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I PART

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CORSO DI DOTTORATO XXIX CICLO

Role of incretin hormones and DPP-IV inhibitors on the control of vascular homeostasis in physiological conditions and their involvement in vascular diseases

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UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

CORSO DI DOTTORATO XXIX CICLO

First part of my PhD project

Role of incretin hormones and inhibitors of DPP-IV on the control of vascular homeostasis in physiological conditions and their involvement in vascular diseases

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Abbreviations*:*

eNOS : endothelial Nitric Oxide Synthase

NO: Nitric Oxide

Cav-1: Caveolin-1

cGMP: cyclic Guanosine Monophosphate

GLP-1: Glucagon-like peptide-1

DPP-IV: Dipeptidyl Peptidase IV

NOD mice: Non Obese Diabetic mice

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

1. INTRODUCTION

The intracellular storage of nutrients and their subsequent release is an important physiological mechanism through which the organism obtains energy in the absence of a continuous feeding. Multiple hormones are involved in controlling the uptake, utilization, storage, and release of nutrients. In particular, glucose, a small, polar and, thus, water-soluble monosaccharide, is an important nutrient which exerts a critical role in the physiological functioning since it represents the mainly energetic source for all cell's biosynthetic reactions. Blood glucose is easily measured and provides an accurate guide to the balance of *insulin* and the *counter-regulatory hormones*. After a meal, complex carbohydrates are broken down to simple sugar in the Gastrointestinal (GI) tract by the action of glucosidase. Simple sugar, as glucose, are then absorbed by GI epithelial cells and transported into the blood to metabolic organs, such as the pancreas. The absorption of glucose from β-pancreatic cells stimulates insulin secretion. Transported into the bloodstream, insulin acts on its own receptor in target tissues, in particular liver, muscle and adipose tissue, to promote glucose uptake and storage as glycogen or triglyceride [1]. Insulin induces an the storage of glucose when there is an excess of this monosaccharide in the bloodstream; in muscle and liver, glucose is stored as glycogen, through the glycogen-synthesis mechanism, while in adipose tissues insulin promotes glucose conversion in triglycerides. On the contrary, when blood glucose concentration is low, pancreas releases *glucagon* from the α-pancreatic cells, which, opposite to insulin, promotes both hepatic production of glucose, through *gluconeogenesis,* and glycogen breakdown, through *glycogenlysis,* increasing blood glucose levels to the physiological condition. Even if, physiologically, insulin and

glucagon are the two most important hormones controlling glucose homeostasis, other hormones are involved in this equilibrium. Insulin action is potentiated by *Glucagon-like peptide-1 (GLP-1)* from the GI tract which enhances insulin release in response to an ingested meal, by *amylin* involved in the suppression of endogenous production of glucose from the liver and *Peroxisome Proliferator-activated Receptors γ (PPARγ)* in adipocytes which sustains insulin effect promoting the conversion of glucose to triglycerides. Moreover, the hormone *leptin* also plays an important role in the neuroendocrine response to energy storage and long-term energy balance suppressing the appetite. Conversely, the lack of leptin, as occurs in prolonged starvation, results in persistently increased appetite and suppression of energy-utilizing functions. On the other hand, glucagon effect is sustained by catecholamine (norepinephrine and epinephrine from the sympathetic nervous system and adrenal medulla), glucocorticoids (cortisol from adrenal cortex) and growth hormone (from the pituitary gland). They oppose the action of insulin as counter-regulatory hormones and raise blood glucose concentrations.

Thus, the balance between insulin and the counter-regulatory hormones is important to maintain the blood glucose levels within a physiological narrow range, 70-120 mg/dl. The alterations of these levels could induce hypoglycemia (glucose levels lower than 70 mg/dl) or hyperglycemia (more than 120mg/dl), both representing pathological condition. Hypoglycemia is dangerous because organs, particularly the brain, depend on a constant supply of glucose for proper functions. The possibility to store glucose or to use triglyceride for energy is completely absent is Nervous System. The brain cannot use fatty acids for energy

because they do not cross the blood-brain barrier, so it needs a continuous supply of the monosaccharide, otherwise, a low intake of glucose leads to permanent damage and death of nervous cells. Conversely, chronic hyperglycemia is toxic to many cells and tissues. When glycemia exceed the value of 126 mg/dl, reaching values of 180-200 mg/dl, means that physiological regulation of blood glucose has been completely altered inducing a pathological state, firstly recognized with appearance of glucose in the urine, and then showing other severe symptoms for diagnosis. This pathological condition is represented by *diabetes mellitus*. Mostly, this disease is caused by the alteration of the physiological insulin-glucagon axes. In particular diabetic patients show an absolute or relative deficiency in insulin with consequent increase in glucagon values and glucose concentration levels. The hyperglycemic condition in diabetes triggers to many other complications, the most common are the cardiovascular disease (CVDs), which represent the major cause of morbidity and mortality in diabetic patients.

Nowadays, antidiabetic drugs which increase the insulin release and its action in controlling blood glucose levels, already ameliorate the cardiovascular profile, however these treatments do not completely restore the physiological vascular reactivity. Nevertheless, modern antidiabetic therapies include particular molecules which have a direct vascular beneficial effect through a blood glucose control independent manner. These class of drugs are represented by Dipeptidyl peptidase-IV (DPP-IV). The molecular mechanism through which these molecules act to restore vascular homeostasis is not yet clear. On this vision has been focused my PhD project, evaluating the beneficial effect of DPP-IV, antidiabetic drugs, in the vascular homeostasis.

1.1 Glucoregulatory hormones

1.1.1 Insulin-Glucagon axis

Insulin and glucagon are two protein hormones produced by the pancreas. The pancreas is a gland attached to the digestive system, formed by an *exocrine* and *endocrine* part. The exocrine pancreas is composed by acinar cells which synthesize and secrete the digestive enzymes (trypsine, chymotrypsin, elastase, amylase and others) involved in breaking down the food into simpler components that can be absorbed by the intestine. Scattered within the exocrine tissue are nearly one million small islands of endocrine tissue that secrete hormones directly into the blood. These tine endocrine glands, collectively called *Islets of Langerhans*, contains different cell types that secrete different hormones: α-cells release glucagon; β-cells release insulin and amylin; δ- release somatostatine and gastrine; and PP cells release pancreatic polypeptide. Insulin and glucagon are the most important pancreatic glucoregulatory hormones. After their synthesis and release in the bloodstream, they reach their target organs, especially the liver, the muscles and the adipose tissue where act to regulate both the intake and production of glucose.

Insulin is a small protein composed of two polypeptide chains containing 51 amino acids released from the β-pancreatic cells. In particular, insulin is synthesized in the rough endoplasmic reticulum as a precursor protein called proinsulin. Proinsulin is then processed into the mature peptide hormone. The increase of blood glucose concentration is the stimulus for insulin release from pancreatic cells. When blood glucose concentration are high, β-pancreatic cells absorb glucose from the bloodstream through a specific plasma membrane transporter GLUT-2. In the cytosol glucose is

phosphorylated to glucose-6-phosphate and thereby enters the glycolytic pathway generating ATP and increasing ATP/ADP ratio in the β-cells. The ATP/ADP ratio modulates the activity of a membrane spanning *ATP-sensitive K^+ channels*. When open, K^+/ATP channels hyperpolarize the cell by allowing an outward flux of K^+ with consequent inhibition of insulin production; on the contrary, when closed, the cell depolarizes and insulin is released. These channels are octameric structures containing four subunits of Kir6.2 and four subunits of the sulfonylurea receptor, SUR1. The kir6.2 tetramer forms the pore of the K^+/ATP channels, while the associated SUR1 proteins regulate the channel's sensitivity to ADP and other pharmacological agents. When Kir6.2 binds ATP the K^+ conductance is inhibited, then voltage-gated Ca^{2+} channels are activated and the K^+ current is directed to the extracellular space. Moreover, the rapid increase in intracellular calcium stimulates insulin exocytosis contained in the granules that constitute the pool of early release (Figure 1.1).

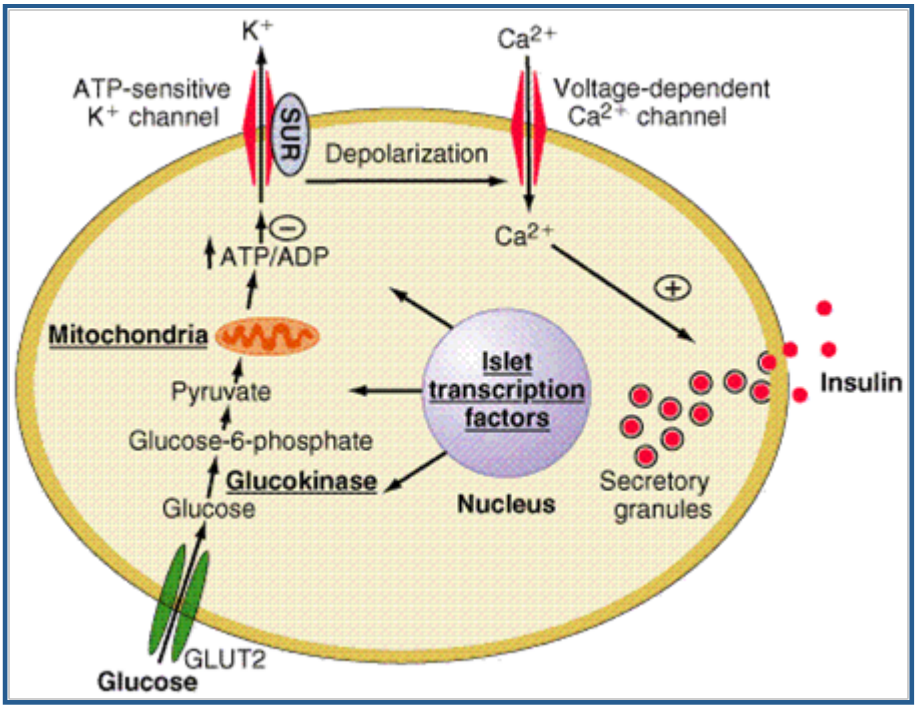


Figure 1.1 **Mechanism of insulin release from β-cells.**

In addition to blood glucose, nutrient sugars, amino acids, and fatty acids increase the intracellular ATP/ADP ratio and thereby stimulate insulin release. Moreover, particularly involved in insulin release are also incretin hormones, such as Glucagon like peptide-1 (GLP-1) and Glucose-dependent insulinotropic polypeptide (GIP). These hormones constitute the endogenous incretin system and they are both secreted from the intestine on ingestion of glucose or other nutrient. There are involved indirectly in the regulation of blood glucose stimulating insulin secretion from pancreatic-β-cells by the inhibition of K^+/ATP channels activity.

Once released insulin exerts its actions through binding to specific receptors present on many tissues of the body, including fat, liver and muscle cells. Insulin receptor is a glycoprotein consisting of four disulfide-linked subunits, including two extracellular α-subunits and two β-subunits.

Each of the β-subunits is composed of short extracellular domain, a transmembrane domain, and an intracellular tail that contains a tyrosine kinase domain. The binding of insulin to the extracellular portion of the insulin receptor activates the intracellular tyrosine kinase, resulting in an auto-phosphorylation of tyrosine on the nearby β-subunit and the phosphorylation of intracellular insulin receptor substrate proteins (IRS) such as the phosphatidylinositol 3'-kinase (PI3-kinase). Since the primary action to induce "glucose disappearance", insulin signaling stimulates the translocation of insulin-responsive glucose transporter, GLUT-4, from intracellular vesicles to the cell surface. GLUT-4 translocation increases the uptake of glucose reducing its levels in the bloodstream. Moreover, especially in liver, insulin is able to activate glycolysis and glycogen synthesis, which are involved respectively in the degradation and store of glucose. Indeed, hepatic cells represent the most important source of endogenous glucose. Here, insulin promotes the phosphorylation of glucose into glucose 6-phosphate, which can be further metabolized and enter the degradation process, or it can be converted into glucose 1-phosphate and enter the route of glycogen synthesis. In accordance to its physiological function to reduce blood glucose concentration, insulin also inhibits the gluconeogenesis process in hepatocytes, suppressing the Phosphenolpyruvate carboxykinase (PEPCK) activity. Since this enzyme does not undergo a post-transcriptional regulation, it can be activated or inhibited only as a result of changes of its mRNA. Insulin alters the acetylation of the gene coding for PEPCK, repressing its expression and then the activity. Leading to its capability to reduce blood glucose levels, insulin simultaneously inhibits glucagon secretion from pancreatic α-cells, thus signaling the liver to stop producing glucose via glycogenolysis and

gluconeogenesis. Moreover, in adipose tissue, insulin promote the store of glucose and fatty acids as triglyceride. This process is enhanced by the activation of other lipogenic enzyme, including pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase and glycerol phosphate acyl-transferase, and the deactivation of hormone-sensitive lipase, which degrades triglycerides.

Conversely, glucagon has a totally opposite effect compare to insulin promoting mobilization glucose. When plasma glucose levels are low, glucagon mobilizes glucose, fat and protein from storage as energy sources. Glucagon acts binding to its G protein-coupled receptor on plasma membrane leading to an increase of cyclic – Adenosin Monophosphate (cAMP) and activation of protein kinase A, a serine/threonine kinase. The primary action of glucagon is on the liver where it stimulates the breakdown of glycogen to glucose and the production of glucose from amino acids. In addition, acting on adipose tissue, it stimulates the release of free fatty acids and directs their metabolic fate in the liver. Rather than being used for the synthesis of triglycerides, these free fatty acids are shunted towards β-oxidation and the formation of ketoacids. Thus, glucagon is both a hyperglycemic and a ketogenic hormone. Besides low glucose and high insulin levels, glucagon secretion is also stimulated by sympathetic nervous system activity, stress, exercise and high plasma levels of amino acids indicating a state of starvation.

Insulin hypoglycemic effect is therefore countered by the hyperglycemic action of glucagon. The increase of one of the two hormones effect varies over time depending on the organism needs, and always has the purpose

of maintaining the blood glucose values in a constant and physiological range (Figure 1.2).

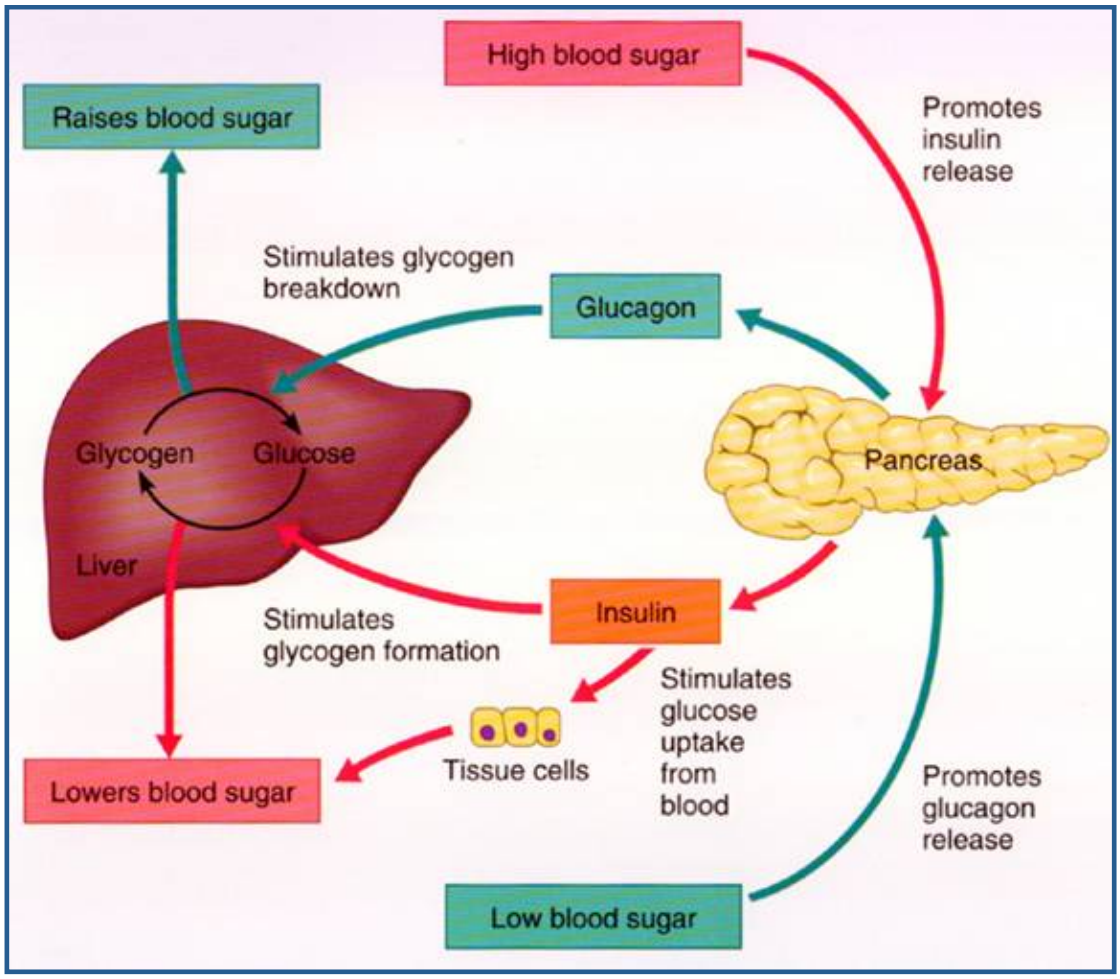


Figure 1. 2 **insulin and Glucagon regulation of bloodstream glucose levels**

1.1.2 Amylin

Amylin, or islet amyloid polypeptide (IAPP), is a 37-residue peptide neuroendocrine hormone. It is co-secreted with insulin from the pancreatic β-cells in the ratio of approximately 100:1 in response to nutrient stimuli [2]. Amylin is processed from an 89-residues coding sequence called Proislet Amyloid Polypeptide (proIAPP). Synthesized in

pancreatic β-cells as a 67 amino acid, proIAPP under goes to post-translational modifications including protease cleavage to produce amylin.

