^{2+} -independent nitric oxide synthase (iNOS or inducible NOS) form which is involved in immune responses and mostly localized in macrophages and neutrophils.

In 1994, Benjamin and Lundberg who were working independently found that great amounts of nitric oxide are produced intragastrically in humans [30, 31]. This NO was found to be generated non-enzymatically by the acid dependent reduction of inorganic nitrite. It was discovered that inorganic nitrate is the substrate for this NO. Nitrate is concentrated and accumulated in saliva and then it is converted to reactive nitrite by commensal bacteria residing in oral cavity [32, 33]. Then nitrite forms NO in the stomach. Some nitrite also escapes the stomach and is absorbed into bloodstream. In blood and tissues there are various pathways to reduce nitrite further to form NO. These include xanthine oxidoreductase (XOR), deoxymyoglobin, deoxyhemoglobin, mitochondrial respiratory chain enzymes and a low tissue pH [34]. The NO formed causes vasodilation and regulates blood pressure in the body and also improves mitochondrial efficiency [35-37].

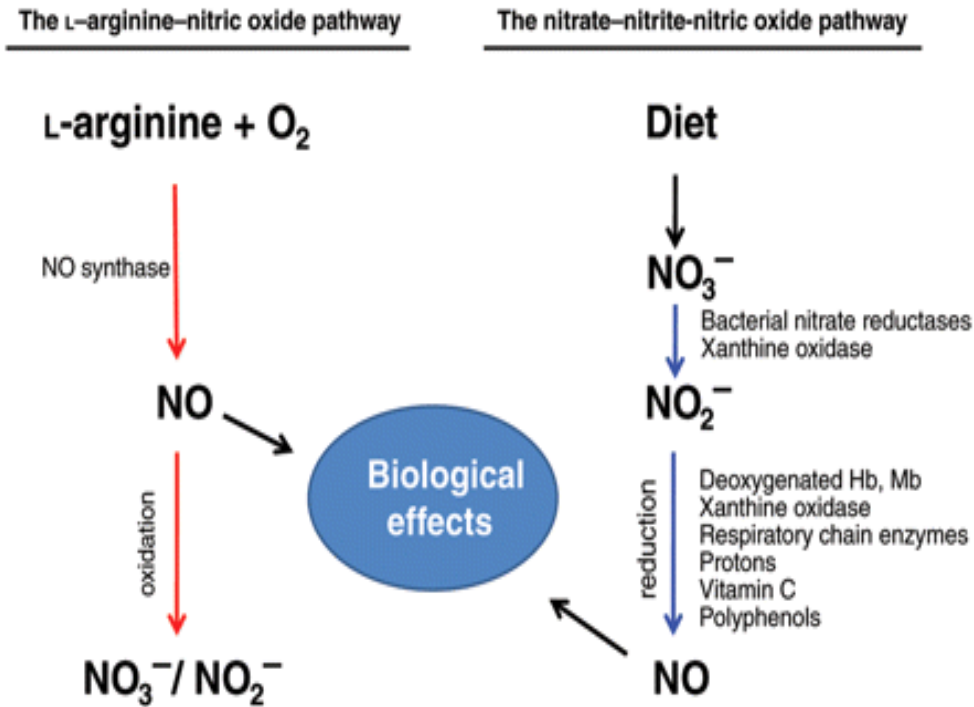


Figure 1. Comparison of two different pathways for nitric oxide formation in mammals. Image from reference [24].

1.3.2. Nitric Oxide, Mitochondria and the Relationship to the Metabolic Syndrome

In the nitrate-nitrite-NO pathway, inorganic nitrate, which can be provided either from the diet or NO oxidation in the body, is reduced to reactive nitrite through commensal bacteria residing in the mouth and to a lesser extent through XOR. Reduced NO bioavailability in the body and increased oxidative stress occur during aging and also lead to development of metabolic disorders, and hypertension.

Mitochondria are crucial players in energy metabolism and therefore any alteration or defect in their function can result in metabolic disorders [38]. Studies have shown that NO improves mitochondrial function and respiration [39]. Moreover, decreased NO generation from eNOS is claimed to be a crucial event in metabolic disorders [40]. NO is a cellular signaling molecule, it regulates a lot of physiological processes in our body

e.g by preventing aggregation of platelets and neutrophils attachment to the vessels' endothelium. It also has cytotoxic and cytostatic features which is of importance in immune regulation and host defence. Other features of NO is that it directly affects mitochondrial respiration [41] and regulates the tone of vascular smooth muscle. Studies have revealed that defects in NOS activity or overall NO bioavailability will result in insulin resistance [42]. Shankar and colleagues demonstrated such relationship between insulin sensitivity and nitric oxide in eNOS knockout mice [43]. Another study in 2007, on healthy newborns' umbilical vein endothelial cells with a genetic family background of type 2 diabetes revealed that these newborns have abnormal intracellular NO synthesis and defects in p53, GLUT1 and eNOS gene expression [44].

In recent years, scientists have studied different ways of regulating NO signaling pathways, including manipulations of the nitrate-nitrite-NO pathway (eg. with dietary interventions) [23, 24, 45]. It has been proven that supplementation of dietary nitrate can reverse some markers of metabolic disease which exist in eNOS impaired mice [46]. Recently a study showed that chronic inorganic nitrite administration in a model of obesity, through higher rates of AMP activated kinase (AMPK) phosphorylation, ameliorated some components of metabolic disorder [47]. Moreover, oxidative stress and hypertension in animal models of cardiovascular and renal disease can be decreased by chronic nitrite treatment [48, 49].

1.3.3. Nitrate and adipose tissue metabolism

Adipose tissue has a significant role in energy homoeostasis. Obesity occurs due to increased adipocyte size and accretion of white adipose tissue (WAT). WAT is a source of energy in the body which can be released through lipolysis [50]. Another component of the adipose organ is brown adipose tissue (BAT) that transfers energy from food into heat during the non-shivering thermogenesis process [51]. Thermogenesis might have anti-obesity properties since it contributes to higher rates of fatty acid catabolism and lipolysis [52]. In general, BAT has larger number of mitochondria in comparison with WAT and recently it has been discovered that dietary nitrate can transform certain

depots of WAT into BAT with increases in mitochondrial function and fatty acid oxidation in hypoxic WAT [53]. One important function of dietary nitrate in adipose tissue is likely to increase cGMP concentrations [54, 55] which in turn regulates energy metabolism through activation of differentiation of adipocyte and lipolysis [56-58]. Indeed, in 2013, cGMP was identified as a molecule that stimulates the browning response in WAT [59]. Later, it was concluded that nitrate triggers the browning response in adipose tissue by cGMP signaling and nitrate-nitrite-NO pathway. A study in 2010 highlighted that dietary inorganic nitrate can reduce body weight and dyslipidemia in eNOS deficient mouse model [46].