Insulin and amylin are co-secreted following a meal. This has been confirmed by some studies in humans which demonstrate that the release of amylin and its plasma concentration is similar to insulin, with a low activity in the fasting state, and an increased response to nutrient intake [2]. Amylin in physiologically involved in the control of glycaemia levels and its glucoregulatory actions complement those of insulin. While insulin regulates the rate of glucose disappearance, amylin's metabolic function is well-characterized as an inhibition of the appearance of nutrient, especially glucose, in the plasma.

As a neuroendocrine hormone, amylin exerts its action binding receptor within the central nervous system. In particular, this hormone slows gastric emptying and inhibits the release of digestive secretion, such as gastric acid, pancreatic enzymes, and bile ejection, resulting in a reduction in food intake. Appearance of new glucose in the blood is also reduced by inhibiting secretion of the gluconeogenic hormone glucagon, but this effect it is not mediated during insulin-induced hypoglycemia. Thus, following a rise of glucose concentration, amylin help insulin to rapidly restore the physiologically condition, also reducing the total demand of the hypoglycemic hormone.

1.1.3 Somatostatin

Somatostatin has 14-and 28 amino acids form that are selectively produced in pancreatic δ-cells, the gastrointestinal tract, and the hypothalamus. In the regulation of glycaemia levels, somatostatin inhibit the secretion of pancreatic insulin and glucagon, also this hormone reduce the GI motility and the release of various GI hormones. The stimuli for pancreatic somatostatin release are similar to those for insulin, such as high plasma levels of glucose, amino acids and fatty acids.

1.1.4 Glucagon-Like Peptide-1 (GLP-1) and Gastric Inhibitory Polypeptide (GIP)

GLP-1 and GIP are incretin hormones released from the enteroendocrine cells of the GI tract. After a meal GLP-1 and GIP are released in the bloodstream and they are particularly direct on pancreatic β-cells, where augments insulin secretion in response to an oral glucose load while on α-cells GLP-1 and GIP suppress glucagon secretion. Moreover, GLP-1 acts in the stomach to delay gastric emptying and at hypothalamus to decrease appetite. Through all these mechanisms both GLP-1 and GIP show their glucoregulatory actions and thus directly participate in the regulation of blood glucose levels.

1.2 Incretin System

By the late 1960s, Perley and Kipnis and have other demonstrated that the ingestion of glucose caused a more potent release of insulin then glucose infused intravenously [3]. This effect is called “incretin effect”, and

is mediated by incretin hormones released from the gut which, not only affect insulin release, but also regulate gastric emptying and gut motility hormones, resulting in a reduction of blood glucose concentration after a meal.

Glucagon-Like Peptide-1 (GLP-1) and Gastric Inhibitory Polypeptide (GIP) represent the two primary incretin hormones. These hormones are both secreted from the intestine on ingestion of glucose or other nutrient to stimulate insulin secretion from pancreatic-β-cells (Figure 1.3).

GLP-1 is produced primarily in enteroendocrine cells (L cells) of the distal small bowel (ileum). GLP-1 is encoded by glucagon gene to form pro-glucagon which is alternatively processed into glucagon in pancreatic–cells or GLP-1 and other peptides in gut L-cells. Blood levels of GLP-1 are low during the fasting state and rise after a meal. On the other side, GIP is a 42-amino-acid hormone secreted from K cells of the upper small intestine. Originally its action was associated to the inhibition of gastric acid secretion [4], later it has been found that GIP administration stimulates insulin secretion in healthy volunteer acting directly on pancreatic islets [5]. Moreover, it has also been shown that the increase of insulin release mediated by GIP is glucose-dependent, and for this reason the hormone has renamed as Glucose-dependent Insulinotropic Polypeptide [6]

Since the immunological inhibition of GIP does not abolish insulin activity in gut extracts, GIP couldn’t be the only incretin hormones regulating insulin release and activity. Indeed, from the L cells of the lower intestine and colon is release GLP-1 as a second incretin hormone acting on pancreatic islets and stimulates insulin secretion from β-cells.

Both GIP and GLP-1 exert their insulinotropic effect by binding to their specific receptors, the GIP receptor (GIPR) and the GLP-1 receptor (GLP-1R) on β-pancreatic cells. These receptors belong to the G-protein coupled receptor family, activating adenylate cyclase and increasing levels of intracellular cyclic adenosine monophosphate (cAMP) in pancreatic β-cells, thereby stimulating insulin secretion glucose-dependently. In addition to their insulinotropic effect, GIP and GLP-1, play critical and several roles in various biological processes in different tissues and organs that express GIPR and GLP-1R, including the pancreas, bone, fat and brain [7]. Nevertheless, these hormones have a short half-life in the circulation, 1-2 minutes, due to enzymatic degradation by Dipeptidyl Peptidase-4 (DPP-4). Through the activity of DPP-4, incretin hormones are degraded in their inactive metabolites, losing their capability to regulate glucose homeostasis. For this reason, trying to reduce the blood glucose levels in pathological condition has been introduced in the pharmacological therapy drugs that act on the incretin system. Some of these drugs are represented by incretin hormones mimetic molecules because they act as agonists of GLP-1 and GIP receptors with a longer half-life, however, mostly recent are the DPP-IV inhibitors. These molecules block the degradation of incretin hormones potentiating and prolonging then their effect in reducing blood glucose concentrations.

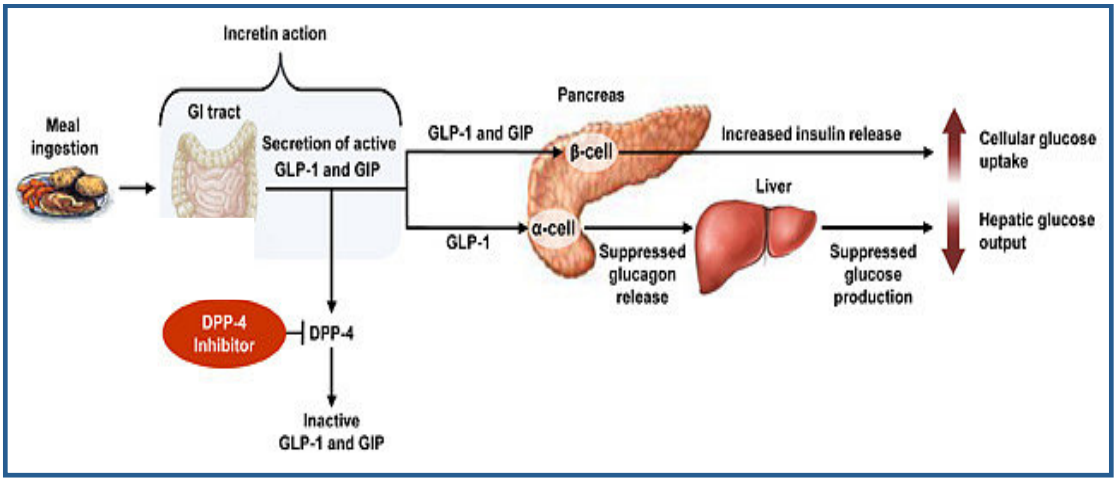


Figure 1.3 ***Incretin system and regulation of blood glucose levels***

1.2.1 Insulinotropic actions of GIP and GLP-1

Several studies have shown that GIP and GLP-1 stimulates insulin secretion from β-pancreatic cells through a glucose dependent way. It has been demonstrated that mice lacking the GIP receptor exhibited intolerance after an oral glucose load [8]. Anyway, in this experimental model the glucose intolerance was not severe, confirming the involvement of another incretin hormone which compensate the lack of GIP signaling. Indeed, several evidences have demonstrated that GLP-1 antagonists drastically reduce insulin secretion after nutrients intake. In addition, mice lacking of GLP-1 showed a severe and fasting hyperglycemic condition after glucose or nutrient load, due to a significantly reduction of insulin [9]. All these evidences confirm the role of GLP-1 and GIP in potentiating insulin secretion and activity. Both these hormones exert their insulinotropic effect by binding their own receptor expressed on the cell membrane of β-pancreatic cells. The bound between incretin hormone and receptor, allows an increase of intracellular cAMP levels, thereby

activating protein kinase A (PKA) and exchange protein activated by cAMP 2(EPAC2). Activation of PKA and EPAC2 pathway induces: alteration of ion channel activity, elevated cytosolic calcium levels and enhanced exocytosis of insulin-containing granules [10]. In particular, PKA shuts down K^+ channels by phosphorylation of the SUR1 subunit and facilitates membrane depolarization [11] with consequent increase of intracellular Ca^{2+} levels through both voltage-gated Ca^{2+} channels (VDCC) and intracellular stores [12]. The increased of Ca^{2+} concentration triggers to two particular events:

- fusion of insulin-containing granules with the plasma membrane releasing insulin in the extracellular environment from β-cells;
- stimulation of adenosine triphosphate (ATP) synthesis in mitochondria, which closes K^+_{ATP} channels increasing cellular depolarization [13].

On the other side, activation of EPAC2 mainly enhances the density of insulin-containing granules near the plasma membrane, facilitating insulin secretion from β-cells [14] (Figure 1.4).

Insulin hormone, as we know, induces the intake of glucose from the circulating blood to the peripheral tissues but fasting state, physical exercises or nutrient intake with the diet, are all conditions with a different request of glucose intake from the blood, so the release of insulin changes following the needs of the organism. Incretin system also supports insulin action in these different conditions, giving its contribution in maintaining glycaemia levels in a constant physiological range.

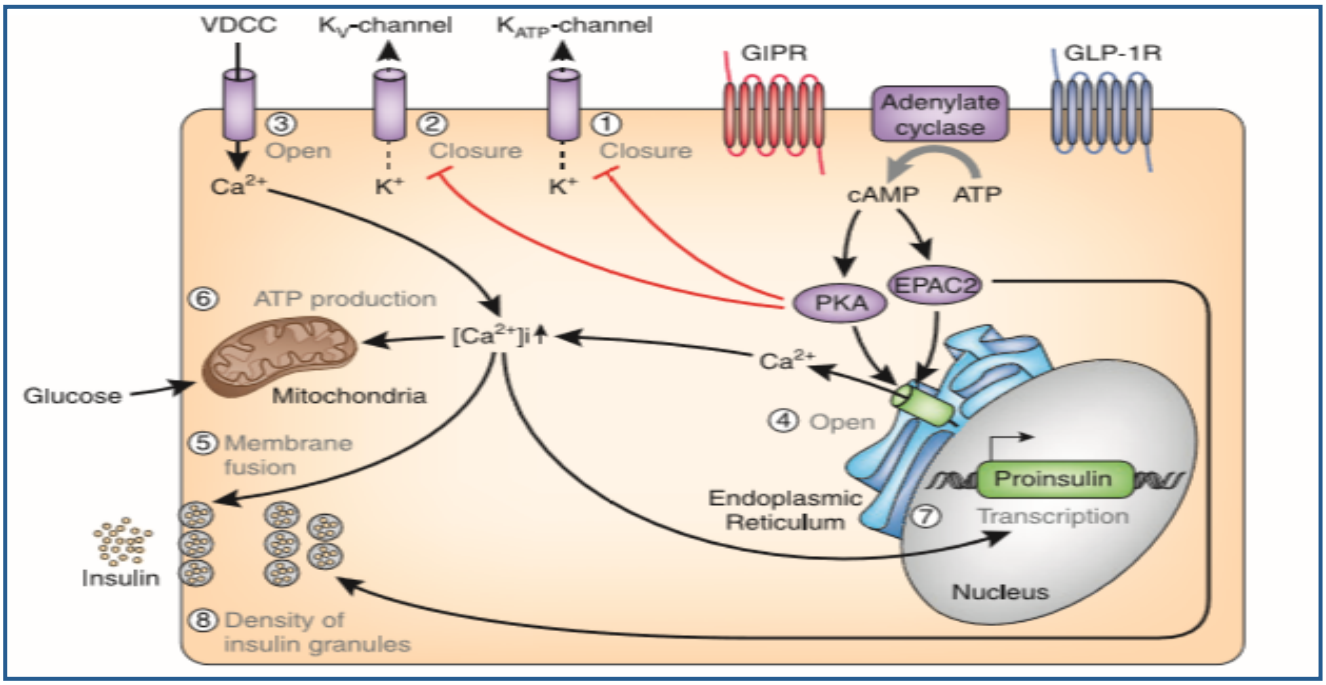


Figure 1.4 ***GLP-1 and GIP molecular mechanism involved in insulin release from β-pancreatic cells***

1.2.2 Non-insulinotropic action of GIP and GLP-1

Incretin hormones exert several physiological effects that can be, or not, related to their capability to increase insulin release and action.

Since incretin hormones receptors are presented in adipocytes experimental evidences have shown that GIP regulates fat metabolism in adipocytes. Indeed, this hormone is able to enhance the insulin-mediated incorporation of fatty acids into triglycerides, activation of lipoprotein lipase and fatty acids biosynthesis in adipose cells [15]

GLP-1 and GIP-mediated control on insulin release and glycaemia levels is also associated to the capability of these hormones in promoting β-pancreatic cells proliferation and survival. In particular GLP-1, in addition

to its effect on expansion of islet cell mass, has antiapoptotic action (Figure 1.5). Farilla L. et al. have shown that GLP-1 reduced the number of apoptotic cells in the pancreas of Zucker diabetic rats [16] and that this effect is independent from glucose control and insulin secretion. Moreover activation of GLP-1R inhibits apoptosis of MIN6 cells (insulinoma cells) exposed to hydrogen peroxide in a cAMP- and PI3-K-dependent manner [17]. Intracellular increase of cAMP levels and the following activation of PI3K leads to the phosphorylation and inactivation of the proapoptotic peptide BAD. BAD is a pro-apoptotic member of the Bcl-2 family that can displace Bax from binding to Bcl-2 and Bcl-xl, resulting in cell death. GLP-1R activation induces Bcl-2 and Bcl-X upregulation with an associated anti-apoptotic affect [18]. These findings support the hypothesis that GLP-1 is not only a growth factor for β-cells but also a powerful antiapoptotic agent ameliorating their function in pathological conditions such as diabetes [19].

Moreover, GLP-1, but not GIP, stimulates gene transcription and insulin biosynthesis in β-cells [20] (Figure 1.5) providing a continual supplies of insulin secretion when the pancreatic vesicles of storage are depleted [21].

The control of blood glucose levels after a meal mediated by GLP-1 is not only related to its effect in insulin release and biosynthesis, but seems to be associated also to its capability in suppressing glucagon biosynthesis and release in pancreatic α-cells (Figure 1.3). Certainly, this is an indirect effect mediated by insulin, but it can't be excluded a direct action of the incretin hormone, since GLP-1 receptors are expressed on pancreatic α-cells [21].

The inhibitory effect of GLP-1 on glucagon secretion also seems to represent an important additional mechanism through which incretin hormones regulate blood glucose levels especially after a meal. However, GLP-1 has been shown to suppress glucagon levels only when glucose plasma levels are above fasting level [22]. This is clinically important because GLP-1 loses its inhibitory effect on glucagon secretion from α-cells at hypoglycemic levels and does not attenuate the counter-regulatory responses to hypoglycemia.

Moreover, the reduction of postprandial glucose excursion mediated by GLP-1 is due to an inhibitory effects of the hormone on gastrointestinal secretion and motility, in particular GLP-1 has been shown to reduce caloric intake and to enhance satiety probably involving central mechanism [23, 24] (Figure 1.5). Experimental studies with rodents made by Van Dijk and colleagues in 1999 have already proved that intra-cerebroventricular administration of GLP-1 significantly reduced food ingestion, whereas concomitant injection of the GLP-1 receptor antagonist abolished completely this effect [25]. Today, 2016, it has been confirmed that Glucagon-like peptide-1 (GLP-1) affects appetite through the Central Nervous System (CNS). In order to investigate if CNS responses to feeding may be mediated by GLP-1, Ten Kulve JS and colleagues have evaluated the effects of endogenous GLP-1 and GLP-1 analogue, liraglutide, on CNS activation to chocolate milk receipt in healthy and diabetic volunteers. The findings obtained showed that in obese diabetic patients endogenous GLP1 and liraglutide improve the observed deficit in responsiveness to palatable food consumption then in healthy individuals, which may contribute to the induction of weight loss observed during treatment [26]. The exact mechanism by which peripheral GLP-1

modulate food intake is not been clarified yet. However the involvement of incretin system is supported by the presence of GLP-1 stores in neurons of nucleus of tracts solitaries, which project into thalamic and hypothalamic regions implicated in the control of food intake [27].

Expression of GLP-1 receptor has been observed also in distinct heart chambers and cell types, including cardiomyocytes [28], endothelial and vascular SMCs and has been associated with myocardial protection in the setting of ischemic/reperfusion injury [29] (Figure 1.5). Moreover, in the cardiovascular protective effects mediated by GLP-1 it has been included also the rapidly metabolite of the incretin hormones, GLP-1(9-36), which, it has been shown, induces vasodilatation via an NO/cGMP-associated mechanism that does not require a functional GLP-1R [30].

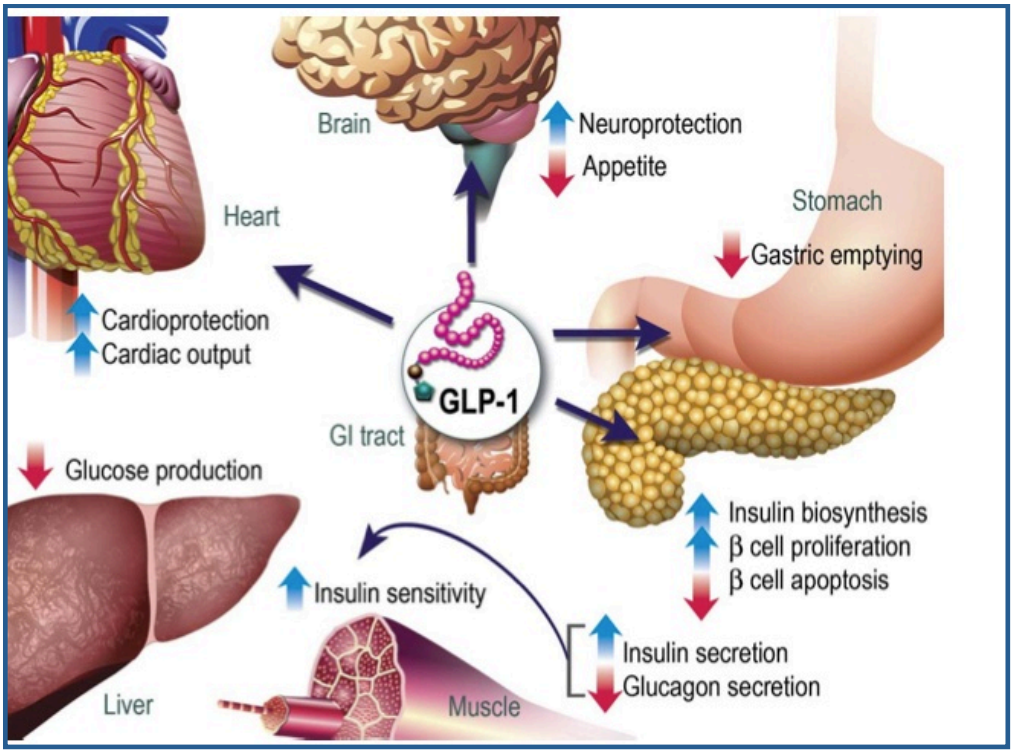


Figure 1.5 ***Non-insulinotropic action of incretin system***

1.3 From the physiological condition to the pathology of diabetes

Glucose regulation is a perfect orchestration of many hormones, both from pancreas and gut, that strongly work together in order to maintain glycaemia levels in a physiological range, 70 – 120 mg/dl. However, blood glucose concentration between meals can increase to 101-125 mg/dl which represents only an "impaired fasting glucose", but can also exceed to values more than 126 mg/dl meaning that the physiological regulation of blood glucose has been altered inducing a pathological state, firstly recognized with appearance of glucose in the urine, and then showing other severe symptoms for diagnosis. This pathological condition is represented by *diabetes mellitus*. The mainly responsible for this disease is the alteration of the physiological insulin-glucagon balance. While the increase of insulin secretion, lowers the blood glucose concentration values, establishing a state of hypoglycemia, on the contrary, the reduction of insulin values and the consequent increase in the glucagon values cause an increase in blood glucose concentration levels, and the triggering of the hyperglycemic state of diabetes.

The term diabetes derives from the greek word *διαβαίνειν*, that means *going through* and identifies some diseases characterized by polyuria (abundant production of urine) and polydipsia (abundant ingestion of water). Commonly the term is used to indicate a chronic condition, characterized by a high concentration of glucose in the blood due to a deficiency (absolute or relative) in the human insulin secretion or function. The use of the adjective diabetes mellitus comes from the Latin "sweetened with honey" and refers to the large amount of glucose that is

found in the urine of patients suffering from the disease. Although the mainly elements which identify the diabetic condition is represented by high blood glucose levels, it is important to highlight that this term is not only use for diabetes mellitus, but also identify another pathological condition which shows similar signs and symptoms, such as an excessive thirst and an excessive urination, called "*diabetes insipidus*". Although they show similar symptoms, diabetes mellitus and insipidus born from different physiological alteration. While the mainly problem of diabetes mellitus is represented by the high blood glucose concentration due to an alteration of physiological insulin secretion and peripheral activity, diabetes insipidus is characterized by the deregulation of the response to antidiuretic hormone (ADH) which causes the inhibition of water reabsorption in the collecting ducts of the nephron, resulting in the production of copious amounts of dilute urine. Laboratory analysis show that in patients with diabetes insipidus the concentration of glucose in the blood and urine remains normal, and this is allow to differentiates diabetes insipidus from mellitus.

1.4 Diabetes Mellitus

In 1774, thanks to Matthew Dobson, it has been discovered that the sweet taste of the urine of patients suffering from Diabetes Mellitus (DM) was due to glucose contained in them [31]. This initial analysis was then followed by other studies that in 1910 suggested that people with DM were actually deficient in a particular substance produced by the pancreas, which he named as insulin, produced by the islets of

Langerhans located in the pancreas. Insulin, thanks to the work of Canadian researchers Frederick Banting and Charles Herbert Best, was isolated in 1921, and following this discovery it has been concluded that diabetes was an endocrine disease linked to a deficiency of insulin. From 1935 to now, many studies have been done and in fact currently the DM disease results from a heterogeneous group of metabolic disorders that have hyperglycemia in common. Hyperglycemia can result from an absolute lack of insulin, like in the type 1 diabetes mellitus (T1DM), also called *insulin-dependent diabetes mellitus (IDDM)*, or from a relative insufficiency of insulin production in the face on insulin resistance, conditions that represents the type 2 diabetes mellitus (T2DM), also called non insulin-dependent diabetes mellitus (*NIDDM*).

1.4.1 Type 1 Diabetes Mellitus

Type 1 diabetes is usually diagnosed in children and young adults. This pathology was previously known as juvenile diabetes, and today is mainly know as “Insulin-Dependent Diabetes Mellitus”.

Type 1 Diabetes Mellitus is a particular form of diabetes characterized by the autoimmune destruction of β-pancreatic cells. In absence of β-cells, insulin is neither produced nor released, and circulating insulin concentrations are near zero leading to pathological consequences. Indeed, in the absence of insulin, tissues fail to take up and store glucose, amino acids and lipids, even when there are high circulating plasma levels of these fuels. Moreover, the unavailability of insulin to promote nutrient entry into cells, coupled with the unopposed actions of counter-regulatory hormones, induces a starvation-like response by the cells and tissues of the body. Thus, glycogenolysis and gluconeogenesis proceed unchecked

in the liver, delivering glucose to the bloodstream even if its levels are already high. Contextually, muscle tissue breaks down protein and release amino acids, which travel to the liver as fuel for gluconeogenesis. In adipose tissue instead, triglycerides are broken down and release into the circulation. The derived fatty acids are then break down in the liver for use as gluconeogenic fuels and for export as ketone bodies that, accumulating in the plasma, increase the normal level of acidity, which, in turn, leads to abdominal discomfort, nausea, vomiting, rapid breathing, confusion and possible coma. All these events leads together to a drastically increase of blood glucose concentration, with reduction of kidney's capacity to reabsorb glucose from the glomerular filtrate and a consequent osmotic dieresis as well as a "sweetening" of urine. This phenomenon causes the typical polyuria and subsequent polydipsia experienced by many diabetic patients. Contextually, although appetite it is stimulated, patients loss their weight because dietary nutrients are inaccessible.

Type 1 Diabetes Mellitus accounts for between 5% and 10% of all diabetes cases [32]. Even if the number of people with DM type 1 is unknown, it has been estimated that globally about 80,000 children develop the disease each year. Moreover it has been assessed that the development of new cases depends on the country and region. Indeed, Japan and China represent the country with the lowest rates of the new cases per year; approximately 1 person per 100,000 per year; finally the highest rates are found in Scandinavia, where rates are closer to 35 new cases per 100,000 per year [33].

While it is well established that the patho-physiology of Type 1 DM is represented by the destruction of β-pancreatic cells, the causes of the

onset of the pathology are unknown. Blood analysis of diabetic patients show the presence of antibodies directed against antigens expressed at the level of cells that produce insulin, known as ICA, GAD, IA-2, IA-2B. For this reason, the Type 1 DM is also ranked among the so-called disease “autoimmune”, that is due to a direct immune reaction against the body itself, in this case against the own β pancreatic cells. This damage, mediated by the immune system, could be related to genetics or environmental factor, or can be associated to the activity of chemicals and drugs. Regarding the genetic factors, at least 20 different chromosomal regions have been linked T1DM susceptibility in humans, using genome screening, candidate gene testing, and studies of human homologues of mouse susceptibility genes. For this reason diabetic pathology can be inherited from parents. Indeed, it has been show that if one of the parents has T1DM the probability that the disease occurs in a child is 5-10%, otherwise if both parents are affected by the pathology the odds for children increased to 23%. Moreover, the risk of developing the disease depends also for the environments, which surround the children. Indeed, for identical twins, when one twin has type 1 diabetes, the other twin only shows the disease in the 30%–50% of the time. Thus for 50%-70% of identical twins where one has the disease, the other will not, despite having exactly the same genome; this suggests environmental factors, in addition to genetic factors, can influence the disease's prevalence. These can also reflects the reason why the rates of the diabetic pathology varies by country and region. Some chemicals and drugs also could be responsible for destroying pancreatic cells. Pyrinuron (Vacor), a rodenticide introduced in the United States in 1976, selectively destroys pancreatic beta cells, resulting in type 1 after ingestion. Pyrinuron was

withdrawn from the U.S. market in 1979 but is still used in some countries. Streptozotocin (Zanosar), an antibiotic and antineoplastic agent used in chemotherapy for pancreatic cancer, kills β-cells, resulting in loss of insulin production. Other pancreatic problems, including trauma, pancreatitis, or tumors (either malignant or benign) can also lead to loss of insulin production.

1.4.2 Type 2 Diabetes Mellitus

Type 2 Diabetes mellitus is the most common form of diabetic pathology, indeed it makes up about 90% of cases of diabetes, with the other 10% due primarily to T1DM and gestational diabetes. T2DM appears in mature ages, typically affects individuals over 40 years old, although young adult and pediatric cases are rapidly increasing. Obesity is the single most important risk factor, and more than 80% of type 2 diabetic patients are obese. The disorder typically develops gradually without obvious symptoms at the onset. At the beginning it can be diagnosed by elevated blood glucose levels, then, when the pathology became severe, appear the typical symptoms of polyuria and polydipsia. Despite the type 1, T2DM in the chronic phase is supported by combination of reduced insulin secretion and insulin sensitivity, defined *insulin resistance*. With increasing age and added weight, tissues that were once normally insulin-responsive become relatively refractory to insulin action and require increasing insulin levels to respond appropriately. In addition, the loss of β-cells through increased apoptosis or for a decreases renewal, cause a failure in satisfies the need of insulin to compensate the insulin resistance. Thus, type 2-diabetes cannot be attenuated by insulin administration, as type 1,

and for this reason it also named “Insulin-Non-Dependent Diabetes Mellitus”.

Insulin levels that are incapable of compensating for insulin resistance result in an imbalance between the actions of insulin and those of the counter-regulatory hormones, which may contribute to hyperglycemia and dyslipidemia as the liver and the adipose tissue inappropriately mobilize fuels from tissue stores. Indeed, insulin resistance occurs primarily within the liver, muscles, and fat tissue. Under physiological conditions, acting on the liver, insulin suppresses the release of glucose. On the contrary, in type 2 diabetes the insulin resistance induces the liver to releases continuously glucose in the blood resulting in higher blood sugar to become hyperglycemia. Furthermore, the decreased sensitivity of fat cells to insulin, activates the process of lipolysis, with consequent increased concentration of free fatty acids in the blood that get worse the pathogenesis of diabetes, reducing the uptake of glucose into skeletal muscle and further promoting the release of glucose by the liver.

The development of type 2-diabetes is caused by a combination of lifestyle and genetic factors [34, 35]. While some of these factors are under personal control, such as diet and obesity, other factors are not, like the increasing age, female gender, and genetics.

Lifestyle factors have an important relevance in the development of type 2 diabetes, including obesity, being overweight, lack of physical activity, poor diet, smoke, stress, and urbanization [35, 36]. Obesity is the most common condition related to the development of diabetic pathology. Indeed, the excessive amount of fat cells in the obese patients leads to an excessive amount of insulin required, and when this request is chronic the

quantity produced start to be not enough to satisfied body's needs. This is the reason why a lack of exercise is believed to be the responsible of the 7% of cases [37, 38].

Moreover, although type 2 diabetes is not considered as an autoimmune disease, elements of both the innate (macrophages and mast cells) and adaptive (T regulatory cells, Th1 cells, CD8+ T cells) immune system are present in obese adipose tissue and may play roles in the pathogenesis of insulin resistance.

Dietary factors also influence the risk of developing type 2 diabetes, indeed consumption of sugar-sweetened drinks in excess is associated with an increased risk [39, 40]. Eating lots of white rice also appears to play a role in increasing risk.

In most cases all of these lifestyle factors are also associated with genetic alterations related to several genes (polygenic disease) involved in insulin production and glucose metabolism. More than 40 genes have now been identified, and most have predominant actions in the β-cells. Few of the identified type 2 diabetes genes confer risk for either obesity or insulin resistance. Therefore, insulin-sensitive patients with type 2 diabetes have a strong predisposition to β-cells failure. Mild or early type 2 diabetes can be also unmasked in predisposed individuals by transient periods of insulin resistance, as occurs during the treatment with glucocorticoids or pregnancy, which show the onset of the gestational diabetes.

1.4.3 Pharmacological treatment of diabetes mellitus

Therapeutic agents are available to modify most steps in the regulation of glucose homeostasis. The major goal of the pharmacological therapy of

diabetes is to normalize metabolic parameters, such as blood glucose, in order to reduce the risk of long-term complication.

For type 1-diabetes, the pharmacologic strategy is to administer a sufficient amount of exogenous insulin to achieve normoglycemia, without inducing hypoglycemia. Human insulin is manufactured by bacterial recombinant DNA technology. Because the insulin molecule has a short half-life of only few minutes in the circulation, for the type 1 diabetes treatment have been formulated many preparation which release the hormone slowly in the way that its activity can be prolonged. The available insulin forms provide 4 rates of onset and duration: *ultra-rapid onset*, *rapid onset with short action*, *intermediate onset and action* and *slow onset with short action*. All insulin preparations contain zinc and the ratio of this molecule is the mainly responsible for the rate of release of the hormone from the site of administration and the duration of action. Although insulin represents the first and also best choice to restore plasma glucose concentration it is important that patients, during the treatment need to follow appropriate dietary and also have to pay attention to all the factors that change insulin requirements, such as physic exercises, infections, stress and deviation from the regular diet in order to avoid a dangerous hypoglycemic event.

On the other side, treatment of type 2 diabetes is more complex and involves several target which can be modify to re-stabilize glucose homeostasis. Firstly, obese patients should to reduce body weight and increase exercises in order to improve the insulin sensitivity. These changes in the lifestyle can be then associated with a pharmacological approach through using oral active antidiabetic drugs:

- Insulin Segretagogues, such as Sulfonylureas and Meglitinides
- Biguanides
- Amylin analogue
- Insulin sensitizers as Thiazolidinediones
- α-glucosidase inhibitor
- Incretin therapy which act trough GLP-1 analogue or, more recent, DPP-IV inhibitor

Insulin Segretagogues

Insulin segretagogues are drugs which act stimulating the release of insulin from pancreatic β-cells, with consequent increase of circulating insulin to levels sufficient to overcome the insulin resistance. This mechanism is typical of Sulfonylureas and Meglitinides, two antidiabetic classes of drugs, used in the therapy of type 2 diabetes. Physiologically, after a meal, the glucose up-taken from the blood into β-pancreatic cells, is metabolized releasing ATP, which consequently inhibits K+/ATP, channels binding the Kir.6 subunits. Indeed, Sulfonylureas bind to K+/ATP channels in β-cells involving another subunit called SUR1. The bind with SUR induces the inhibition of the channel as well, with membrane depolarization, Ca2+ influx and fusion of insulin-containing vesicles with the plasma membrane releasing insulin in the blood stream. The increased release of insulin in type 2 diabetic patients compensates the increased tissues request of the hormone in the condition of an increased insulin-resistance. Nevertheless, this action mediated by Sulfaniluree has to be continuously monitored since one of the major adverse affect is the hypoglycemia resulting from the over-secretion of insulin. Thus, these

medications should be used continuously in patients who are unable to recognize or respond appropriately to hypoglycemia, such as those with impaired sympathetic function, mental status changes, or advanced age. Like Sulfonylureas, Meglitinides stimulate insulin release by binding SUR1 and inhibiting the β-cell K+/ATP channel. Although Sulfonylureas and Meglitinides both act on the SUR1 subunit, these two class of drugs bind to distinct regions of the SUR1 molecule. Obviously, since the the molecular effect is the same, also the adverse-effect profile is similar in these two class of anti-diabetic, with a high risk of hypoglycemic episodes.