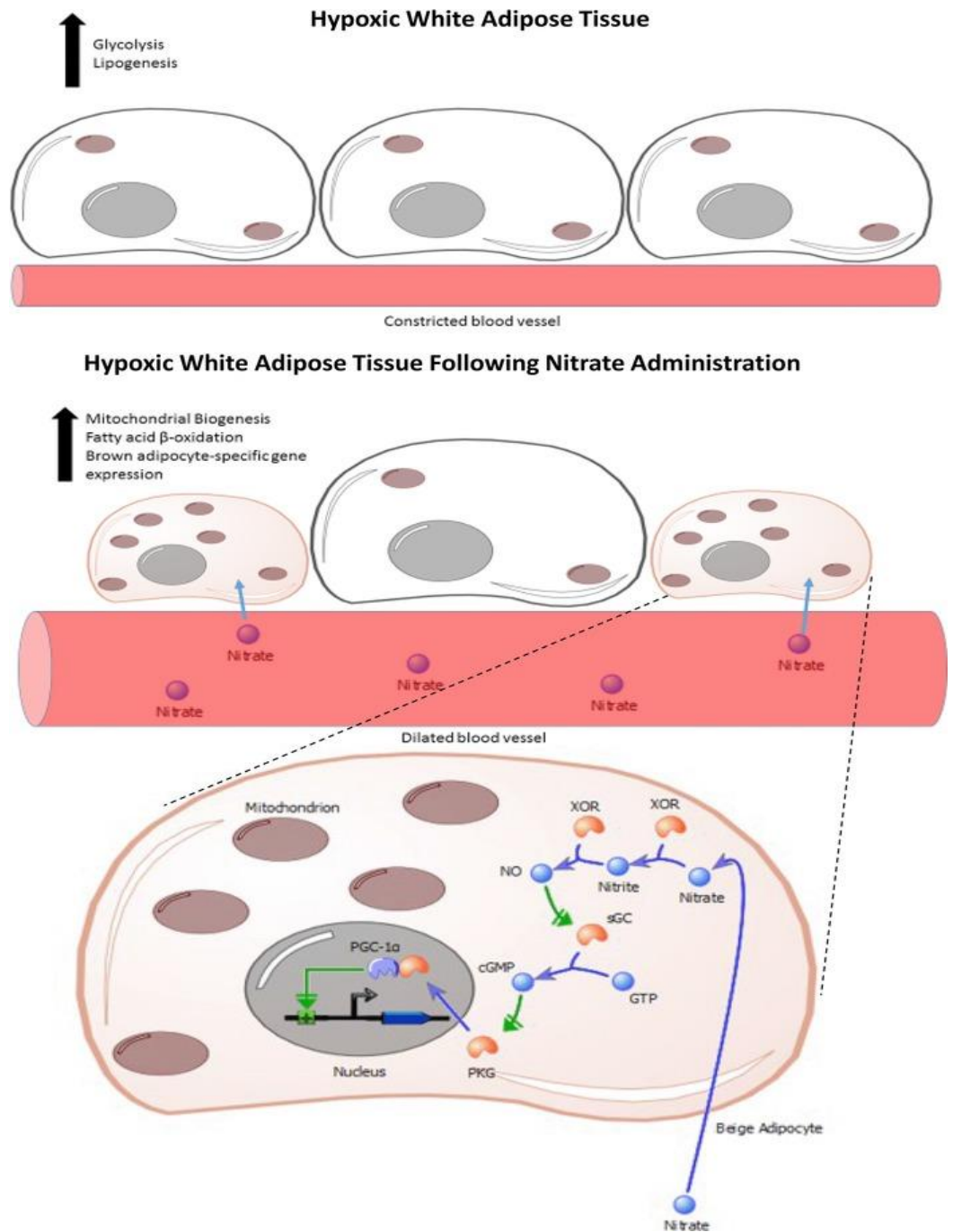


Figure 2. Impact of nitrate on WAT. Nitrate activates browning response in WAT through cGMP-PKG pathway and elevated expression of PGC-1 (transcriptional activator), PKG and protein kinase G. Image from reference [60].

1.3.4. Nitrate and hypertension

Dietary inorganic nitrate can attenuate hypertension which is a major feature of metabolic disorder and CVD [61]. Hypertension elevates the risk for cardiovascular disease several fold depending on severity [62] and according to National Diabetes Statistics Report in 2014, 71% of diabetic patients have hypertension. Decreased amounts of NO is closely linked with endothelial function impairment and consequently hypertension and CVD [63-65]. Therefore, consumption of dietary inorganic nitrate is emerging as a potential new strategy to decrease endothelial dysfunction, hypertension, CVD and metabolic syndrome by providing NO from nitrate-nitrite-NO pathway [66]. A study in 2006 demonstrated that dietary nitrate supplementation for 3 days decreases blood pressure both diastolic and systolic [67]. Another study conducted in 2011 showed that dietary inorganic nitrate improves high blood pressure in patients with peripheral artery disease [68]. Finally, a recent study investigated the effect of chronic treatment of nitrate-containing beetroot juice during a 4-week period in hypertensives and found that it sustainably reduced blood pressure and improved endothelial function [69].

The kidneys regulate blood pressure through secretion of vasoconstrictors (angiotensin II) and vasodilators (NO) [70]. Another potential way for dietary nitrate to exert its beneficial effects in hypertension is to specifically target the renal microcirculation [49].

According to a recent study, inorganic nitrate also inhibits the production of erythropoietin and consequently decreases haematocrit and blood pressure [71]. Indeed, a pre-hypertensive state and hypertension is contribute to increased haematocrit [72, 73].

1.3.5. Dietary nitrate and the heart

During hypoxia the activity of numerous mitochondrial enzymes may decrease in the heart [74, 75]. It has been speculated that dietary nitrate intake may contribute to

improvement in mitochondrial function, respiration rate and consequently can protect the heart against hypoxia related damage [76]. Moreover, nitrate may increase the concentration of L-arginine in the heart by decreasing expression of the enzyme arginase which thereby would enhance the production of NO from NO synthases. Increased arginase activity contributes to mitochondrial dysfunction and cardiac pathologies mainly due to the fact that the enzyme steals substrate (L-arginine) from NO synthases, resulting in a decreased NO bioavailability [77-80]. Hence, it is believed that dietary inorganic nitrate might be used as a therapeutic agent in heart failure [81, 82].

1.4. Adenosine receptors

It has been more than 40 years since adenosine receptors (a type of G protein-coupled receptors) were discovered. There are four types of adenosine receptors with different functions;

The A1 receptor is widely expressed in all the body specially, in the brain and excitatory nerve endings [24] and exert their effects in various organs and cells. A1 receptors regulate neuronal activity through blockage of neurotransmitter release.

The A2A receptor is present in immune cells, leukocytes, blood platelets, heart, lung and blood vessels thymus and in the brain [83, 84]. Activation of A2A receptor, alters the cyclic AMP–protein kinase A (PKA) pathway. Moreover, A2A receptors regulate inflammation, myocardial blood flow and oxygen consumption, dopamine and glutamate release in the brain and the control of cancer pathogenesis.

The A2B receptor stimulates AMPK activity and is less sensitive to adenosine compared to other adenosine receptors. Therefore, they exert their effect during hypoxia, ischaemia or inflammation in which the adenosine concentration is elevated.

The A3 receptors contribute in many physiological functions and intracellular signaling pathways. A3 receptors signaling have anti-inflammatory, anticancer and cytoprotective effects.

1.4.1. The A2B adenosine receptor and the metabolic syndrome

Adenosine receptor has been an interesting target in drug discovery and development field since 1940s since it has an important role in many diseases.

The A2b adenosine receptor controls glucose homeostasis [85] in the body and has protective effects against impaired glucose metabolism. It also enhances bone cell differentiation [86, 87] and regulates hyperlipidaemia and atherosclerosis. A study in 2014 showed that the A2b adenosine receptor deletion affected glucose and lipid metabolism in mice [88]. A2BR are expressed in most immune cells and regulate macrophage activation. Therefore, it seems the insulin resistance can be increased by their signal elevation in diabetes due to increase in production of pro-inflammatory mediators. A2BR antagonists may be beneficial to treat insulin resistance in diabetes.

1.4.2. A2B-knockout mice

One of the major metabolic organs in the body is the liver, which is the principle site of glycogenolysis, glyconeogenesis, gluconeogenesis, lipolysis and lipogenesis [89]. A study in 2002 showed that abnormal liver function is a marker of risk for T2DM, and insulin resistance [90].

The A2BKO mouse model is one of the best metabolic disease animal models and it was made by targeted deletion of the A2B receptor gene from the mouse genome which leads to abnormalities in the liver, development of metabolic disease and type 2 diabetes symptoms. A2BKO mice are characterized by high NOX activity and low AMPK phosphorylation in their liver [40]. It has been proved that activation of NOX activity is

closely linked with AMPK inhibition in colon cancer cells [91] and elevated NADPH oxidase activity in liver can reduce glucose uptake and leads to hyperglycemia [92]. Some quiet recent experiments revealed that, A2BKO mice on HFD have impaired insulin signaling with abnormal, inflamed and steatotic livers [93, 94]. Another study in 2014 showed, A2BKO mice which had been fed with normal diet had higher levels of triglyceride biosynthesis and liver gluconeogenesis [88] and finally, a study which was performed in our research group represented A2BKO mice are a great model of metabolic disease and dietary nitrate can reduce blood glucose level [40]. It is still unknown why A2BKO mice develop such symptoms but one of the potential hypothesis is that A2 receptors are linked to the NOS dependent generation of NO in different organs and cells [48, 95]. This type of mice also showed inflammation of the vascular system [96] and they usually suffer from vascular leakiness [97]. It is surprising that A2BKO mice have strong phenotype [96], although A2B receptors have low affinity for adenosine [98].

In general, it seems that A2BKO mice constitute a novel model of metabolic disease and is suitable model for testing the effect of inorganic nitrate or nitrite on metabolic syndrome.

1.5. AMPK as a metabolic regulator

One of the important metabolic regulators which has a great impact on liver glucose homeostasis is Adenosine monophosphate kinase (AMPK) [99, 100] which is recently suggested to be a novel target of the nitrate-nitrite-NO pathway [47, 101, 102]. Typically, under hyperglycemic conditions AMPK activation is decreased. Moreover, metformin and AICAR, which are two well-known anti-diabetic drugs, provoke AMPK activation [103, 104] and consequently reduce NOX activity in several cells and organs [105-108].

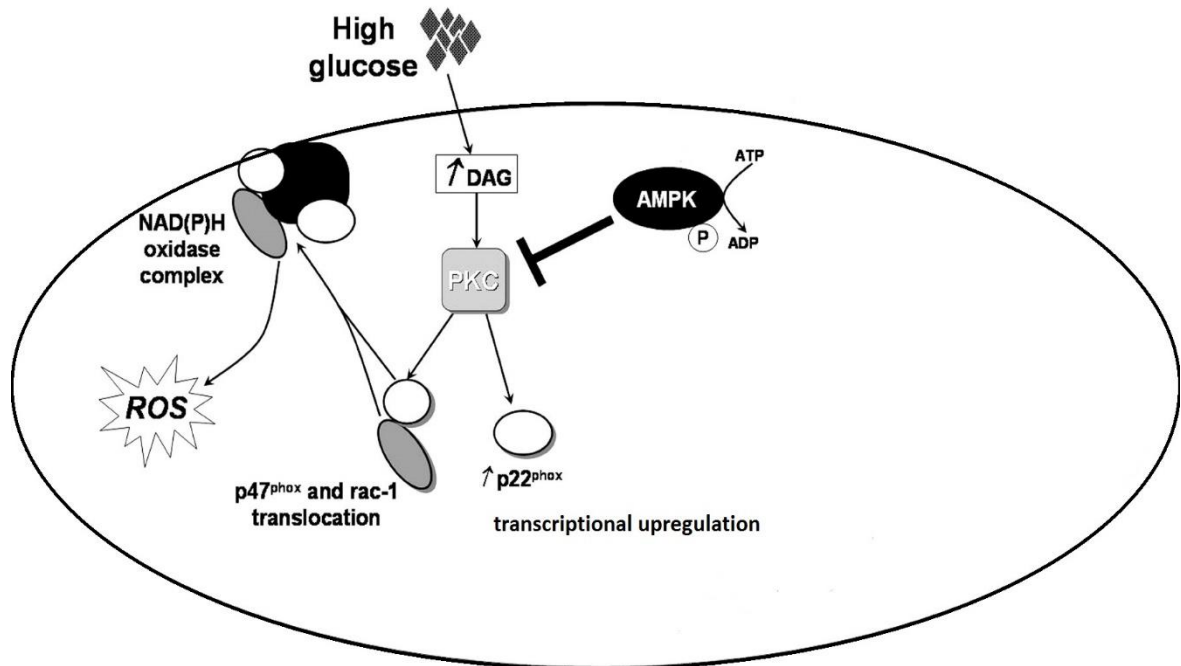


Figure 3. Post-translational modifications triggered by AMPK on PKC can reduce oxidative stress mediated by NOX (modified from [107]).

1.6. Diabetes

Diabetes mellitus is a cluster of diseases associated with high blood glucose concentration that is due to the body's dysfunction to produce and/or respond to insulin. This situation is defined by the hyper-glycaemia level that can increase the risk of retinopathy, nephropathy and neuropathy. According to the WHO classification, two types of diabetes are exist, type 1 and type 2. Diabetes is associated with poor life quality, decreased life expectancy and significant abnormality due to diabetes related complications (nephropathy, retinopathy and neuropathy) and greater risk of developing macro-vascular complications including peripheral vascular disease, stroke and ischaemic heart disease (www.who.int/diabetes). Different pathogenetic processes are associated with diabetes including, processes which destroy the pancreatic beta cells and result in insulin inferiority and those that cause resistance to insulin action. Abnormalities in the metabolism of proteins, carbohydrates and fats are come from

reduced insulin action on target organs and tissues, which is due to insensitivity or lack of insulin (Report of a WHO Consultation, 1999). Based on data from The International Diabetes Federation in 2015, 387 million people suffer from diabetes which is supposed to boost to 592 million by 2035. According to the statistics, 77% of diabetic patients are live in poor or middle income countries and in 2014, about 5 million people died from diabetes.

1.6.1. Type 1 Diabetes

Approximately 5 to 10% of all diabetic patients are diagnosed with type 1 diabetes; however. In type 1 diabetes, pancreatic beta-cells are destroyed which leads to diabetes mellitus and insulin administration is necessary to prevent the progression of ketoacidosis, coma and death. In order to manage type 1 diabetes several actions must be taken including, proper insulin administration, blood glucose monitoring, following healthy diet and regular screening for diabetes-related complications such as micro-vascular and macro-vascular disease, which is a main morbidity and mortality factor of type 1 diabetes [109]. Family history is one of the significant risk factors for type 1 diabetes. Moreover, any injury or diseases or infections in the pancreas can damage the pancreas and consequently lead to development of diabetes due to its inability to produce insulin [110].

1.6.2. Diabetes in Pregnancy (Gestational Diabetes)

In general, during pregnancy the body becomes more resistance to insulin in order to provide more glucose for the fetus and therefore, pancreatic beta cells should secrete more insulin. Gestational diabetes is a type of diabetes which emerges in some pregnant women for the first time due to inability of beta cells in the pancrease to provide more insulin for this demand. Overweight and obese women and those women who have a strong family background in diabetes are at the higher risk for gestational diabetes

during their pregnancy. Gestational diabetes can harm the health of both the mother and the baby and they may develop T2DM in future [111] therefore, it is important to diagnose and treat it as soon as possible.

1.6.3. Type 2 Diabetes

Type 2 diabetes is an important progressive metabolic disease which consists of multiple defects including, insulin resistance, insulin secretion defects, excess glucagon secretion and/ or GLP-1 (Glucagon-Like Peptide-1) deficiency and possible resistance. Insulin resistance in liver increases basal hepatic glucose production which result in fasting hyperglycemia, muscle insulin resistance causes postprandial glucose disposal (80% of glucose uptake) in muscles. Mitochondrial dysfunction may play a role in muscle insulin resistance. Moreover, adipocyte insulin resistance may result in elevated FFA level after glucose ingestion that can cause impair insulin secretion and insulin resistance in liver and muscle. Insulin secretion defects can be observed due to severe beta-cell dysfunction in the pancreas which can become worse with insulin resistance. ER stress, transcription factor expression and C/EBP β expression in beta-cells in the pancreas are associated with beta-cell failure [112]. Moreover, Islet amyloidosis is responsible for progressive loss of beta-cells [113]. Elevated plasma glucagon concentration is another associated factor in T2DM which contributes to increased rate of hepatic glucose production in T2DM patients. GLP-1 receptor agonists reduce plasma glucagon, enhance insulin secretion, slow gastric emptying and decrease food consumption by increasing satiety and therefore, GLP-1 deficiency may lead to T2DM [114].

There are plenty of complications associated with T2DM which are as follows, cardiovascular disease, diabetic microangiopathy, diabetic neuropathy (nerve damage, skin ulcers, open wounds), diabetic retinopathy (blindness, macular oedema), diabetic nephropathy (kidney failure), diabetic ketoacidosis, diabetic gastroparesis (delayed gastric emptying) and diabetic ulceration (foot injury, amputation).

Type 2 diabetes is highly depends on our lifestyle and genetic risk factors. 85–90% of all diabetic patients are diagnosed with T2DM and it usually occurs in middle-aged or older adults who are over 45. However, younger age groups including adolescents, young adults and even children, increasingly develop T2DM in recent years. Most of the T2DM patients do not have any symptom at first stages of the disease. Early symptoms of T2DM are fatigue, excess thirst and urination, hunger, blurred vision and bladder, kidney, skin, or other inflammation or infections that takes time to heal.

T2DM can be controlled by a combination of healthy diet, sufficient physical activity and weight loss. Following unhealthy diet, obesity, inadequate physical activity, aging, family background of diabetes and genetic factors are type 2 diabetes' risk factors.

As type 2 diabetes is a progressive syndrome, lifestyle changing will not be enough and therefore, patients will need oral anti-diabetic medications and/or insulin injections over time.