Biguanides

The insulin-resistance condition in T2DM causes a greater glucose production from the liver, increasing hyperglycemia and its pathological consequences. For this reason, another pharmacological approach in this pathology is represented by drugs, which are able to reduce the hepatic glucose production, such as the Biguanides. The one from this class of drugs, which is the most efficient and used in the anti-diabetic therapy, is the *metformine* that acts to decrease glucose production in the liver by activating the energy-regulating enzyme AMPK. By triggering hepatic AMPK, *metformine* inhibits gluconeogenesis, fatty-acid synthesis and cholesterol synthesis. Through this mechanism, *metformine* increases insulin signaling and is affective in lowering blood glucose levels in type 2 diabetic patients. Nevertheless, because to its capability in decrease the flux of metabolic acids, a serious adverse effect of *metformine*, as of all the Biguanides, is the lactic acidosis due to the accumulation of lactic acid to dangerous levels for the patients. On the other hand, since Biguanides

do not directly affect insulin secretion, their use is not associated with hypoglycemia, as happen for Sulfaniluree and Meglitinides.

Amylin analogue: Pramlintide

In the post prandial phase, β-pancreatic cells co-secreted with the insulin another glucoregulatory hormone, the amylin, which specifically participates in the regulation of blood glucose levels after the meal sustaining the activity of insulin. Type 1 and type 2 diabetes are both characterized by a relatively lack of amylin, thus in order to restore the glucose metabolism in these patients, has been introduced a new drugs in the anti-diabetic therapy, represented by *pramlintide*, an analogue of amylin. *Pramlintide* was designed as a stable analogue of the human amylin and, as the endogenous hormone, is able to slows the gastric emptying, reduces postprandial glucagon and glucose release, and promotes satiety. Through these effects, *pramlintide*, can sustain the action of the remaining amount of insulin present in the diabetic patient, or can collaborate with other antidiabetic drugs, such as Sulfonylureas, Biguanides and other in order to improve their hypoglycemic effect and improve the glucose metabolism.

Insulin sensitizers: Thiazolidindiones

Rosiglitazone and *pioglitazone* are the two currently available Thiazolidindiones (TZDs) used in the therapy of type 2 diabetes. These drugs are insulin “sensitizers” and enhance the action of insulin at target tissues without directly affect insulin secretion. The Thiazolidindiones are synthetic ligands, which stimulate the peroxisome proliferator-activated receptor-gamma (PPARγ-receptor). PPARγ is a nuclear receptor, which regulates the transcription genes encoding protein involved in

carbohydrate and lipid metabolism. By activating PPARγ, TZDs promote fatty-acid up-take and storage in the adipose tissue rather than in skeletal muscle and liver. The decrease in muscle and liver fat content makes tissues to be more sensitive to insulin increasing the up-take of glucose from the blood. Moreover, these drugs inhibit the hepatic gluconeogenesis suppressing hepatic glucose production typical of the disease.

α-glucosidase inhibitors

Complex starches taken through the diet, such as oligosaccharides and disaccharides, have to be metabolized to smaller monosaccharides in order to be assimilate in the bloodstream. This is a necessary process, which happens in the intestine, and it's mediated by the enzyme α-glucosidase. *Acarbose* and *miglitol* are carbohydrate analogs that act within the intestine to inhibit α-glucosidase. This effect results in a slow of monosaccharide's absorption with consequent reduction of the postprandial hyperglycemia. Both drugs can be used as monotherapy, but since continuous regulation of blood glucose levels in diabetic patients cannot be maintain by the only action α-glucosidase inhibitors, they are used also in combination with other antidiabetic drugs. The primary adverse effect includes flatulence, diarrhea and abdominal pain resulting from increased fermentation of unabsorbed carbohydrate by bacteria in the colon. Moreover, hypoglycemic event in patients treated with α-glucosidase inhibitors should be controlled directly with oral glucose because the absorption of the other complex carbohydrates is delayed.

GLP-1-based "Incretin" therapy

Incretin system plays an important role in the regulation of blood glucose levels through the action of incretin hormones, GLP-1 and GIP, released

from the gut after a meal. Incretin hormones show hypoglycemic actions through an increased release of insulin and reduction of circulating glucagon, but these effects last very few since their short half-life. Indeed, GLP-1 and GIP after their release are metabolized in inactive molecules in 2-3 minutes by Dipeptydil peptidase-IV enzyme (DPP-IV). Presently, the available incretin-based therapies for diabetes mellitus include the GLP-1 receptor agonists or incretin analogs, which are resistant to DPP-4 degradation, and DPP-4 inhibitors; the mechanisms of action of both involves the increase of the endogenous effects of GLP-1. Since one of the physiological effect of incretin hormones is to increase the release of insulin from the pancreas, these drugs are only used in type 2 diabetes, and not type one where the capability of β-pancreatic cells to release insulin has been compromised. *Exenatide* is a GLP-1 analogue, originally isolated from the salivary gland of the Gila monster. This molecules, has been approved for use in type 2 diabetics in the United States in 2005. As a GLP-1 analogue, *exenatide* has several modes of action that benefits patients with diabetes: it increases secretion of insulin by pancreatic β-cells in a glucose-dependent manner; it suppresses secretion of glucagon by pancreatic α-cells; it slows gastric emptying and thereby slows the rate of nutrient entry into the circulation with consequent reduction of the appetite. Despite the efficient action of these class of drugs in maintain the glycemia within physiologically and safety levels, there is another way to improve and sustain the hypoglycemic effect of the incretin system, the use of DPP-IV. DPP-IV is the endogenous enzyme responsible of incretin hormones degradation with consequent reduction of their physiological effects. The inhibition of DPP-IV prolongs the half-life of incretin hormones, in particular of the endogenous GLP-1, potentiating its

hypoglycemic effect. Indeed, inhibition of DPP-IV and the consequent increase of circulating GLP-1, induces an enhanced release of insulin from pancreas in a glucose-dependent manner and a decrease of glucagon concentrations. The most common DPP-IV inhibitors used in T2DM are *sitagliptin* and *saxagliptin*, which were Food and Drug Administration (FDA) approved in 2006 and 2009 respectively. Moreover, another inhibitor recently introduced in therapy and equally effective is *linagliptin*, FDA approved in 2011.

1.4.4 DPP-IV enzyme and its inhibitors

DPP-4 is a 766 aminoacid transmembrane glycoprotein, also known as adenosine deaminase complexing protein 2 or CD26, and DPP-4 inhibitors are cyanopyrrolidines with key interactions with the DPP-4 complex that allow for competitive inhibition. This enzyme is expressed on the surface of several cell types such as monocytes, lymphocytes [41] and vascular endothelial cells [42]. Specifically, DPP-IV is a serine aminopeptidase enzyme, which inactivates GLP-1, GIP and other proteins in vivo via dipeptide cleavage of the N-terminal amino acid. In order to show its catalytic activity is necessary the dimerisation of the enzyme for its activation, than occurs the glycosylation to finally induce its physiologically effects. Moreover, DPP-IV acts on several proline or alanine containing peptides, such as growth factors, chemokines, neuropeptides and vasoactive peptides [43]. For this reason, since DPP-IV not only regulate the activity of incretin hormones on blood glucose regulation, but also of other endogenous mediators, inhibition of this enzyme mediates a wide range of pleiotropic effects, both positive and negative, independent of GLP-1. The currently available DPP-4 inhibitors

include *sitagliptin, saxagliptin, linagliptin, vildagliptin and alogliptin*. The first three are approved in the USA and throughout much of the world for the treatment of T2DM; *vildagliptin* has been approved for use in Europe and Latin America and *alogliptin* for use in Japan. Other members of this class are in phase III clinical trials, and include *dutogliptin* and *gemigliptin*. Currently, the drugs are predominately used as second- or third-line agents, typically after *metformin* or the combination of *metformin-sulfonylurea* or *metformin-thiazolidinedione*. Moreover, DPP-4 inhibitors are well tolerated, with hypoglycemia only occurring when used in combination with sulfonylureas therapy. Indeed, they are also approved for use as monotherapy, mainly used in this setting in those who cannot tolerate or have active contraindications to metformin (e.g. chronic kidney disease). DPP-4 inhibitors may also have a unique role in elderly patients who face several precautions regarding the use of other anti-hyperglycemic drugs due to their potential toxicities.

1.4.5 Symptoms and complications of Diabetes mellitus

The symptoms of diabetes mellitus are several and, in particular they depend on whether the pathology has the characteristics of T1DM and T2DM. In type 1-diabetes the onset of the disease is sudden. At the beginning, patients complain a widespread fatigue (asthenia), an intense thirst (polydipsia) and increase the amount of urine. These first symptoms are then followed by weight loss, dry skin and tendency to get sick frequently, such as the susceptibility to infections. While symptoms of T1DM are immediate and clear since the beginning of the disease, on the other side, T2DM has a slower onset, so the disease is usually diagnosed after clinical investigation carried out for other reasons. All these

symptoms are signs of the diabetic process and when the pathology well confirmed, is not good treated, they are accompanied by several clinical *acute* or *chronic* complications. Acute complications occur in short-term from the onset of the diabetes, while chronic complication comes after long time from the beginning of the pathology. In the first group are included:

- ***Diabetic ketoacidosis***, characterized by excessive concentration of ketone bodies in the blood due to insulin deficiency with consequent excess of glucagon. As we already know, under physiological conditions triglycerides are stored in VLDL (with particular lipoprotein transport function); in the conditions of fasting and excess glucagon accompanied to insulin deficiency, the cells are unable to use the plasma glucose as an energy source, exploit triglycerides and fatty acids transported in the blood. The latter, through the reaction of beta-oxidation, produce energy, but also high amounts of a molecule (acetyl-coenzyme A), from which the ketone bodies such as acetone, acetic acid and beta-hydroxy-butyric acid are obtained. Subsequently, these ketone bodies are poured in blood and cause the so-called diabetic ketoacidosis. Moreover, the inability to use glucose stimulates the production of hormones that in turn have a hyperglycaemic effect facilitating the endogenous glucose production via hepatic gluconeogenesis and glycogenolysis, further aggravating the hyperglycemic state and diabetic ketoacidosis. Moreover, hyperglycemia and ketosis induce a lowering of the pH of the blood, resulting in manifestations of nausea, vomiting, dehydration, polyuria, polydipsia, hypotension, drowsiness and coma.

- ***Hyperglycemic hyperosmolar state***, is observed mostly in elderly patients in whom the diabetic condition is aggravated by recurring events (eg. Infections or stroke) and the ability to drink is reduced making impossible the compensation of water loss due to osmotic diuresis. It follows severe neurological symptoms due to dehydration of the cells, with convulsions and motor deficit. Laboratory tests show marked glycosuria in absence of ketone bodies in urine and extremely high blood sugar, usually above 1000 mg / dl, about twice as many values in ketoacidosis coma.

On the other side, the long-term or chronic complications include:

- ***Diabetic retinopathy*** is a damage to the small blood vessels supplying the retina with loss of the sight. In addition, people with diabetes are more likely to develop eye diseases such as glaucoma and cataracts.

- ***Diabetic nephropathy,*** a progressive reduction of the kidney filtering function that, if untreated, can lead to renal failure until the need for dialysis and / or kidney transplant.

- ***Diabetic neuropathy,*** which is one of the most frequent complications and according to WHO is manifested at different levels in 50% of diabetics. It can cause loss of sensation, of varying intensity pain and limb damage, requiring amputation in severe cases. It can lead to malfunctions of the heart, eyes, stomach and is a major cause of male impotence.

- ***Diabetic foot,*** which is correlated to the modifications of the structure of blood vessels and nerves that cause ulceration and problems in the lower extremities, especially the foot. This may necessitate amputation of limbs and statistically is the leading cause of amputation of the limbs of non-traumatic.

- ***Cardiovascular disease (CVD)***: From the point of view of the cardiovascular medicine, diabetes is considered a cardiovascular disease. Several epidemiological and pathological data demonstrate that diabetes is an independent risk factor for CVDs [44], listed as the mainly cause of death in of people with diabetes.

1.5 Vascular tone

In combination with cardiac output, vascular tone determines the adequacy of perfusion of the tissue of the body. The importance of vascular tone is underscored by the wide spectrum of diseases are associated with altered vascular tone. A complex of molecular mechanisms is involved in the regulation of vascular homeostasis in the face of different stimuli. In particular, regulators of vascular tone act by influencing the actin-myosin apparatus of vascular smooth muscle cells. As in other muscle cells, the actin-myosin interaction leads to contraction and is regulated by intracellular calcium (Ca^{2+}) concentration. Stimulation of vascular smooth muscle cells can increase the cytoplasmic Ca^{2+} concentration through two mechanisms: first, Ca^{2+} can enter the cell by way of voltage-gated Ca^{2+} -selective channels in the sarcolemma, then the increase of cytoplasmic Ca^{2+} can be elicited by the release of intracellular Ca^{2+} from sarcoplasmic reticulum. Vasoconstriction, the contraction of vascular smooth muscle, is commonly initiated by the opening of voltage-gated L-type Ca^{2+} channels in the sarcolemma during plasma membrane depolarization. When Ca^{2+} channels are open, a calcium ions flux goes into the cytoplasm and activates cytoplasmic calmodulin (CaM). The Ca^{2+}-CaM complex binds to and activates myosin chain kinase, which

subsequently phosphorylates myosin-II light chains. When the light chain is phosphorylated, the myosin head can interact with an actin filament, leading to the smooth muscle contraction.

On the other side, the opposite reaction of the vessel to the contraction is the vasodilatation, the relaxation of the vascular smooth muscle. Vasodilatation occurs upon de- phosphorylation of the myosin light chain. De-phosphorylation is potentiated when Guanylyl Cyclase (GC) is activated inside the smooth muscle cells. Indeed, activated GC increases the production of cyclic guanosine 3’5’-mophosphate (cGMP) which stimulates cGMP-dependent protein kinase, called PKG inducing phosphorylation and than activation of myosin light chain phosphatase, involved in the de-phosphorylation of myosin light chain. De-phosphorylation of myosin light chain inhibits the interaction of the myosin head with actin, leading to smooth muscle relaxation.

Stimulation of vasoconstriction and vasorelaxation is governed by a wide variety of mechanisms, which involve an interaction between vascular smooth muscle cells and vascular endothelial cells. In particular, as a major regulator of vascular homeostasis, the endothelium has the important role of maintain equilibrium between vasodilation and vasoconstriction, moreover it is involved in regulate migration and proliferation of smooth muscle cells, fibrinolysis, thrombogenesis as well as the adhesion and aggregation of platelets [45]. Many vascular diseases are associated to an endothelium dysfunction which cause an alteration in all the molecular mechanism involved in vascular homeostasis maintaining.

1.5.1 Components of the vascular wall

Blood vessels are composed of three layer: an intimal monolayer of endothelial cells, medial vascular smooth muscle and the adventitia, also called tuninca externa (Figure 1.6)

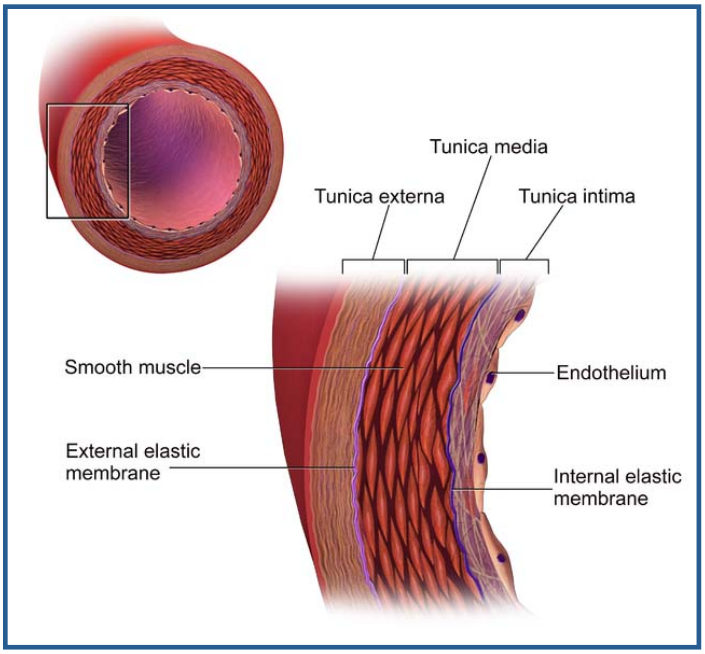


Figure 1.6 ***Structure of a blood vessel wall***

Endothelial cells constitute the interior surface of the blood vessels of the entire vascular tree, from the heart to the smallest capillaries. The endothelium plays an essential role in the regulation of vascular tone, modulating both the basal and dynamic diameter of the vessels through the release of vascular mediators and by initiating endothelium-dependent hyperpolarization. Endothelial cells monolayer is then surrounded by the tunica media composted by an elastic membrane attached to a thick layer of smooth muscle cells, which are responsible of the contraction and vasodilatation of the vessels. Contraction of vascular smooth muscle cells

can be due by mechanical or pharmacological stimulus. Mechanical stimuli are represented by the increase of intraluminal pressure (myogenic tone) or the stretch of the vessels. On the other hand, pharmacological approach involves the bind of particular molecules to smooth muscle cells surface receptors. All these signaling lead to an increase of intracellular calcium levels and consequent vasoconstriction. The outermost layer of the blood vessel is characterized by the tunica externa, or tunica adventitia, composed entirely from connective fibers and surrounded by an external elastic lamina which functions to anchor vessels with surrounding tissues. This layer is often thicker in veins preventing collapse of the blood vessel, and also as veins are often more superficially located providing protection from damage.

1.5.2 Endothelial function

The endothelium is a monolayer of cells covering the vascular lumen. For many years this cell layer was thought to be relatively inert, considering it just a barrier between circulating blood and the underlying tissues. Now it has been recognized that endothelium is indispensable in preserve the physiological vascular homeostasis through a paracrine, endocrine and autocrine action [46]. Indeed, the endothelium participates in multiples vascular functions, such as the regulation of vessel integrity, vascular growth and remodeling, tissue growth and metabolism, immune response, cell adhesion, angiogenesis, fibrinolysis and thrombogenesis of platelets and vascular permeability [47]. Beside all these functions, endothelium shows its pivotal role in the regulation of vascular tone, controlling tissue blood flow [48]. The research in the past few decades has elucidated several molecular pathway through which the endothelium controls the

vascular tone. In particular, endothelial cells are responsible of release mediators which participate in the maintenance of vascular homeostasis [49]. Some of these are: the endothelium derived hyperpolarization factor (EDHF), Nitric Oxide (NO) [50] and prostacyclin (PGI2) [51], which are responsible of a vasodilatory and antiproliferative effects, while endothelin-1 (ET-1) [52], angiotensin II and reactive oxygen species (ROS) are the main mediators involved in the vasoconstriction events [53]. Moreover, endothelium-derived mediators also exert opposite effects on coagulation process: endothelial derived NO and PGI2 induce antithrombotic pathway inhibiting platelets aggregation, while other molecules, such as the Von Willebrand factor, and plasminogen activator inhibitor-1 which respectively promote platelets aggregation and inhibit fibrinolysis [54, 55].

However, the crucial player in the regulation vascular homeostasis is the Nitric Oxide, thus the disturbing of endothelium integrity and consequently NO release leads to endothelium dysfunction.

1.5.3 eNOS and NO

The role of endothelial cells in regulating vascular tone was first recognized with the observation that *acetylcholine* caused vasoconstriction when applied directly to de-endothelialized blood vessels, but induced vasodilatation in physiological vessels. The hypothesis that is still valid in the field is that the activation of the muscarinic receptor on endothelial cells by acetylcholine, induces the production of a relaxant molecule in endothelial cells, which after diffusing to subjacent vascular smooth muscle cells, activates the Guanylyl Cyclase and the subsequent vasorelaxing pathway. At the beginning this putative vasodilatory

compound was termed endothelial-derived relaxing factor, or EDRF. Then, with the discovery of nitroglycerin, the identity of EDRF passed to the Nitric Oxide (NO). Nitroglycerin is an organic nitrate commonly prescribed for angina pectoris. The effects of this molecule starts after its metabolization in the body in order to release NO which is responsible for the relaxation of vascular smooth muscle.

NO, a free radical gas produced by Nitric Oxide Synthase (NOS) family of proteins, is a ubiquitous second messenger of the cardiovascular system involved in the regulation of different physiological process such as vasodilation, anti-coagulation, vascular remodeling and angiogenesis. Three different isoforms NOS have been discovered in mammalian: 1): neuronal NOS (nNOS or NOS-1), 2) inducible NOS (iNOS or NOS-2) activated in an inflammatory state and 3) endothelial NOS (eNOS or NOS-3). *In-vitro* and *in-vivo* studies using NOS inhibitors, NO donors or using mice deficient in each of three mammalian isoforms have shown the importance of NO in the cardiovascular system. Indeed, the absence of all the three NOSs isoforms caused a drastically reduction of endothelium-dependent vasorelaxation in murine arteries and also a increased the risk of cardiovascular diseases in these mice, including hypertension, cardiac hypertrophy, diastolic heart failure, arteriosclerosis and myocardial infarction leading to an earlier death of the animals [56]. This highlights the importance of Nitric Oxide in the regulation vascular physiological homeostasis.

Placed in endothelial cells, eNOS is a multi-domain enzyme consisting of an N-terminal oxigenase domain and a C-terminal reductase domain with a CaM binding the region connecting the two domains. Several co-factors

are required for the synthesis of NO, namely tetrahydrobiopterin (BH_4), Nicotinamide Adenine Dinuclotide Phosphate (NADPH), Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN); the enzyme also contains binding sites for CaM and heme. The substrate necessary for the NO formation is L-arginine. eNOS, as all the three NOS isoforms, is synthesized as a monomer which needs to form dimmers in order to bind BH_4 and the substrate L-arginine to catalyze NO production. When the complex Ca^{2+}-CaM binds to the CaM binding domain in eNOS, the enzyme is activated and interacts with another monomer [57]. The zinc ion (Zn) is responsible for connecting the monomers at the heme group, which are resistant to the dimerization. This connection is further stabilize by BH_4 [58]. Following the formation of this complex, an electron flux occurs from NADPH to FAD and FMN at the reductase domain towards the oxigenase domain where they are accepted by heme iron. At this point, eNOS can utilize the donated electron and molecular oxygen to catalyze the conversion of L-arginine to form L-citrulline and NO [57] (Figure 1.7).

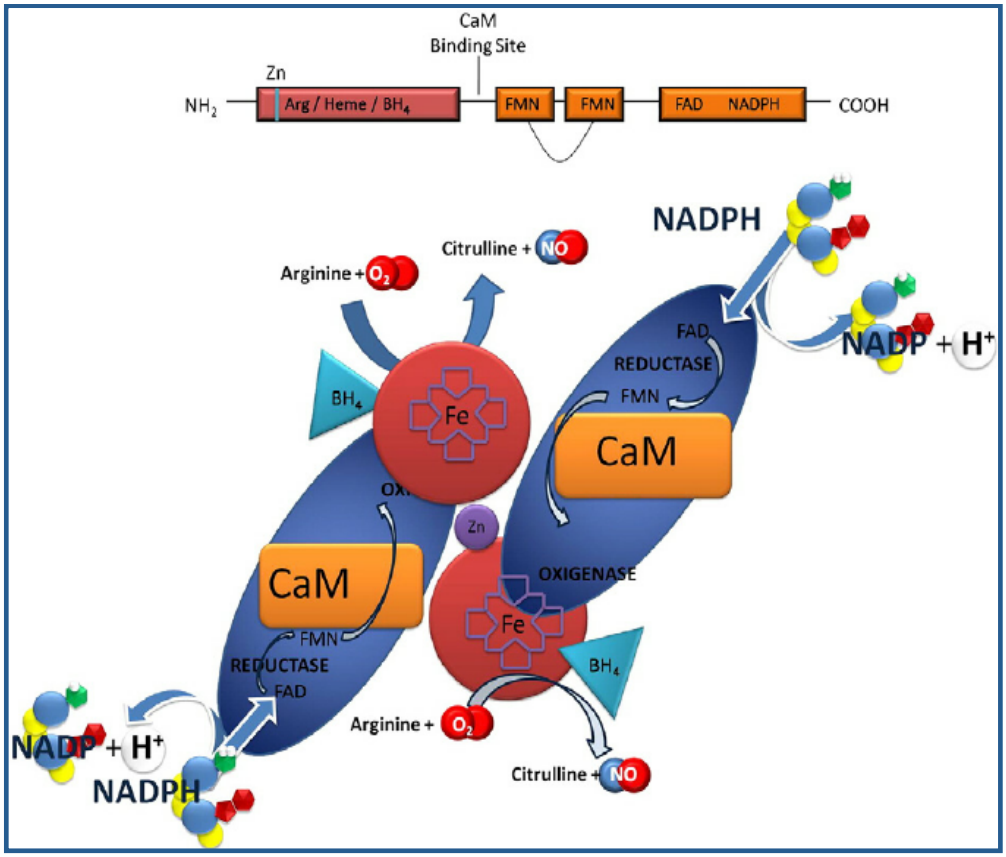


Figure 1.7 ***eNOS and the biosynthesis of Nitric Oxide (NO)***

However, eNOS can also acts as a monomer, but in this condition it is only able to generate O^{2-} instead of NO from their oxigenase domain. This condition is called un-coupling and causes an increase of the oxidative stress and a reduction of NO production [59] (Figure 1.8).

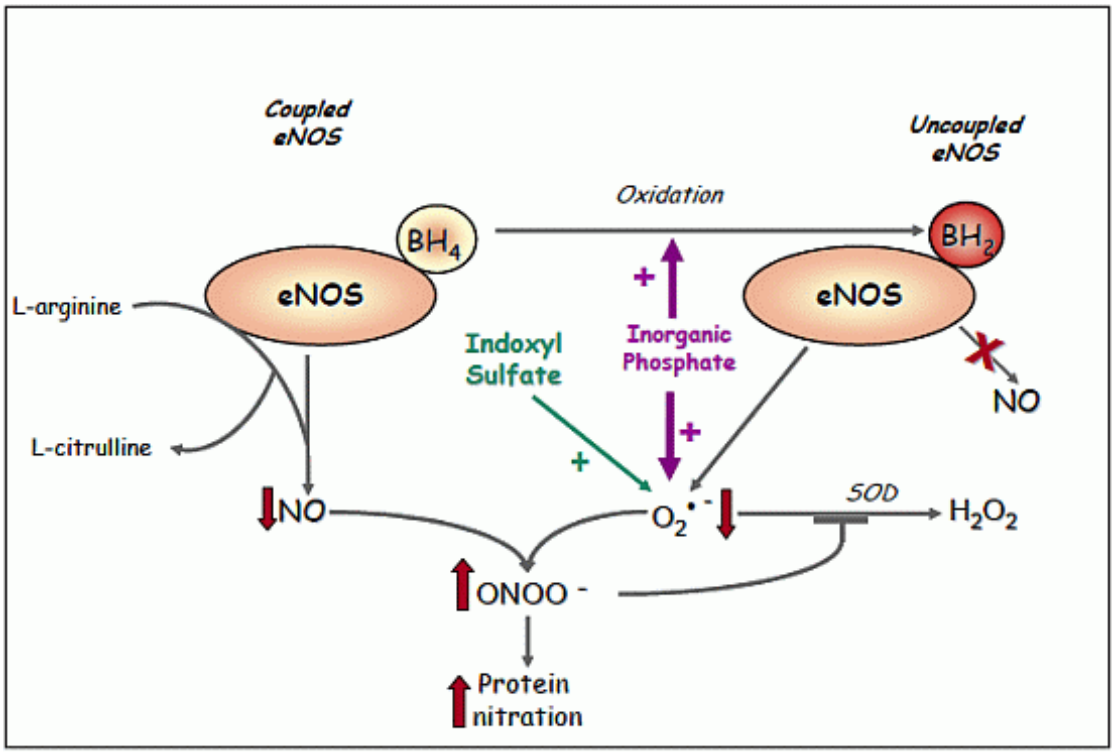


Figure 1.8 ***Uncoupled eNOS***

1.5.4 Cav-1 and eNOS interaction

Vascular production of NO is predominantly regulated by eNOS activity specifically in endothelial cells. It is important to know that in basal condition, eNOS is expressed in the caveolae, cell-surface plasma membrane invaginations, which are present in several different cells including endothelial, adipocytes and muscles cells. These portions of the plasma membrane are marked by the presence of particular protein called caveolin (Cav-1, -2, -3), which are important in their formation. In caveolae, eNOS interacts with caveolins. Among the three isoforms of caveolin, caveolin-1 (Cav-1) principally interacts with and regulates eNOS by inhibiting its enzymatic activity [60, 61]. Several stimulus that are able to activate eNOS, serve to break down the bond between Cav-1 and the

enzyme. Indeed, different endogenous receptor-operated substances like acetylcholine, bradykinin and histamine acting on their own endothelial receptor, increase intracellular Ca^{2+} levels and by the formation Ca^{2+}-calmodulin complex calmodulin is directed to replace caveolin in its interaction with eNOS [62]. Thus eNOS binding calmodulin dissociates from Cav-1 and recovers its activity. At this point eNOS is a free enzyme in the cytosol of endothelial cells, which can be phosphorylated and activated to increase NO bioavailability. On the contrary, once Ca^{2+} concentrations return to basal levels, Cav-1 re-interacts with eNOS gaining again its place in caveolae (Figure 1.9). On this basis, Cav-1 represents an important modulator of eNOS activity, and several studies have demonstrated how the interactions between Cav-1 and eNOS plays a role in vascular homeostasis regulation [63]. Experiments on mouse aorta rings mounted in insolated organ bath, have shown that a cell-permeable peptide containing the region of caveolin-1 that binds to eNOS, was able to inhibit acetylcholine-induced vasodilation [64] while the completely absence of Cav-1 mice, allowed a significantly enhancement of the relaxation response induced by Acetylcholine, which was reversed by the inhibition of eNOS [65]. Since NO is also involved in vascular permeability, other experiments have been performed to evaluate the effect of Cav-1-eNOS interaction on edema. As expected, the injection of caveolin-1 scaffolding domain-containing peptide was able to ameliorate the edematous response in mice [64], while the loss of Cav-1 expression markedly increases the vascular permeability [66]. This hyper-permeability was reversed by eNOS inhibition, demonstrating the necessity of Cav-1 for a proper tonic inhibition of eNOS.

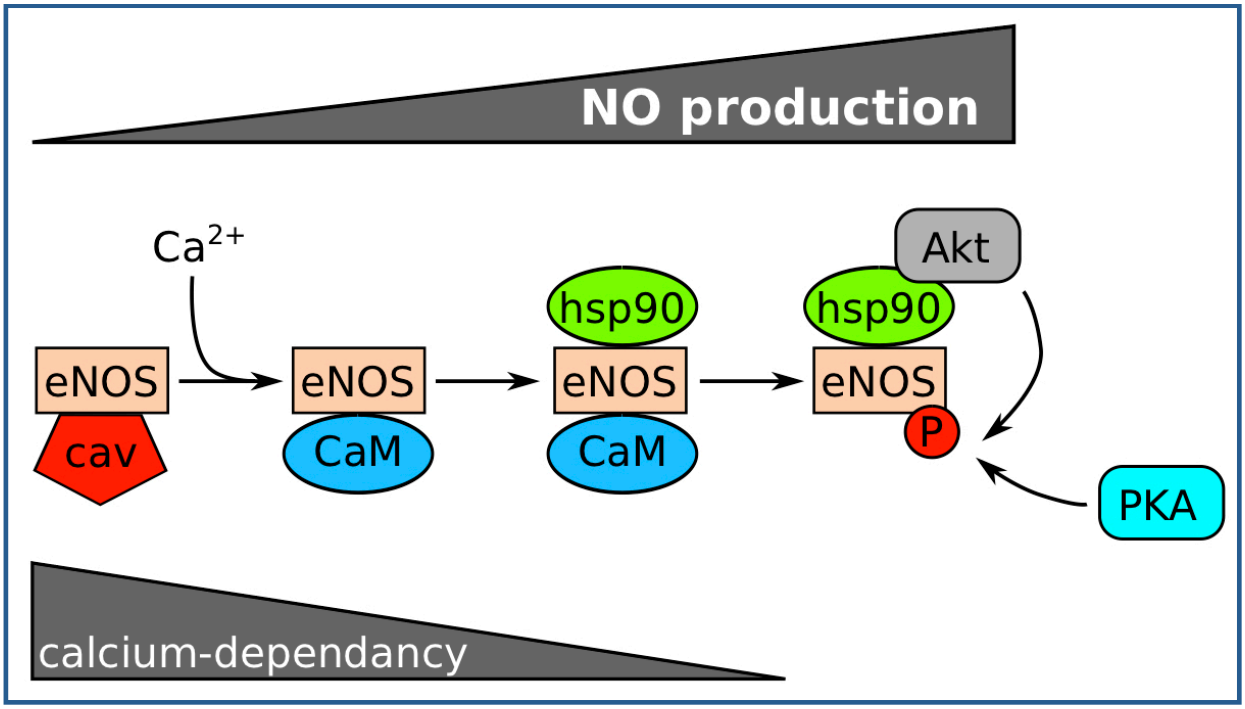


Figure 1.9 ***Inhibitory effect of Cav-1 on eNOS activity***

1.5.5 Endothelial dysfunction

Endothelial dysfunction is strictly correlated to the reduction of eNOS expression or activity, which leads to a decrease of NO bioavailability [67]. Oxidative stress is one of the major mechanisms involved in the alteration of endothelial cells function. In endothelial cells reactive oxigen species (ROS) are responsible to quench NO with formation of a peroxynitrite [68], which as cytotoxic oxidant is responsible for nitration and consequent lost of protein functions. Moreover, peroxynitrite leads to the reduction of the eNOS cofactor BH_4 in BH_2 causing the uncoupling eNOS process [53]. In this condition eNOS goes from its oxigenase function, which produce NO, to its reductase activity generating more ROS with consequent exacerbation of the oxidative stress.

eNOS activity in endothelial cells is also modulated by circulating factors like insulin [69]. This hormone is able to regulate vascular homeostasis with a vasodilator action exerted through the phosphoatidylinositol-3-kinase (PI-3K)/AKT pathway-dependent eNOS activation [69]. Moreover insulin prevents ROS production and sustains eNOS activity by increasing BH_4 [70], for these reason insulin resistance condition can be associated to a reduction of eNOS activity, NO bioavailability and following endothelial dysfunction. Oxidative excess is also linked to a pro-inflammatory state of the vessel wall with an up-regulation of adhesion (VCAM-1 and ICAM-1) and chemotactic (MCP-1) molecules [71]. This inflammatory condition in vascular endothelial wall further reduces NO bioavailability and increase endothelial dysfunction.

A relatively new and attractive mechanism that leads to reduced NO production is Asymmetric Dimethyl arginine (ADMA), an endogenous competitive inhibitor of eNOS that has been linked to endothelial dysfunction [72, 73]. ADMA is a product of protein turnover and is eliminated by excretion through the kidneys excretion or metabolism induced by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline in hepatic cells. Patients suffering of chronic renal failure or hepatic dysfunction are characterized by high ADMA plasma levels which lead to a reduction of effective renal plasma flow and increased renovascular resistance and blood pressure, with further reduction of heart rate and cardiac output [74-76]. Moreover, plasma ADMA levels are also increased in patients affected by hypercholesterolemia and hypertension [77, 78] and can be also associated to increased cardiovascular risk factor, such as carotid intima-media thickness, concentric left ventricular hypertrophy and left ventricular dysfunction [79, 80]. However, it has been shown that

infusion of l-Arginine, the physiological substrate of eNOS and competitor of ADMA is able to normalize endothelial function and cardiovascular homeostasis [81].