1.6.3.1. Genetics of T2DM

Several genes are associated with T2DM including, TCF7L2 (associated with decreased response to GLP), PPAR γ (decreases insulin sensitivity and increases T2DM risk), CAPN10 (encodes an intracellular Ca²⁺-dependent cysteine protease and affects on insulin secretion), HNF4A (encodes hepatocyte nuclear factor 4 alpha receptor), adenosine receptor genes particularly, ADORA2B gene (encodes an adenosine receptor and is located on chromosome 17, close to the Smith-Magenis syndrome region) and Potassium channel genes which consist of ABCC8 and KCNJ11. ABCC8 encodes SUR1 subunit which is connected to the Kir6.2 subunit (encoded by KCNJ11) [115-117].

1.6.3.2. Diagnostic criteria for T2DM

T2DM may be diagnosed through diabetes symptoms such as polyuria and polydipsia or/and blood glucose concentration of 200 mg/dL and higher or fasting blood glucose level of 126 mg/dL and higher or HbA1c test which should be equal or greater than 6.5% to indicate diabetes. If the individuals have no diabetes symptoms, a single blood glucose measurement is not enough and therefore, confirmatory blood glucose measurement is required.

1.6.3.3. Pharmacological treatment of T2DM

Pharmacological products to treat T2DM are anti-hyperglycemic and they focus on decreasing blood glucose levels and glucose absorption in body, increase insulin secretion and organ sensitivity. Diabetic pharmacological agent are as follows, Biguanides (metformin), Insulin, Amylinomimetics, Thiazolidinediones, Sulfonylureas, Meglitinides, DPP-4 inhibitors, α -glucosidase inhibitors and GLP-1 receptor agonists. The first-line treatments for T2DM consists of metformin [118]. Moreover, exercise and various hypoglycemic agents can decrease oxidative stress in diabetic patients by improving mitochondrial function and decreasing NOX activity [119]. Furthermore, it has been proved that some medications including ACE inhibitors , AT1-receptor blockers and statins, improve vascular function in cardiovascular disease patients due to their ability to stimulate nitric oxide production [120].

1.6.3.3.1. Metformin

Metformin is an oral anti-hyperglycemic agent which reduces blood glucose level through suppressing hepatic glucose production, it also enhances insulin sensitivity and reduces HbA1c levels in NIDDM. However, it has some adverse reactions including, abdominal discomfort, stomach upset, diarrhea and lactic acidosis which can result in

death. The side effects may reduce by consumption of smaller divided doses. Prescription of metformin should be avoided for those with acute heart failure, severe renal function defect, liver disease and heavy alcohol ingestion and also it should not be used after application of iodinated contrast dyes because of the lactic acidosis risk. Use of metformin may induces weight loss in individuals and it does not lead to hypoglycemia itself but it accelerates the hypoglycemic impact of other medications such as insulin and sulfonylureas. Metformin can trigger AMPK phosphorylation by promoting AMP aggregation which consequently leads to increasing the activity of insulin receptor and insulin receptor substrate (IRS-2) and also enhancing translocation of glucose transporters. Phosphorylated AMPK inhibits the synthesis of fatty acids and enhances beta oxidation. Metformin possibly enhances insulin secretion and also insulin sensitivity by lowering free fatty acids and glucose concentrations and by preventing lipid storage in insulin sensitive tissues.

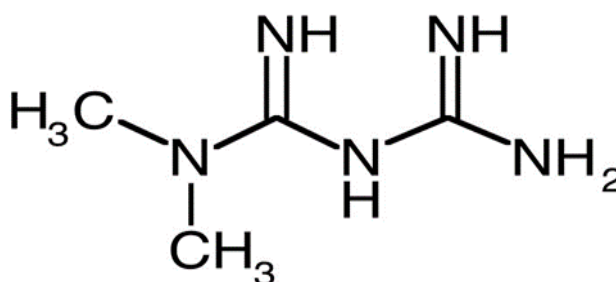


Figure 4. Metformin structure

1.6.3.3.1.1. Lactic acidosis

Lactic acidosis is a kind of metabolic acidosis and it lowers the pH of the body. It is a medical condition in which the lactate level, particularly L-lactate, increases in the body. MALA (Metformin-associated lactic acidosis) is an infrequent but fatal adverse effect of metformin therapy and it has up to 50% mortality. However, the metformin's

independent impact on lactic acidosis development still remains unclear. As it was mentioned, prescription of metformin should be avoided for those with acute heart failure, liver disease, renal function defect and high alcohol ingestion.

Normally, glucose is metabolized by most cells in the body to form water and carbon dioxide. Cells produce ATP by glycolysis in which glucose breaks down to pyruvate and then the pyruvate is oxidized into water and carbon dioxide through oxidative phosphorylation and Krebs cycle by mitochondria. The hydrolysis of ATP results in energy generation for the cells, hydrogen ions and Pi (inorganic phosphate). Normally, the mitochondria use these free hydrogen ions and Pi to produce more ATP, but during cellular hypoxia, ATP hydrolysis results in collection of free hydrogen ions and Pi in the cytosol and also the rate of glycolysis increases in the cells. Excess produced pyruvate is converted into lactate which causes lactic acidosis and decreases the blood pH. Metformin promotes anaerobic metabolism by decreasing the reducing agents transportation and pyruvate dehydrogenase activity in mitochondria. The anaerobic metabolism leads to elevated lactic acid generation due to boosted conversion of pyruvate to lactate.

1.6.3.3.2. Insulin

Insulin hormone is another treatment for both types of diabetes mellitus patients which treats diabetes by tight glycaemic control. As a medication, insulin can be derived from pancreases of animals such as beef and pork or is genetically made by genetically modified bacteria to be able to be replaced with native human insulin. Insulin can be injected subcutaneously or intravenously and the dose is adjusted according to the patient's weight and sensitivity to insulin. Individuals who were diagnosed with type 2 diabetes mellitus may also need to get insulin therapy when other anti-diabetic medications cannot manage their blood glucose levels. There are four injectable insulin available for diabetic patients including, rapid acting insulin in which the effect will be seen in a few minutes and lasts for some hours. Short acting insulin or regular insulin in which the effect will be seen after couple of minutes and lasts up to 6 hours. Another kind is intermediate acting insulin in which the effect will be seen after about 3 hours

and last till 18 hours and finally, long acting insulin which has about 8 hours delay to start to work but the effect remains for an entire day. The most common side effect of insulin is hypoglycemia and its possible symptoms consists of headache, weakness, sweating, fast heartbeat, extreme hunger, fatigue, intense anxiety, general sense of confusion, tremors, irritability, rapid breathing and fainting. Moreover, other side effects such as enlargement of insulin injection site (hypertrophy) and rash in the body can be seen in insulin treated patients. Diabetic ketoacidosis (DKA) is an insulin complication, which can occur due to inadequate insulin administration and can lead to diabetic coma or death. In DKA, excessive urination in response to high glucose causes severe dehydration and the body doesn't have enough insulin to use glucose. Therefore, the body starts to breakdown of existing protein and fat stores in order to provide energy, which produces ketones and release in into the blood and ketones affect the blood acidic and cause DKA. Symptoms of diabetic ketoacidosis are nausea, vomiting, and abdominal discomfort. Hyperosmolar hyperglycemic nonketotic syndrome (HHNS) is another insulin treatment complication which is less occur than DKA. In HHNS, frequent urination due to high glucose levels can cause extreme dehydration and consequently leads to diabetic coma and death in older or obese individuals with type 2 diabetes [121].

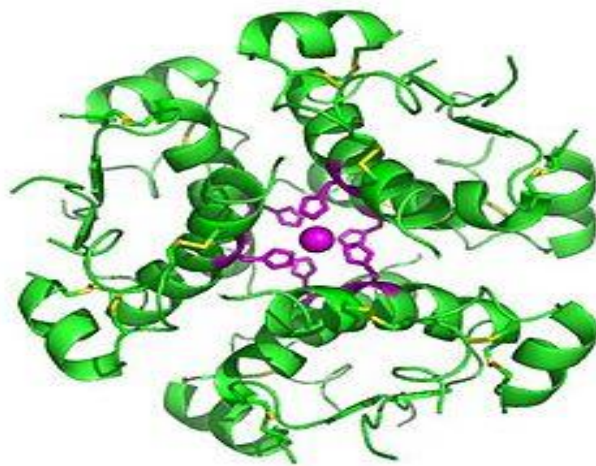


Figure 5. Insulin 3D structure

1.6.3.3.3. Amylinomimetic

Amylinomimetic is a synthetic analog of amylin, which is a neuro-hormone secreted simultaneously with insulin from beta cells in the pancreas and is always used with insulin in diabetic patients but the injections are given separately. It suppresses post-prandial glucagon secretion, increases satiety, and slows gastric emptying without altering nutrient absorption. Amylinomimetic's main adverse effects are nausea, anorexia and vomiting.