NO bioavailability is also reduced by the increase of homocysteine in endothelial cells responsible of the increase of the oxidative stress. Cardiovascular problems are associated to high levels of homocysteine in the blood since this molecule is responsible to increase plasma concentration of ADMA by inhibition of DDAH enzyme [81, 82].

Endothelium and vascular function is also affect by the renin-angiotensin system (RAS). Ang II also causes an increase of ROS by stimulating NADPH oxidase and promoting vascular inflammation [83]. These mechanisms are potentiated in the hypertensive conditions and represent the reason of the vascular dysfunction observed in these patients. Indeed, in hypertensive human patients, interruption of the renin-angiotensin system with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers restores endothelial function [84].

Thus, all of these mechanisms are exacerbated in several pathological conditions such as hypertension, hypercholesterolemia with elevated oxidized LDL and low HDL-cholesterol, hypertriglyceridemia, atherosclerosis, renal and hepatic dysfunction, hyperglycemia and diabetes. Vascular dysfunction is the common factor in all these diseases and the therapeutic approach does not only include the treatment of the underlying disease which albeit only in some conditions, but it tries also to act on the components which trigger to the endothelium dysfunction.

1.6 Diabetes as the main risk factors of cardiovascular diseases

From the point of view of the cardiovascular medicine, diabetes is considered a cardiovascular disease. Several epidemiological and pathological data demonstrate that diabetes is an independent risk factor for CVDs, listed as the cause of death in 65% of people with diabetes. Diabetic patients often manifest large number of vascular occlusion compared with age-matched healthy people, which lead to an increase in the development of peripheral and cerebral-vascular diseases. The main consequence of the vascular occlusion is the ischemia. Ischemia is caused by a restriction of blood supply to tissues, which causes a reduction of oxygen and glucose for the cells and also an inadequate removal of metabolites. These processes are important in order to maintain cells alive, thus their alteration leads to a progressive cellular apoptosis. In order to compensate to this vascular the organism spontaneously develops new collateral blood vessel as a response to the vascular occlusion, trying to maintain the vitality of the tissue after the ischemia. This mechanism of neo-vascularization is called *angiogenesis* and is a common clinical observation that it results impaired in diabetic patients. The main growth factors involved in the generation of new blood vessels is the endothelium-derived VEGF. VEGF-mediated angiogenesis mechanism is active in ischemic tissue in order to compensate for the suppression of blood flow. Using the experimental model of Non Obese Diabetic (NOD) mice, which spontaneously develops a diabetic pathology similar to the human insulin dependent diabetes mellitus, Rivard and his colleagues in 1999 have shown that VEGF levels in ischemic tissues from

these mice are lower than the control. On the contrary, when NOD mice were injected with a replication-deficit adenovirus coding for murine VEGF, it has been demonstrated a significant improvement of blood flow and capillary density [85]. This confirms that diabetic condition, alters the VEGF-mediated angiogenesis pathway, leading to peripheral and central vascular occlusion and to a worsening of previously cardiovascular disease.

The link between diabetes and vascular diseases is today well confirmed and involves the activation of different molecular mechanisms regulating the vascular homeostasis. Plasma glucose is responsible of the advanced-glycation end products (AGEs). AGEs are proteins or lipids that become glycated as a result of exposure to sugars. In the context of the vascular diseases, AGEs induce a crosslinking of collagen, which can cause vascular stiffening, and entrapment of low-density lipoprotein particles (LDL) in the artery walls. At this point, AGEs cause glycation and oxidation of LDL, which represent the major factors in the development of atherosclerosis [86]. Finally, AGEs can bind to Receptor for Advanced Glycation End-products (RAGE) causing oxidative stress as well as activation of inflammatory pathways in vascular [87]. Hyperglycemia causes an increase of intracellular AGEs formation and also increment the expression of RAGE exacerbating the vascular damage [88].

Diabetes conditions is also associated increased levels of Free Fatty Acids (FFAs) which impair the endothelial function [89]. FFAs facilitate the activation of protein-kinase C (PKC) in the vasculature which is known to be associated with vascular alterations such as increase in permeability, contractility, extracellular matrix synthesis, cell growth and apoptosis,

angiogenesis, leukocyte adhesion and cytokines activation [90]. In particular in the pathological state of diabetes, PKC activates NADPH oxidase intensifying the oxidative stress [91]. Moreover, FFAs are also associated to AGEs formation and their interaction with RAGE [91].

Insulin resistance, which mainly characterized the diabetes, in particular type 2, is also responsible of the exacerbation of the vascular dysfunction in these patients. Insulin, acting on a tyrosine-kinase receptor in endothelial cells, is responsible to activate of eNOS/NO pathway which not only regulate the vasodilation but also inflammatory and thrombotic mechanism in the vascular wall. Moreover, insulin modulates eNOS activity by regulating the expression of BH_4 [70]. Thus, absence of this signaling is responsible to further increase oxidative stress and endothelial impairment. Conversely, since insulin receptor (IR) is strongly associated to eNOS activation, it has been demonstrated that eNOS plays one of the major role in the regulation of insulin sensitivity due to the functions of NO in peripheral tissues [92]. Previous studies have also shown that mice lacking eNOS are more likely to develop insulin resistance, while modulation of eNOS phosphorylation affects insulin sensitivity [93]. In this context, is possible that eNOS phosphorylation could be a novel target for the treatment of insulin resistance helping also the rescue of the endothelial homeostasis.

On the basis of this evidences, it appears obvious that NO bioavailability reduction is the common factor in all these altered vascular mechanism, and it is the mainly responsible of the endothelial dysfunction and CVD observed in diabetic patients. Salheen and colleagues confirmed these hypotheses when in 2015 they demonstrated that STZ-induced T1DM rats

show a reduction of basal production of NO in mesenteric artery compared to the healthy animals confirming by a reduced contractile response to the NOS inhibitor L-NNA [94].

Treatment with antidiabetic drugs reduces plasma glucose levels and increases insulin sensitivity being *per se* responsible to ameliorate vascular homeostasis. However lowering glucose levels is not sufficient to switch off intracellular pro-oxidant environment or completely restore the physiological vascular reactivity, indeed, vascular damage can perpetuate despite the achievement of improved glycemic control. In vitro experiments on cultured endothelial cells as well as in vivo studies on diabetic animals, have shown that after plasma glucose levels normalization, ROS and oxidative marker continued to increase vascular stress [95]. For this reason, new therapies are not only focused to lower hyperglycemia, but also to ameliorate vascular functions by directly acting on the endothelial molecular mechanisms responsible of the impairment of eNOS/NO pathway. In this regard, it has been show that a particular class of antidiabetic drugs, DPP-IV inhibitors, induce beneficial effect on vascular homeostasis regulation, beyond their capability to control glucose levels DPP-IV increasing incretinic system function.

1.6.1 Incretin therapy and its incidence on vascular homeostasis regulation

Incretin system acts by improving glucose metabolism through the upregulation of insulin secretion and suppression of glucagon release. Incretin analog and DPP-IV inhibitors potentiate the anti-hyperglycemic effect of incretin system, however it has been shown that beyond the regulation of blood glucose levels, these drugs have cardio-protective

actions, reduce atherosclerosis, and show beneficial vascular effect by improving the endothelial function in diabetic patients [96, 97].

In vitro studies on Human Umbilical Vein Endothelial Cells (HUVEC) have demonstrated that *tinigliptin* as a molecule belonging to DPP-IV family has antioxidant proprieties by reducing ROS concentration and oxidative stress in hyperglycemic conditions. This data has been also confirmed by clinical studies, which have evaluated the effect of *teneligliptin* and *sitagliptin* treatment in T2DM diabetic patients. Even if both two DPP-IV inhibitors induce the same effect on bloodstream control*, teneligliptin* also improves endothelial function by reducing the oxidative stress markers reactive oxygen metabolites (ROMs) and reduces the incidence of the chronic kidney disease, while *sitagliptin* doesn't affect the vascular profile. Thus, the beneficial effect of *tiniligliptin* on vascular homeostasis is independent of blood-glucose lowering effect. [98].

In vitro studies have shown that the activity and expression of DPP-IV in endothelial cells appears to be increased when glucose levels are elevated [99]. For this reason the use of DPP-IV appears to be able to protect endothelial cells from the negative effects due to the hyperglycemic condition. It's well know that endothelial cells show, not only DPP-IV enzyme, but also the GLP-1 receptor (GLP-1R) [100] and inhibition of DPP-IV induces a significantly increase of GLP-1 levels promoting its interaction with the GLP-1R expressed in endothelial cells. This interaction leads to an increase of cAMP in endothelial cells and a subsequently activation of K_{ATP} channel, regulating the vascular tone [101]. Since, NO is the mainly indicator of vascular endothelial function integrity and eNOS/NO pathway is impaired in diabetic condition, some

studies have focused their attention on the effect of incretin therapy on eNOS/NO signaling. Several experimental studies have confirmed that inhibitors of DPP-IV have favorable effects on systemic blood pressure and the control of vascular tone as well as endothelial growth and that these effects are not dependent on the activation of the GLP-1R [102, 103]. Indeed, Shah et al have demonstrated that *alogliptin*, a DPP-IV inhibitor, induces vasodilatation in isolated vessels, which is not altered by GLP-1R antagonist [104]. STZ-induced diabetes in rats causes a progressive development of endothelium function impairment [105] related to a drastically decrease of NO production from ECs which has been confirmed by a reduction of Ach-induced vasorelaxation in isolated vessel and a reduction of basal contraction induced by NOS inhibitor Nω-nitro-L-arginine (L-NNA) [94]. Additionally, in order to investigate the effect of diabetes on vascular smooth muscle relaxation it has been evaluated the Sodium Nitroprusside-induced vasorelaxation (SNP), as a NO donors, on isolated mesenteric arteries. The response to SNP in this study is similar in normal and diabetic rats, indicating that hyperglycemia only affects NO-endothelium production but not its effect on VSMCs. However in this mirror of diabetes-induced vascular dysfunction, treatment with *linagliptin* improves Ach-induced relaxation in mesenteric arteries, probably by preventing the generation of superoxide anions via the antioxidant effect of *linagliptin* [106], reducing eNOS uncoupling and improving the bioavailability of NO in vascular endothelial cells [94]. Moreover, Salheen and colleagues demonstrate that basal contraction induced by NNA increases in mesenteric artery from diabetic rats treated with *linagliptin* compared the control, confirming that the DPP-IV inhibitor has an

important role in improving NO bioavailability and ameliorating endothelial vascular function [94].

1.7 Aim of the PhD Project

Incretin and DPP-IV inhibitors therapies have been deeply correlated to an improvement of vascular endothelial function in diabetic condition. However, despite all the evidences, the molecular mechanism through which incretin and DPP-IV inhibitor improve NO bioavailability and ameliorate endothelial function is not yet clear. Before starting my PhD project, we have assessed some experiments to evaluate a possible cross talk between DPP-IV and the pathway L-Arginine/eNOS/NO using cells Human Embryonic Kidney (Hek), stably transfected with the enzyme eNOS (HEK-eNOS). Hyperglycaemic condition caused a drastically reduction of NOx production from the cells, as an indirect index of eNOS activity, both in basal and after the exogenous stimulation of Calcium ionophore. These first data were in accordance with an increase of Cav-1 expression and a reduction of eNOS in Hek-eNOS cells. Incubation of the cells with linagliptin showed a greater increase of NOx production and a reduction of CAV-1 expression, while eNOS expression was not affected. These preliminary data were the first confirmation for us that linagliptin had a role in CAV-1/eNOS/NO signaling regulation. On this evidence the aim of my project is to clarify role of incretin hormones and DPP-IV inhibitors in the control of vascular homeostasis, both in physiological and physio-pathological focusing on the modulation of eNOS/CAV-1 as a possible molecular mechanism responsible for the beneficial vascular effect of DPP-IV inhibitors.

2. METHODS

2.1 Animals

All animal procedures were performed according to the Declaration of Helsinki (European Union guidelines on use of animals in scientific experiments) and following ARRIVE guidelines and authorised by local animal care office (Centro Servizi Veterinari, Università degli Studi di Napoli "Federico II"). This work has been carried out using: NOD/Ltj and age-matched CD-1 control mice (Charles River, Calco, Italy). GLP-1R$^{-/-}$ mice and Guanilate Cyclase-α1 ($GC_{\alpha1}^{-/-}$) mice has been also used and supplied by Boheringer Ingelheim (Ingelheim, Germany) and Prof. P. Brouckaert from the University of Ghent (Belgium), respectively. Mice were kept in an animal care facility under controlled temperature, humidity and light/dark cycle conditions, with food and water *ad libitum*.

2.2 Reagents

Calcium ionophore A23187, Trizma base, Trizma HCl, Sodium chloride (NaCl), Glycine, Methanol, Nonidet-40 (NP-40), cocktail of protease and phosphatase inhibitors, Sodium Ortovanodate (Na_3VO_4), TritonX, Sodium-deoxycholate, Sodium Dodecyl Sulphate (SDS), Ammonium Peroxydisulfate (APS), Acrylamide, N N,N,N-Tetramethyl ethylenediamine (TEMED), 2-mercaptoethanol, EDTA, Luminol (a-8511), p-coumaric acid, Tween-20 and all the salts of Krebs solution were purchased from Sigma-Aldrich (Italy, Milan). Antibodies Anti-caveolin-1, Anti-eNOS and Anti-GAPDH were purchased from Santa Cruz respectively Biothecnology Inc (Santa Cruz, California, USA), from BD Biosciences and Sigma-Aldrich (Italy, Milan). All reagents for cell culture were purchased from Lonza.

Fluo-4 NW Calcium Assay Kit was purchased from Invitrogen, Carlsbad, California). The Linagliptin was kindly provided by Boehringer (Germany).

2.3 Non Obese Diabetic (NOD) mice

Not Obese Diabetic mice (NOD) are an experimental animal model characterized by a marked susceptibility to hyperglycemia leading to the development of a diabetic pathology similar to human type 1 diabetes, *IDDM*. Measuring once a week glycosuria levels these animals were classified into three groups, in relation to the severity of the diabetic pathology (Figure 9):

- The NOD or NOD I, the animals at this stage not yet show signs of diabetic pathology and present levels of glycosuria less than 20 mg / dl (0 <gl <20 mg / dl);
- NOD II, such a stage is reached at about the thirteenth week of age and of glycosuria levels are between 20 mg / dl and 500 mg / dl (20 <gl <500 mg / dl);
- NOD III, the animals, the twenty-second week of age, showing signs of a full-blown diabetic condition with urine glucose levels over 500 mg / dl (gl> 500 mg / dl).

NOD I: 0<glicosuria<20 mg/dl = low or null glycosuria

NOD II: 20<glycosuria<500 mg/dl = high glycosuria

NOD III: 500<glycosuria<1000 mg/dl = severe glycosuria

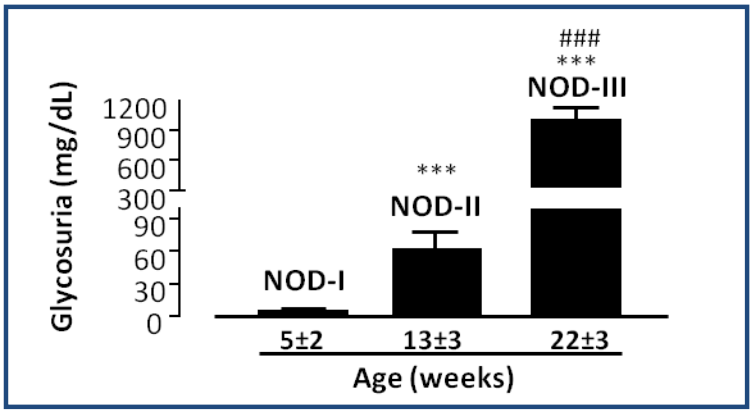


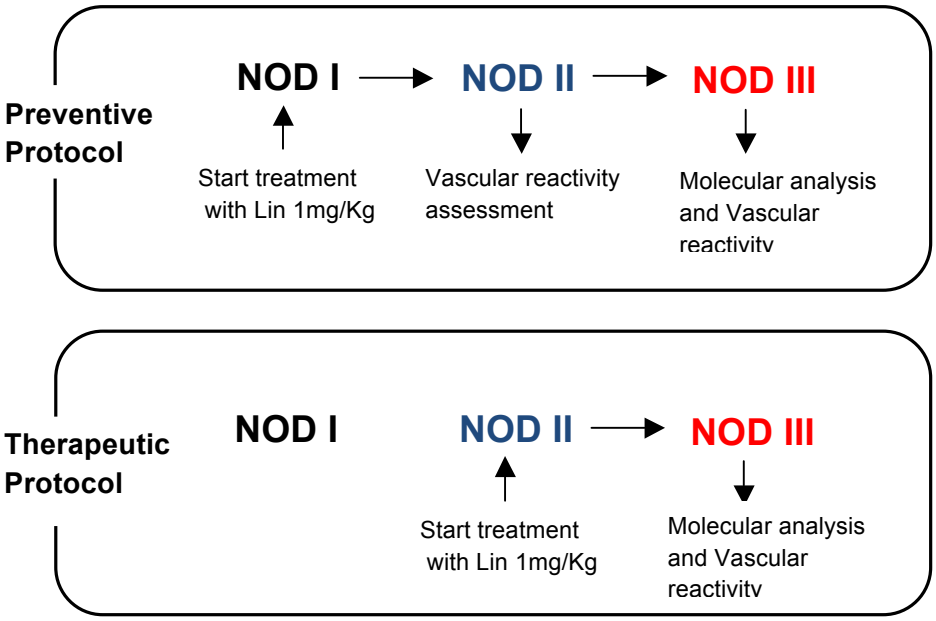
Figure2.1 ***Gradually increase of NOD glycosuria levels during diabetes development***

2.4 In-vivo treatment with Linagliptin in NOD/Ltj mice

NOD mice are treated with vehicle or with *linaglitin* (Boehringer Ingelheim) at a dose of 1mg/kg/day by gavage, following two different protocols:

- ***Preventive protocol***: treatment with *linagliptin* occurs when the animals are considered still healthy, and continues until they reach the stage of NOD II and NOD III, monitoring glycosuria levels during the therapy
- ***Therapeutic protocol***: treatment occurs when animals start to show the symptoms of diabetes with glycosuria levels corresponding to stage II, and the administration of *linagliptin* continues to the stadium of NOD III.