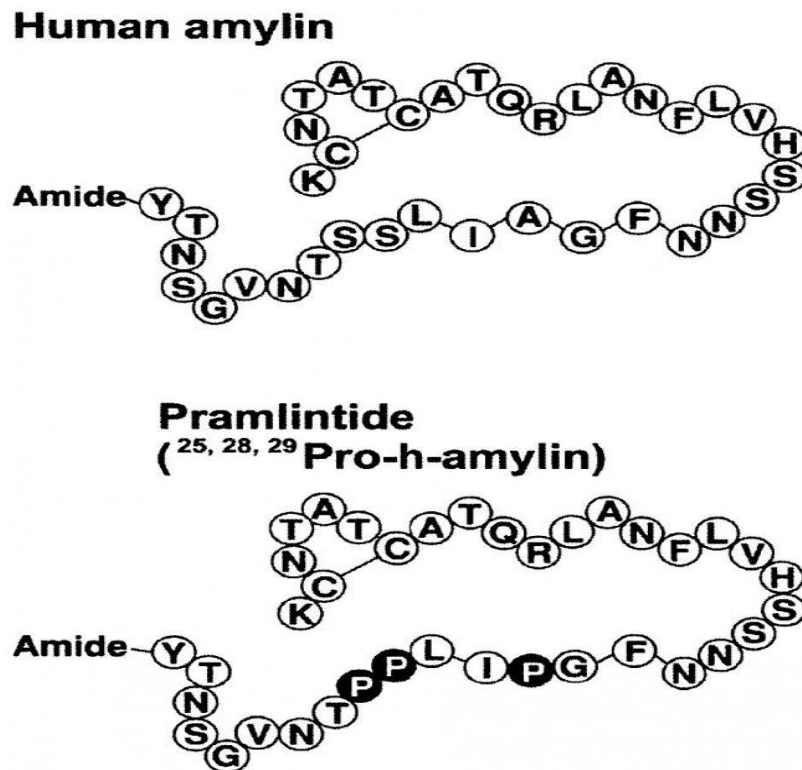


Figure 6. Human amylin and Pramlintide structure

1.6.3.3.4. Thiazolidinediones

Thiazolidinediones such as pioglitazone and rosiglitazone are another possible treatment for T2DM. They bind to the PPAR- γ receptor which regulates gene

expression and increase insulin sensitivity by reducing the amount of free fatty acids which are present in body circulation. However, TZDs can cause fluid retention, weight gain, heart failure and myocardial Infarction.

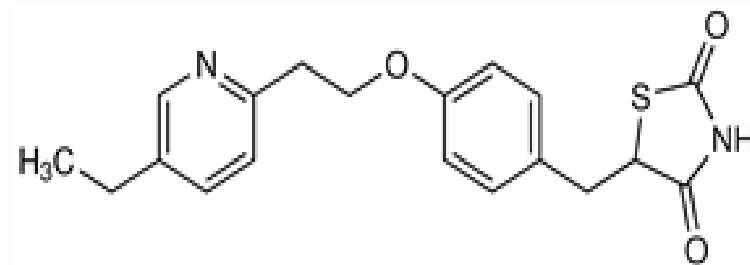


Figure 7. Pioglitazone structure

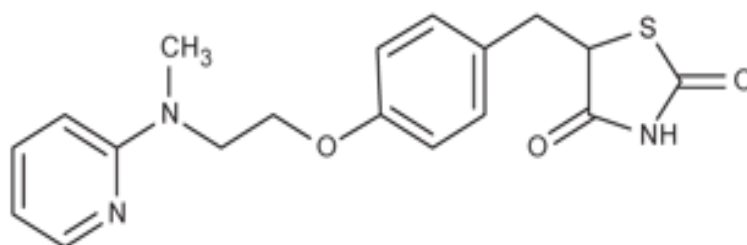


Figure 8. Rosiglitazone structure

1.6.3.3.5. Sulfonylurea

Sulfonylurea (Gliclazide) is an anti-diabetic drug which is broadly consumed in treatment of T2DM. It binds to sulfonylurea receptors (SUR) on pancreatic β -cells and stimulates insulin secretion by closing the potassium channel which is dependent to ATP (KATP) which depolarize the membrane of cells and cause opening of calcium channels. SUR's main adverse effect is hypoglycemia.

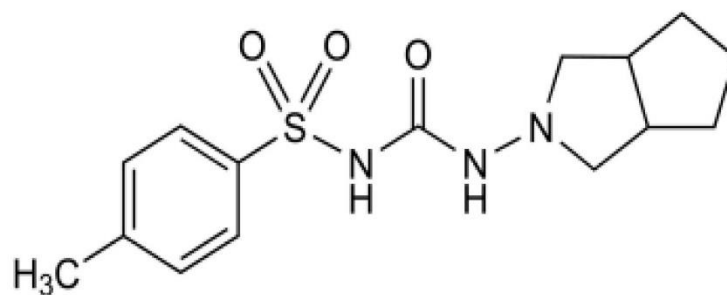


Figure 9. Gliclazide structure

1.6.3.3.6. Megletinides

Another therapy option for T2DM is Megletinides which consists of nateglenide and rapeglenide. These drug's binding site is close to the sulfonylureas' binding site and they stimulate insulin secretion. Megletinides' main side effects are GI side effects.

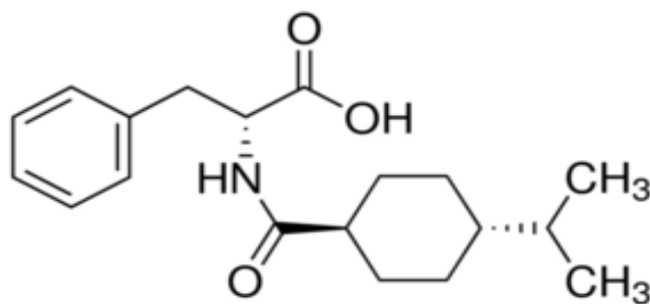


Figure 10. Nateglenide structure

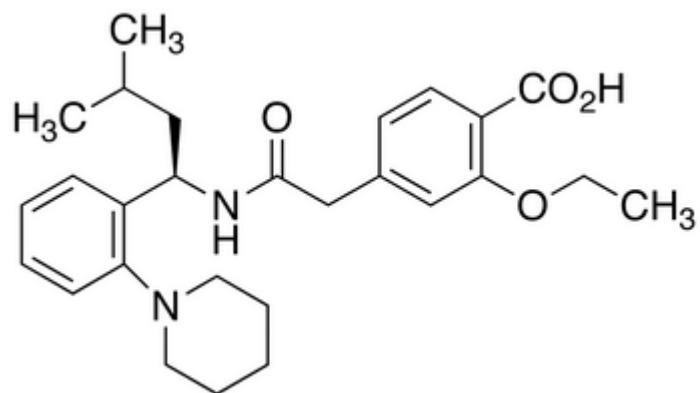


Figure 11. Rabeprazole structure

1.6.3.3.7. α -Glucosidase Inhibitors

α -Glucosidase Inhibitors act in the small intestine and prevent breakdown of sucrose and complex carbohydrates. The common side effects are flatulence, bloating, abdominal discomfort and diarrhea.

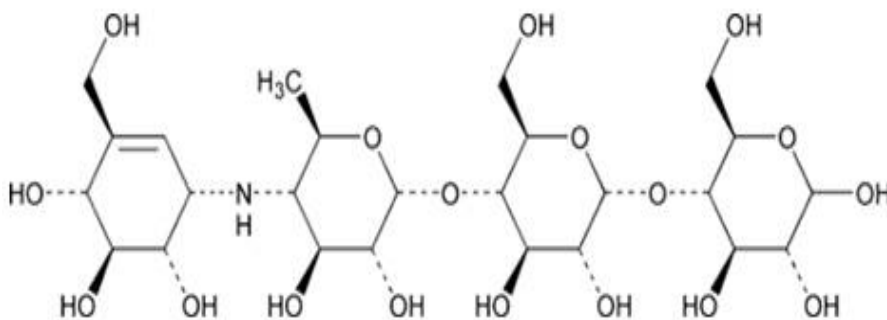


Figure 12. Acarbose structure

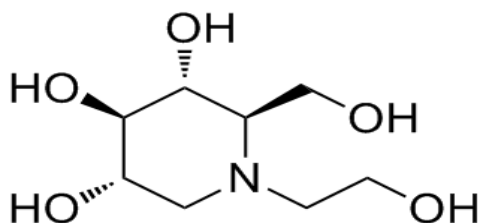


Figure 13. Miglitol structure

1.6.3.3.8. Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

Another class of T2DM drugs which is called dipeptidyl Peptidase-4 (DPP-4) Inhibitors. They partially decrease the elevated glucagon postprandially and stimulate insulin secretion. Urticaria and facial edema are the rare SARs of dipeptidyl Peptidase-4 (DPP-4) Inhibitors. Different types of DPP-4 Inhibitors are shown in figures 14-17.

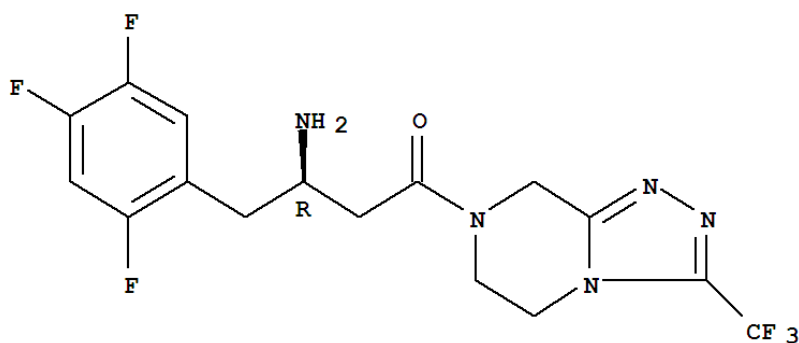


Figure 14. Sitagliptin structure

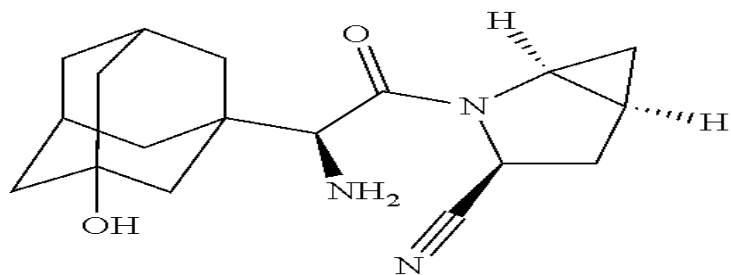


Figure 15. Saxagliptin structure

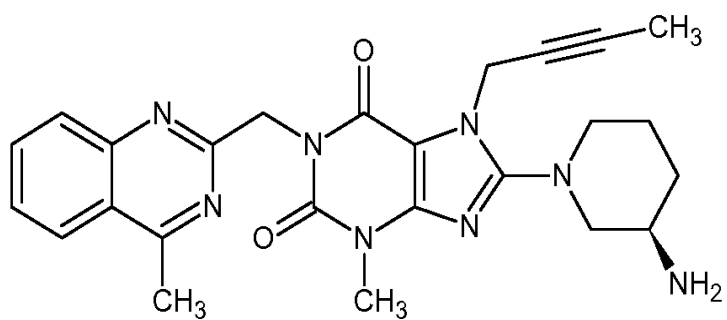


Figure 16. Linagliptin structure

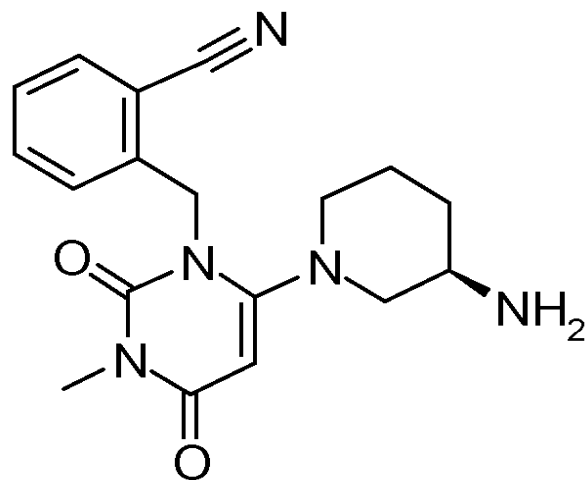


Figure 17. Alogliptin structure

1.6.3.3.9. GLP-1 receptor agonists

GLP-1 agonists including, Liraglutide and Exenatide are another possible treatment for T2DM. Exenatide is the synthetic analog of exendin, and it is more resistant to DPP-4. They suppress postprandial glucagon secretion, stimulate insulin secretion, reduce postprandial glucose, increases satiety, slow gastric emptying and promotes weight loss. The GLP-1 agonists' main adverse effects are nausea, vomiting, diarrhea.

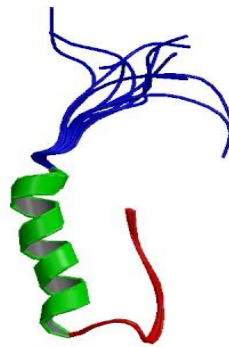


Figure 18. Exenatide 3D structure



Figure 19. Liraglutide 3D structure

2. Aims

Study I: Nitrate improves mitochondrial function which makes it a potential component to prevent lactic acidosis that produced due to elevated lactate production in mitochondria. The aim of proposed study was to determine whether acute pre-treatment of nitrate can prevent lactic acidosis induced by a high dose of metformin.

Study II: The aim of study II was to explore the synergistic effect of combining low dose of metformin and inorganic nitrate to improve metabolic functions and decrease blood glucose concentration in a mouse model of metabolic disease (A2BKO mice).

3. Materials and methods

3.1. Experimental animals

Study I: 3-month male Wistar rats (Average 350 g of body weight) were purchased from Charles River Laboratory in Germany. There were 6 rats per group.

Study II: 18 aged (Average 14 month, 45 g of body weight) A2BKO mice per each treatment group were used. The mice were 11 times back crossed to a C57BL/6J background and adenosine A2B receptor gene was deleted in them. Moreover, 6 aged (Average 12 month, 30 g of body weight) wild type mice from heterozygous breeding pairs were used in order to demonstrate the phenotype in the A2BKO mice. For all experiments both sexes were used.

All the animals were housed based on standard temperature (21-22°C), humidity and illumination (12 hours light/darkness). Animals were kept in their new cage environment at least one week before the initiation of the experiments and adequate amounts of food and water were provided for them in order to adjust to new

environment. All the experiments were authorized by the Stockholm's Ethics Committees for animal experiments (Stockholms Norra Djurförsöksetiska Nämnd).

3.2. Chemicals

Metformin (1, 1-Dimethylbiguanide hydrochloride) and sodium nitrate (NaNO_3) was provided from Sigma Chemical. The lactate was detected by Cobas b 123 Sensor Cartridge BG/ISE/GLU/LAC (Roche Diagnostics GmbH).

3.3. Experimental protocols

Study I: Rats were anesthetized individually with isoflurane and we catheterized their femoral vein with polyethylene tubing for metformin infusion. The animals were pre-treated with intraperitoneal injection of placebo (NaCl , 0.9%) or inorganic nitrate (NaNO_3 ; 10 mg/kg body weight). Metformin infusion to femoral vein was performed by syringe pump. Dissolved metformin in saline was administered to the animals for 2 h. The constant metformin infusion dose was 125 mg/h/kg and the administration rate was 8.0 ml/h/kg. The tail blood was sampled at 0, 30, 60, 90 and 120 minutes during the infusion to determine blood lactate level.