In both protocols, as a result of the treatment, the animals were sacrificed and the thoracic aorta was removed to evaluate asses molecular analysis and evaluate the vascular reactivity by ex-vivo studies in baby baths for isolated organs.



2.5 Isolated organ bath studies

2.5.1 Vascular tissue preparation

Males NODI-III, CD-1, GLP-1 Knock Out (KO) and Guanilate Cyclase (GC) KO mice were used to evaluate the vascular reactivity on aorta and carotids vascular tissues. Mice were anaesthetized with enflurane (5%) and then killed in CO2 chamber (70%). Aorta and carotid vessels were rapidly harvested, and adherent connective and fat tissue were removed. Aorta rings of 1-1.5 mm length were cut and placed in organ baths 3.0 ml of volume, while carotids rings 3.0 mm length were mounted 5 ml volume chamber. Both the vascular rings were filled with oxygenated (95% O_2 - 5% CO_2) Krebs solution and kept at 37°C. Aorta and carotid rings were connected to an isometric transducer respectively 7006-Ugo Basile (Comerio, Italy) and DMT Wire Myograph, and changes in tension were continuously recorded with a computerized system (DataCApsule-17400, Ugo Basile and Mac-Lab Recording System). The composition of the

Krebs solution was as follows (mM): 118 NaCl, 4.7 KCl, 1.2 MgCl2, 1.2 KH2PO4, 2.5 CaCl2, 25 NaHCO3 and 10.1 glucose. The rings were initially stretched until a resting tension of 1.5 g was reached and then were allowed to equilibrate for at least 30 min; during this period the tension was adjusted, when necessary, to 1.5 g and the bath solution was periodically changed. In a separate set of experiments, aortic and carotid rings were denuded from endothelium by gently rubbing the internal surface of the vascular lumen.

2.5.2 Experimental protocol

In each set of experiments, rings were firstly challenged with phenylephrine (PE, 1μM) until the responses were reproducible. In order to verify the integrity of the endothelium, cumulative concentration-response curves to Acetylcholine (Ach, 10nM – 30μM) were performed with 1μM PE pre-contracted rings. Rings not reaching a relaxation response of at least 75% were considered , while vessels reaching a vasodilatation less the 20% were considered endothelium-denuded.

Ach and Isoprenaline (Iso) curves (10nM – 30μM) has been performed on aorta rings from NOD I, NOD II and NOD III mice in basal conditions and following the preventive and therapeutic treatment with Linagliptin 1mg/Kg, to evaluate vessel's vasodilatation response both endothelium dependent and independent respectively.

Aorta and carotid rings from NOD III, CD-1, GLP-1 KO and GC KO mice were contracted with PE (1μM) and, once the plateau was reached, cumulative concentration-response curves to Linagliptin (LIN) and Sitagliptin (SIT) (100nM – 30μM) has been performed. Moreover, concentration-response curves of Linaglipton has been assessed on aortic

and carotid rings in presence or absence of endothelium, and after incubation with N^5-(1-iminoethyl)-L-ornithine dihydrochloride, L-Nio (NOS inhibitors) 10μM for 15 minutes and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, GC inhibitor) 10μM for 15 minutes and on vessels denuded from endothelium.

Krebs solution was used as the vehicle for all isolated organ bath experiments.

2.6 Cell culture experiments in a normal and high-glucose environment

Human Embryonic Kidney cells stabled transfected with eNOS (Hek-eNOS cells) and Bovine Aortic Endothelial Cells (BAEC) were cultured in Dulbecco Modified Eagle's Medium (DMEM) containing Fetal Bovine Serum 10%, HEPES (1M), Na-Piruvate (100mM), L-Glutamine (200mM), G418 and solution of Penicillin-Streptomycin (10.000 units of Penicillin and 10 mg Streptomic/ml). Cells at 80% of confluence were starved with Medium prepared without FBS. Hek-eNOS cells incubated with Linagliptin (0.1 – 10 – 100 nM for one hour and then treated for 2 hours with 25 mM D-glucose solution (Normal Glucose) or 50mM D-glucose solution (High Glucose). Immediately thereafter, cells were stimulated with Calcium Ionophore A23187 10μM for 30 minutes. Cells were then separated from the supernatant and utilized for western blot analysis (eNOS and Cav1 expression) while the medium was collected and used for nitrite/nitrate (NOx) assay. The same protocol was utilized for BAEC increasing the time of incubation of High Glucose to 3 hours.

2.7 Western Blot analysis

Hek-eNOS cells and aorta vessels, harvested from NOD III after the respective treatments described above, were individually homogenized in modified RIPA buffer (Tris HCl 50 mM, pH 7.4, triton 1%, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, phenylmethanesulphonylfluoride 1 mM, aprotinin 10 µg/ml, leupeptin 20 mM, NaF 50 mM) using a polytron homogenizer (two cycles of 10 sec at maximum speed) on ice. After centrifugation at 12,000 rpm for 15 min, protein concentration was determined by Bradford assay using BSA as standard (Bio-Rad Laboratories, Milan, Italy). Denatured proteins of each sample were separated on 10% SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in PBS-tween 20 (0.1%, v/v) containing 3% non fat dry milk for 1 hour at room temperature, and then incubated with anti-eNOS (1:500), anti Cav-1 (1:1000) or anti-GAPDH (1:1000) overnight at 4°C. The filters were washed with PBS-tween 20 (0.1%, v/v) extensively for 30 min, before incubation, for 2 hours at 4°C, with the secondary antibody (1:5000) conjugated with horseradish peroxidase antimouse IgG. The membranes were then washed and immunoreactive bands were visualized using an Enhanced Chemiluminescence Substrate (ECL; Amersham Pharmacia Biotech, San Diego, CA, USA). Images were obtained using ImageQuant-400 (GE Healthcare, Chicago, IL, USA). The housekeeping protein GAPDH (1:5000, Sigma-Aldrich, Milan, Italy) was used for normalization.

2.8 NOx Assay

NOx Assay was performed according to Thomsen [107], with modification. Cultured Medium of Hek-eNOS and BAEC cells were collected and incubated for 75 minutes with acid washed (0.24M HCl) cadmium powder (Sigma Aldrich) to reduce nitrate in nitrite. After the incubation period, the samples were centrifuged. The obtained supernatants were then used to quantify NOx levels trough a spetto-fluorometric assay with the following wavelengths: excitation 365 λ and emission 450 λ. NOx levels in each samples were interpolated trough a standard curve of $NaNO_2$ (50-2000nM).

2.9 Immunoprecipitation study

Immunoprecipitation analysis was performed on BAEC (300µg total protein) Lysates were pre-cleared by incubating with protein A/G-Agarose (SantaCruz Biotechnology, Hidelberg, Germany) for 1 hour at 4°C and the incubated under agitation for 18 hours at 4°C with the relevant antibodies. Subsequetly, samples were incubated for 1 hour at 4°C with fresh beads, washed in PBS and then the antigen-antibody complexes were collected by centrifugation. SDS-PAGE and immunoblotting were performed as described above, and mouse monoclonal anti-eNOS (1:500, BD-Trasduction Laboratories, Milano, Italy) and rabbit polyclonal anti-caveolin-1 (CAV-1, 1:1000, SantaCruz Biotechnology) were used as primary antibodies.

2.10 Measurement of intracellular Ca^{2+}

Changes in the intracellular Ca^{2+} concentration were measured in BAEC using Fluo-4 NW Calcium Assay Kit (Molecular probes, Invitrogen, Carlsbad, California).

BAEC cells were plated in 96-well microplates (polyd-lysine-coated plates) to near confluence in a concentration of 20000 cells/well. Dye solution Fluo-4, composed by Fluo-4 NW dye mix and Probenenecid) was prepared following the manufacturer's instructions. After cultured overnight cells with DMEM, they were washed with PBS and incubated with 100µl of the dye solution Fluo-4 at 37°C for 30 minutes, and then at room temperature for an additional 30 minutes. At the end of incubation, Fluo-4 solution has been replaced with a stabilizing solution pH 7.4 composed as follow: Hepes 20nM,NaCl 120mM, KCl 2 mM. $CaCl_2$ 2mM, $MgCl_2$*H_2O 1mM and Glucose 5mM. Ca^{2+} concentration were measure in basal conditions and after stimulation of Linagliptin 1-10-100nM and Bradykinin and A23187 were used as positive control. Fluorescence (excitation 485 nm, emission 525 nm) was measured for 3 minutes in a microplate fluorometer (Promega, Madison, WI, USA). Data were compared with BAEC stimulated with vehicle and expressed as relative fluorescence.

2.11 Statistical analysis

All data were expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnet's post-test, or two-way ANOVA followed by Bonferroni's post-test (GraphPad software, San Diego, CA, USA). Differences with p values <0.5 were taken to indicate statistical significance.

3. RESULTS

3.1 Diabetic conditions alter Acetylcholine and Isoprenaline-induced vascular homeostasis regulation

The development of T1DM in NOD mice is associated with a progressive impairment of vascular relaxation. Indeed, in aortic rings Ach induced a concentration dependent vasodilatation, which reached a maximum value (Emax) of 74.69±3.56% relaxation at a concentration of 30μM, while this effect was significantly reduced in NOD II and NOD III (Figure 3.1, A-B). The same results have been also observed with Iso. Diabetic conditions caused a significantly impairments of Iso-induced vasodilatation (Figure 3.1, C-D)

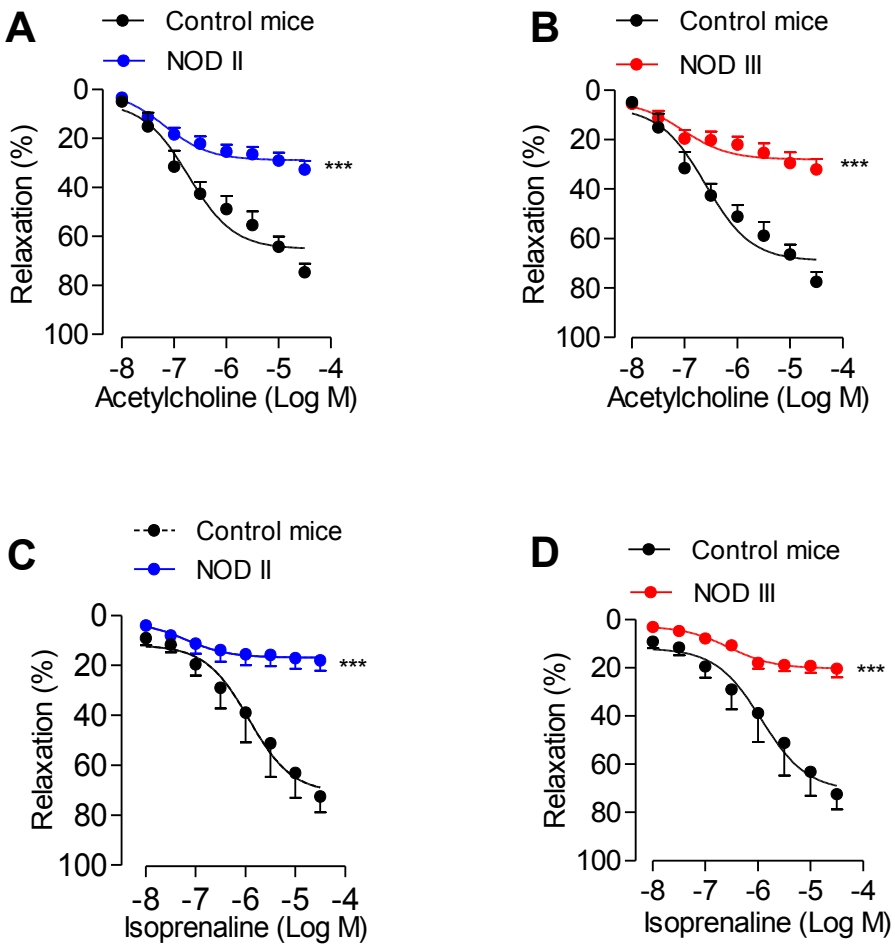
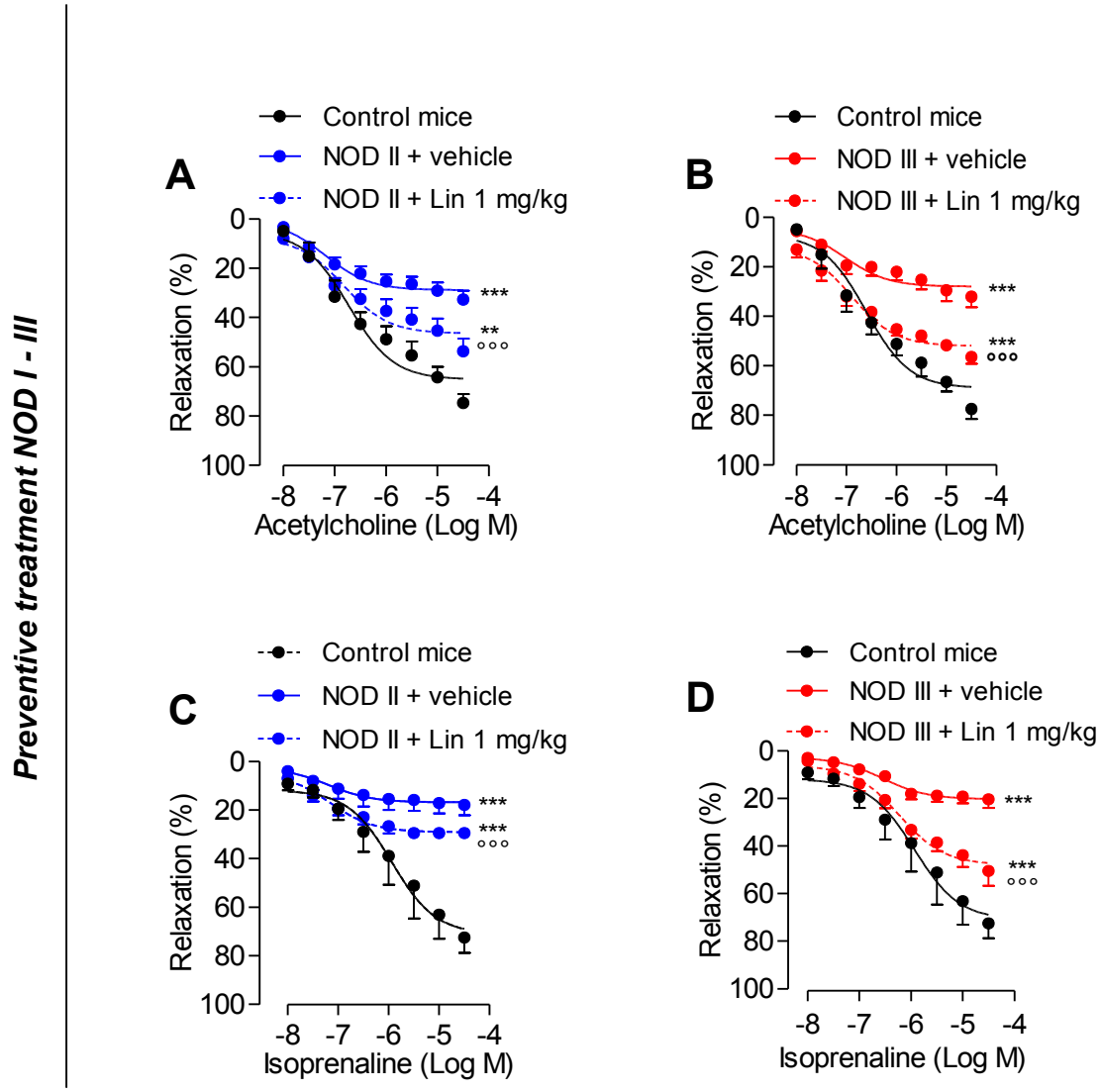


Figure 3.1***(A-B)*** *Ach-induced vasodilation in NOD II and NOD IIImice compared to control mice (EC50 Ach: 248,7nM vs 65,4nM and 93,4nM, CD1 vs NOD II and III, ***p <0.001, n = 4). (C-D) Vasodilation curve of Isoprenaline incontrol, NODII and NOD III mice (EC50 Iso: 1,166µM vs 64, 2nm and 279,8nM, CD1 vs. NOD II and III, ***p <0.001 n = 4)*

3.2 In-vivo treatment with Linagliptin ameliorates Ach and Iso-vascular response in diabetic mice without affecting the development of diabetes