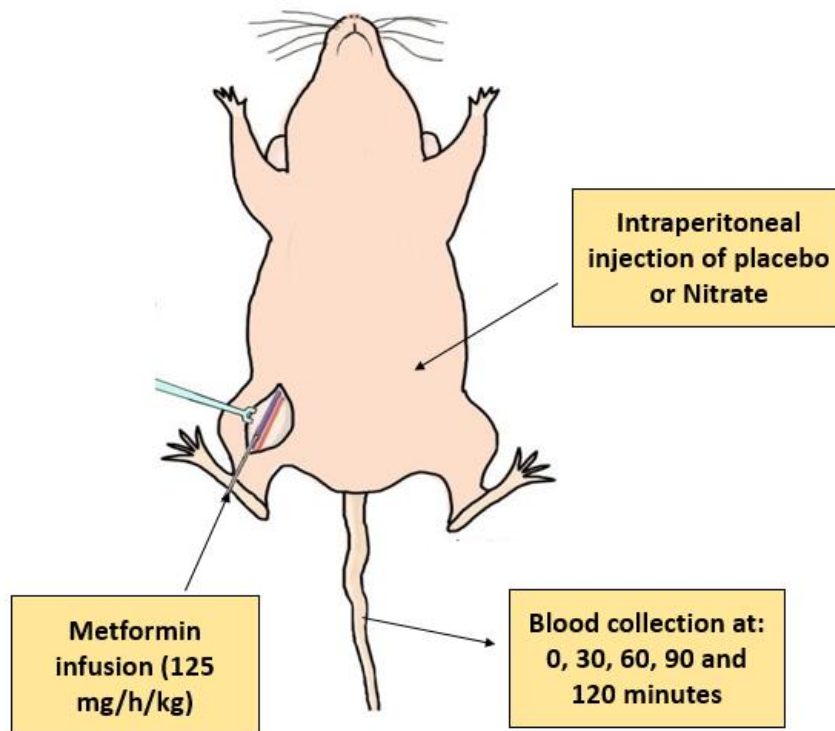


Figure 20. Metformin infusion into rat's femoral vein (modified from [122]).

Study II: A2BKO mice were pre-treated with acute intraperitoneal injection of placebo (NaCl 0.9%) or the anti-diabetic drug metformin ($C_4H_{11}N_5$; 3.8 mg/kg body weight) or combination of inorganic nitrate and metformin ($NaNO_3$; 10 mg/kg body weight, $C_4H_{11}N_5$; 3.8 mg/kg body weight), 60 minutes before IP-GTT. After 1 hour intraperitoneal injection of D-glucose was performed (2 g/kg body weight) which was followed by tail blood collection at -60, 0, 15, 30, 60, and 120 min. The mice were fasted for 5 hours (no food but free access to water) before the initiation of the experiments and separated in single cages 1 hour prior the experiment. Finally, blood glucose level was determined by a glucose meter (Free Style Lite, Abbot Diabetes CareInc, CA).

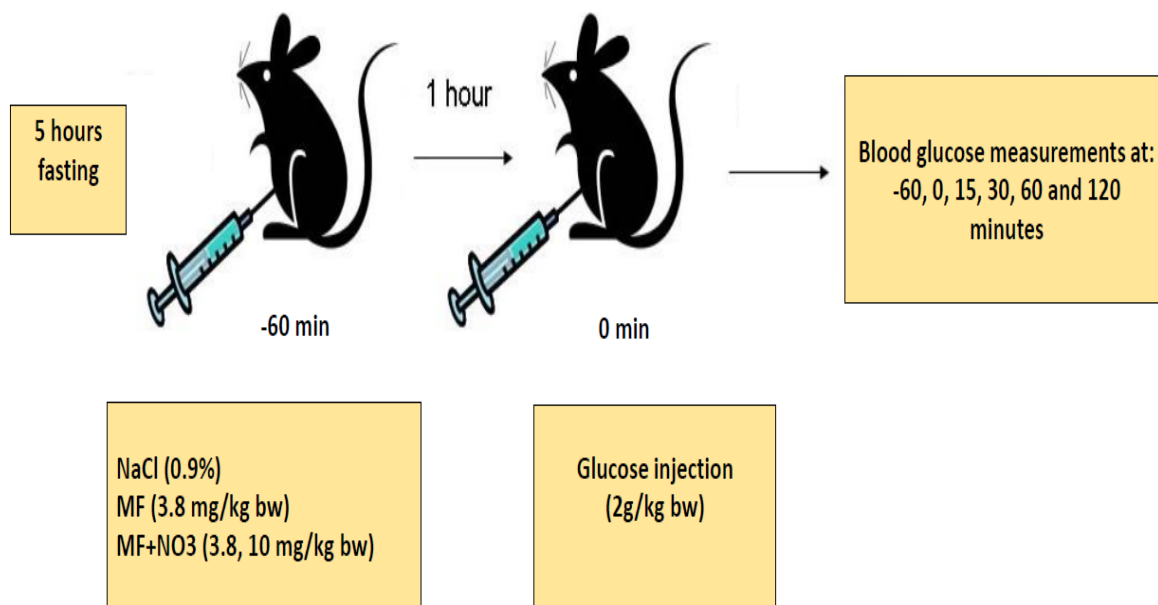


Figure 21. Intra-peritoneal glucose tolerance test (IPGTT) protocol in mice.

3.4. Plasma nitrate and nitrite

Plasma levels of nitrate and nitrite were determined by HPLC (ENO-20 NO_x analyzer). The samples of plasma were centrifuged at 4°C 10000 g for 10 minutes after their extraction with methanol (1:2). Reversed-phase ion-pairing chromatography was used to separate nitrate and nitrite which was followed by conversion of nitrate to nitrite using reduced copper and cadmium. In order to form diazo compounds, nitrite was derivatized by Griess reagent and detected at 540 nm.

3.5. Statistical Analysis

Data are revealed as means \pm SEM. Unpaired or paired t-test were applied in order to perform single comparisons between normally distributed data and one-Way ANOVA coupled with Bonferroni's post-hoc test was performed to statistically compare different treatments groups to the control group and to be able to do more than one comparison with the same variable (GraphPad Prism 6 Software). Statistical significance threshold was $p < 0.05$.

4. Results and Discussion

Study I: In study I, we investigated whether inorganic nitrate could prevent lactic acidosis caused by acute administration of metformin in rats. The two potential mechanisms for development of lactic acidosis, induced by metformin, are as follows: 1) Metformin augments the lactate production in peripheral tissue [123]; and 2) Inhibition of mitochondrial respiration by metformin consequently can inhibit lactate transport, metabolism and clearance in the liver, heart and muscle. As NO directly affects mitochondrial respiration [41], we hypothesised that inorganic nitrate which consequently converts to NO, may prevent lactic acidosis by counteracting the mitochondrial effects of metformin. Therefore, lactic acidosis was induced by constant intravenous infusion of metformin at the dose of 125 mg/h/kg in rats. The blood lactate concentration at different time points is shown in Figure 22. The lactate concentration in blood started to increase significantly after 30 minutes of metformin infusion and continued to increase until 120 minutes after which the animals were killed. As shown in figure 22, there was no significant difference between the placebo pre-treatment group and the nitrate pre-treated group (P value 0.24).

Therefore, we conclude from this study that pre-treatment with inorganic nitrate cannot prevent lactic acidosis induced by acute administration of metformin in rats.

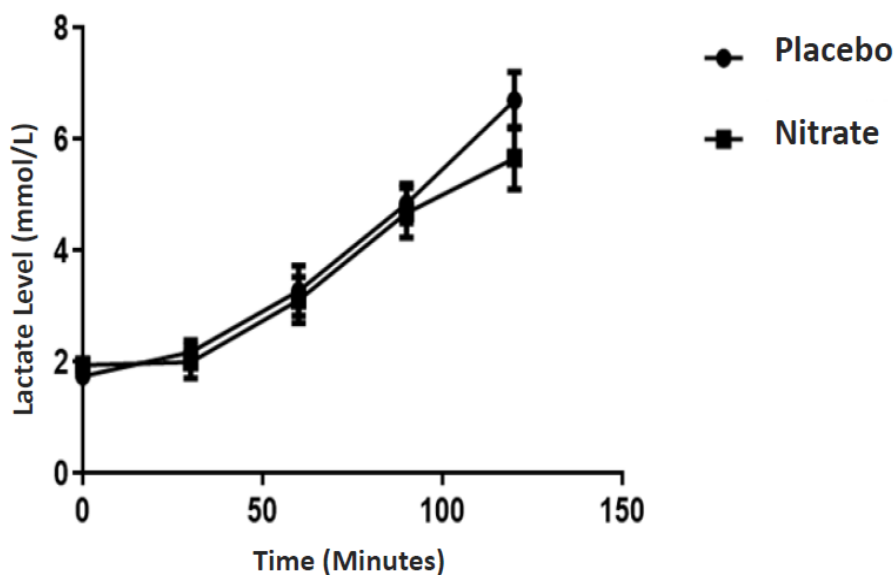


Figure 22. Blood lactate concentration at different time points in Wistar rats pre-treated with placebo (NaCl, n=6) or inorganic nitrate (NaNO₃, n = 6) and then infused with metformin to cause lactic acid acidosis.

Study II: As inorganic nitrate targets multiple aspects of the metabolic disease, the main aim of study II was to explore whether nitrate in combination with the anti-diabetic drug metformin, has a synergistic effect and can decrease blood glucose levels more. This combination, if successful, may be beneficial to control progression of type 2 diabetes by enabling lowering of the required dose of metformin (less risk of side effects) with preserved effects.

In agreement with our previous findings, as shown in figure 23, A2BKO mice which is a solid animal model of metabolic syndrome with hyperglycemia, poor glucose clearance, obesity and hepatic inflammation, displayed impaired glucose clearance compared with age-matched Wild type controls [85]. In our previous study, inorganic nitrate was able to decrease blood glucose level by targeting NOX activity and AMPK activation in the liver of aged A2BKO mice [40]. It increases phosphorylation levels of

AMPK and decreases NOX activity which are important regulators of glucose homeostasis and clearance in the liver [99, 100].

According to figure 24, intraperitoneal administration of a low dose of metformin was associated with improved glucose clearance at 30 min in A2BKO mice compared to control group with the P value of <0.05 , whereas the overall glucose clearance (Total area under the curve) was not significantly affected ($p=0.06$)

Combination of metformin and inorganic nitrate was associated with improved glucose clearance at 15, 30 and 60 minutes after injection of glucose in A2BKO mice compared to the control group.

Comparing the low dose of metformin with the combination of nitrate and metformin, it was noted that in the combination group glucose disposal was numerically better at 15, 30 and 60 minutes after injection of glucose, and the total AUC trended to be reduced. The reason for the lack of a clear synergistic effect can only be speculated upon but may be due to the fact that the tested compounds act through the same mechanism in targeting NOX activity and AMPK phosphorylation. Moreover, a glucose tolerance test is not always the most affected parameter in metformin treatment particularly at low doses and therefore other parameters such as insulin tolerance test, NOX and AMPK activity in tissues would be required.

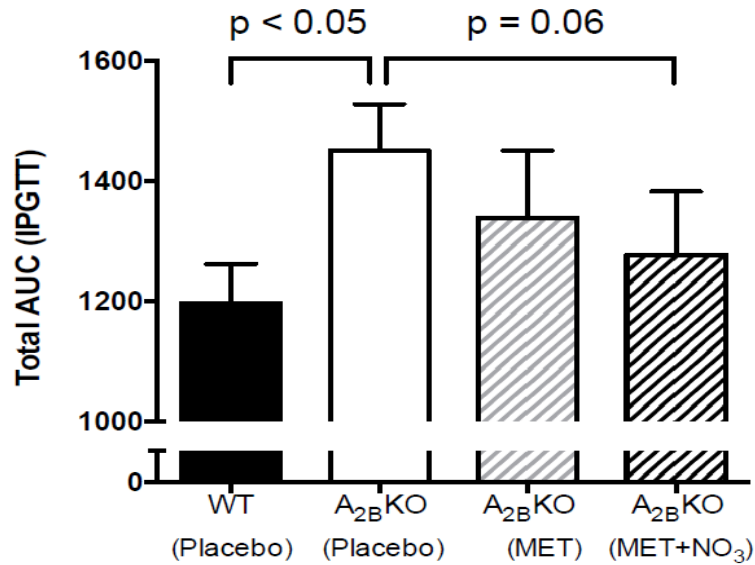


Figure 23. Intraperitoneal glucose tolerance test (IPGTT) in wildtype (WT, n=6) and adenosine A_{2B} receptor knockout mice (A_{2B}KO, n=18), and effects of acute treatment with metformin (MET, n=18) alone or in combination with inorganic nitrate (NO₃, n=18).

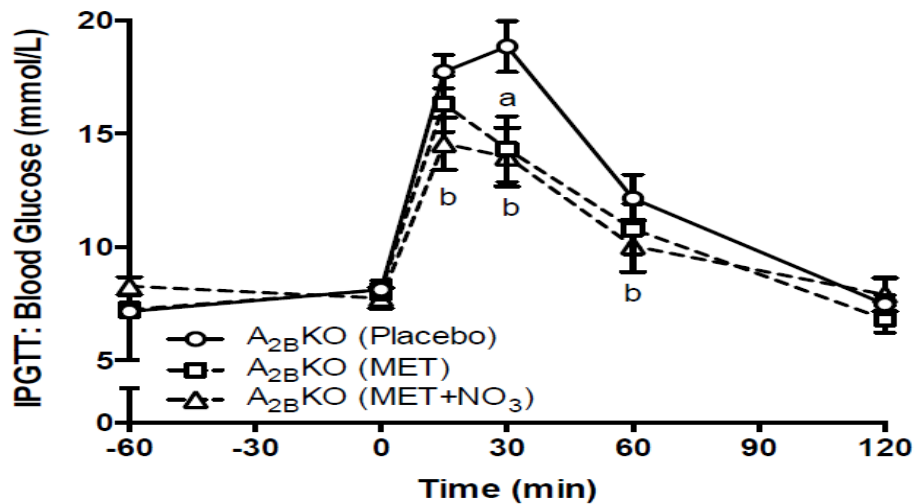


Figure 24. The effect of combination of metformin and inorganic nitrate on plasma glucose levels in A_{2B}KO mice after placebo (NaCl, n=18), metformin (MET, n=18) or combination of metformin and inorganic nitrate injection (MET + NO₃, n=18).

Nitrate and nitrite in plasma

As expected, both plasma nitrate (Figure 25) and nitrite (Figure 26) levels in A2B $-/-$ mice which were pre-treated by combination of metformin and nitrate increased significantly in comparison with saline and metformin pre-treated mice. ($p < 0.05$)

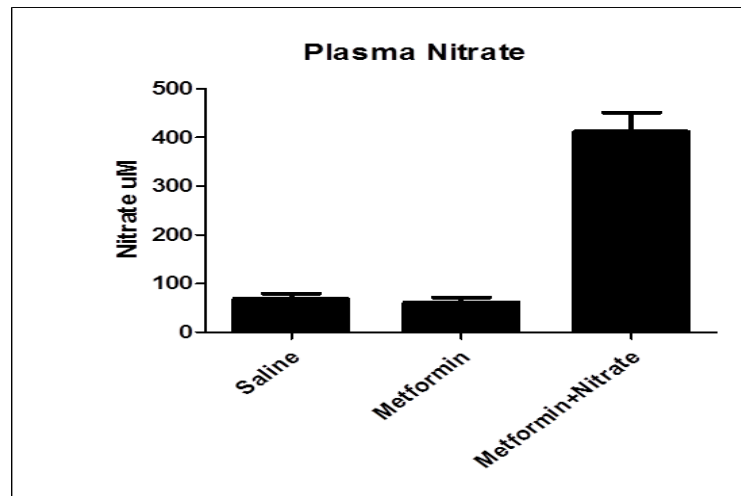


Figure 25. Nitrate levels in plasma samples of different pre-treated groups of A2BKO mice ($n = 6/\text{group}$).

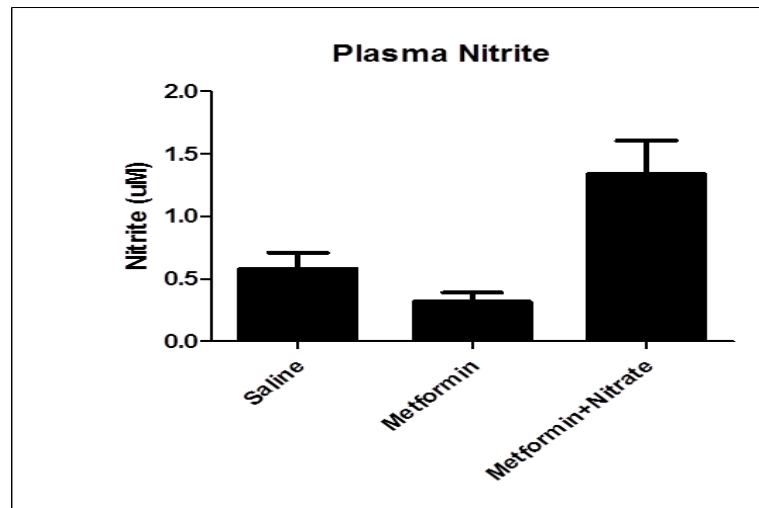


Figure 26. Nitrite levels in plasma samples of different pre-treated groups of A2BKO mice ($n = 6/\text{group}$).

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