NOD II and NOD III mice *in-vivo* treated with Linagliptin following both preventive (NOD I → NOD III) and therapeutic (NOD II → NOD III) protocol show an increase of both Ach and Iso

vasorelaxant effect, compared with mice were as been injected only the vehicle, even if the vasodilatation is not completely restored to the physiological condition (Figure 3.2, A-F). Moreover, glycosuria levels measurement during both preventive and therapeutic treatment with Linagliptin reveals that the vascular effect is totally. This effect is totally independent from the intrinsic action of the drugs in controlling blood glucose levels, since the development of the diabetic pathology in NOD mice in not reduced. (Figure 3.2, G-H)



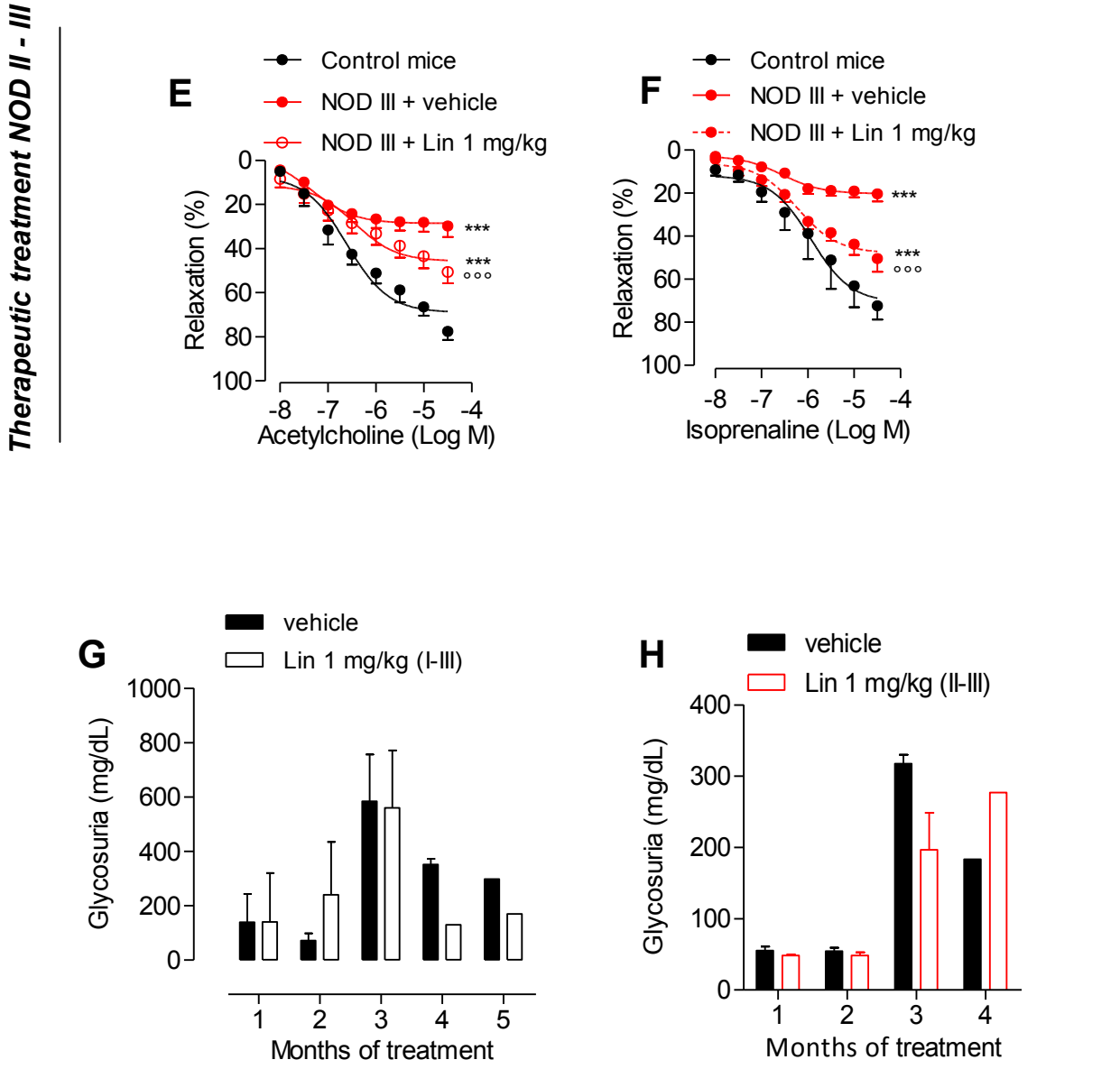


*Figure 3.2 Ach-induced vasodilatation in **(A)** NOD II and **(B)** NOD III mice after preventive treatment with Linagliptin compared to mice terated with vehicle (EC_{50} Ach NOD II: 149nM vs 65,4nM, Lin 1mg/ml vs vehicle, °°°p<0.001, n=20; NOD III: 128nM vs 93,4nM, Lin 1 mg/ml vs vehicle, °°°p<0.001, n=12) **(C-D)**: Vasodilatation curve of Isoprenaline in NODII and NOD III mice respectively after preventive treatment (EC_{50} Iso NOD II: 69,4nM vs 64,2nM, Lin 1 mg/ml vs vehicle, ***p<0.001, n=19; NOD III: 592,4nM vs 279,8nM, Lin 1 mg/ml vs vehicle, ***p<0.001, n=12). **(E)** Ach and **(F)** Iso-induced vasodilatation in NOD III mice after therapeutic treatment with Linagliptin ((EC_{50} Ach: 359nM vs 46,96nM, Lin 1mg/ml vs vehicle, *** p<0.001, n=5; EC_{50} Iso: 1,255µM vs 433,6nM, Lin 1 mg/ml vs vehicle, ***p<0.001, n=4). Glycosuria levels changes during Linaliptin administration following **(G)** preventive and **(H)** therapeutic protocol*

3.3 Treatment with Linagliptin increases eNOS and reduces Cav1 expression in NOD III mice

Since Ach and Iso-induced vasodilation involve Nitric Oxide (NO) release, which is the main endothelium-derived vasorelaxing mediator, we have evaluated the expression on eNOS and its plasma membrane protein regulator CAV-1 on homogenized aorta from Linagliptin treated NOD III. Several studies have previously shown that in diabetic condition the impaired endothelium-dependent vasodilatation is associated to an increased expression of CAV-1 [108]. Following this evidence, we have evaluated the effect of Linagliptin treatment on CAV-1 and eNOS expression in aorta harvested from NOD III mice. Linagliptin, in both preventive and therapeutic regimen, modulates the expression of these two proteins towards to a more active state. Indeed, in aorta homogenates eNOS analysis shows a trend of enhanced expression following Linagliptin treatment, associated to a reduction of CAV-1 (Figure 3.3, A-B) These data, taken together, suggest that Linagliptin exerts a positive action on eNOS/NO signaling by modulating CAV-1.

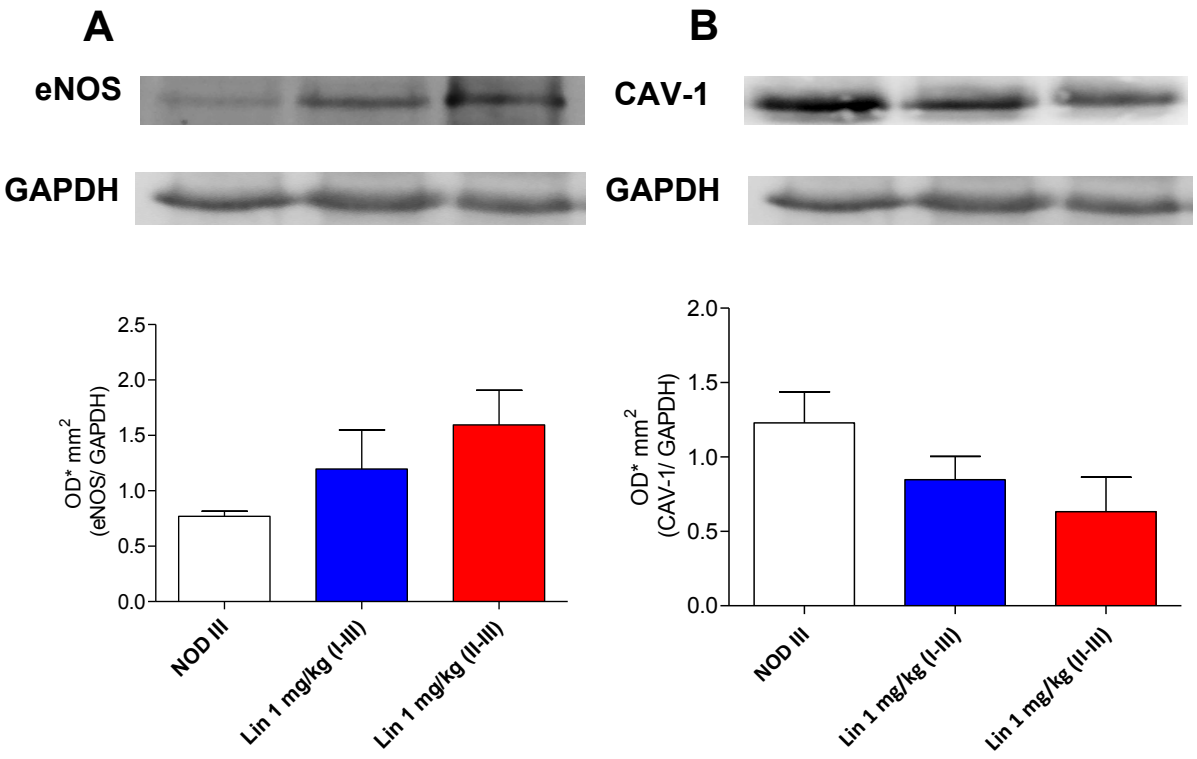


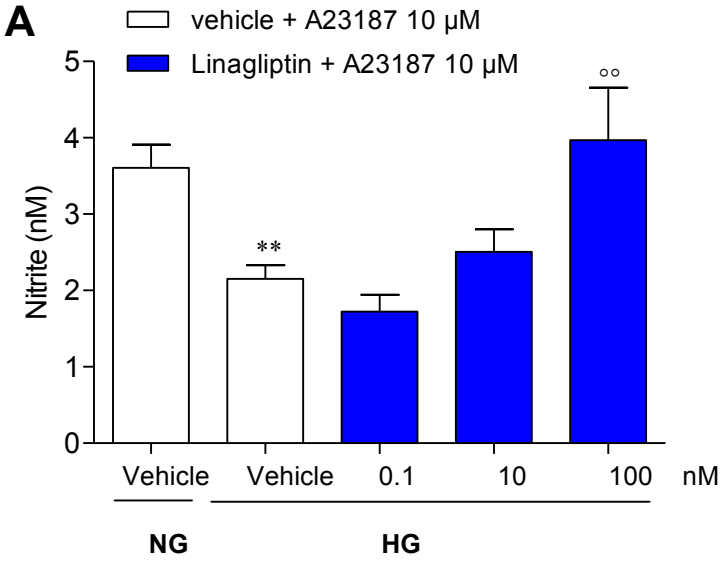
Figure 3.3 ***(A-B)*** *Western blot images of eNOS and CAV-1 performed on aorta homogenates harvested from NOD III mice after treatment with vehicle and Linagliptin.* ***(A)*** *eNOS expression in homogenized aorta from NOD III mice treated with Linagliptin following preventive (OD mm^2 eNOS/GAPDH: 0.77 ± 0.045 vs 1.198 ± 0.35 n=3) and therapeutic (OD mm^2 eNOS/GAPDH: 0.77 ± 0.045 vs 1.59 ± 0.31 *p<0.1 n=3) regimen.* ***(B)*** *Effect of Linagliptin treatment on CAV-1 expression in NOD III aorta (Preventive treatment, OD mm^2 Cav1/GAPDH: 1.23 ± 0.21 vs 0.84 ± 0.15, NOD III + vehicle vs NOD III + Lin, n=3; Therapeutic treatment, OD mm^2 Cav1/GAPDH: 1.23 ± 0.21 vs 0.63 ± 0.23, NOD III + vehicle vs NOD III + Lin, n=3)*

3.4 Linagliptin promotes eNOS activation and increases NO production in vitro model of endothelial cells

Starting with Human Embryonic Kidney cells stably transfected with endothelial enzyme eNOS, we have demonstrated that high glucose environment affects eNOS activity (reduction of NO production) in this *in-vitro* model. Indeed, in normal glucose environment, upon calcium ionophore A-23187 challenge, HEK-293 produces about 3.6 ± 0.30 nM of nitrite. When cells are cultured in high glucose levels, the NO release is

significantly reduced by 40% while Linagliptin treatment (1-100nM) in high glucose environment restores the physiological pattern observed in normal glucose environment (Figure 3.4, A). Indeed, Linagliptin gradually increases NOx levels in a concentration-dependent manner, with a maximal effect at 100nM. These data are supported by Western Blot analysis which prove that reduction of NO content in high glucose environment is associated to an increase of CAV-1 expression compared to control condition. However, after treatment with Linagliptin 100 nM, homogenized Hek-eNOS cells show a reduction of CAV-1 with consequent eNOS activation and enhanced NO levels in cell's medium (Figure 3.4, B).

Overall these data indicate eNOS/NO signaling as the molecular target for the beneficial action of Linagliptin on endothelial function.



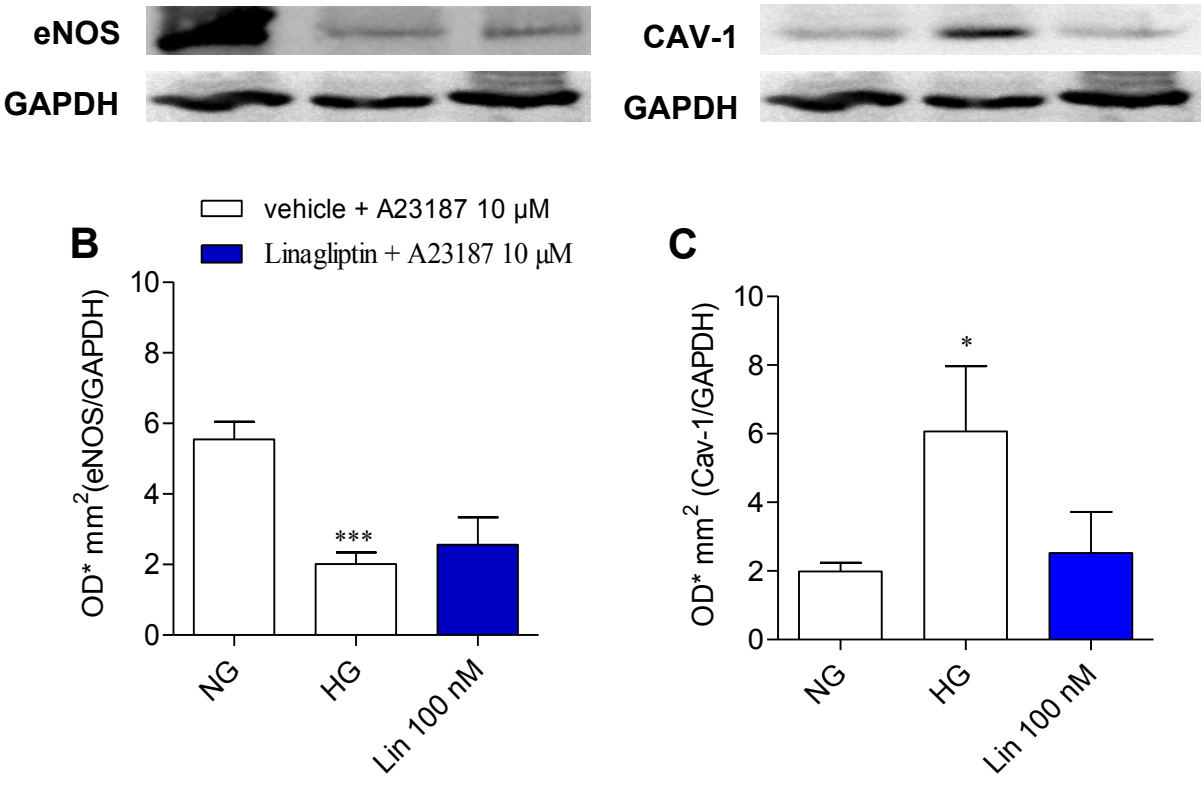


Figure 3.4 ***(A)*** *Effect of Linagliptin (0.1-1-10 nM) on Ca2+ ionophore A23187-induced NOx levelsin Hek-293-eNOS cells in Normoglycaemic (NG) and Hyperglacaemic (HG) environment (NOx concentration: 3.61nM ±0.30 vs 2.15nM ±0.17 as vehicle in NG vs vehicle in HG, **p<0.01, n=8; 2.15nM ±0.17 vs 3.96nM ±0.69, HG vs HG+Lin 100nM, °°p<0.01, n=3).* ***(B-C)*** *Western Blot images of eNOS and CAV-1 protein in Hek-293-eNOS cells in the HG conditions after treatment with Linagliptin 100nM. Data are compared with Hek-293-eNOS cells in the NG or HG condition treated with vehicle (B: OD mm^2 eNOS/GAPDH, 5.54 ±0.25 vs 2.014 ±0.33 vs 2.56 ±0.77 as NG vs HG vs HG+Lin 100nM, ***p<0.001 n3; C: OD mm^2 Cav1/GAPDH, 1.98 ±0.3 vs 6.06 ±1.9 as NG vs HG, *p<0.1 n=3; 6.06±1.9 vs 2.52±1.2, HG vs HG+Lin 100nM, °p<0.1 n=3).*

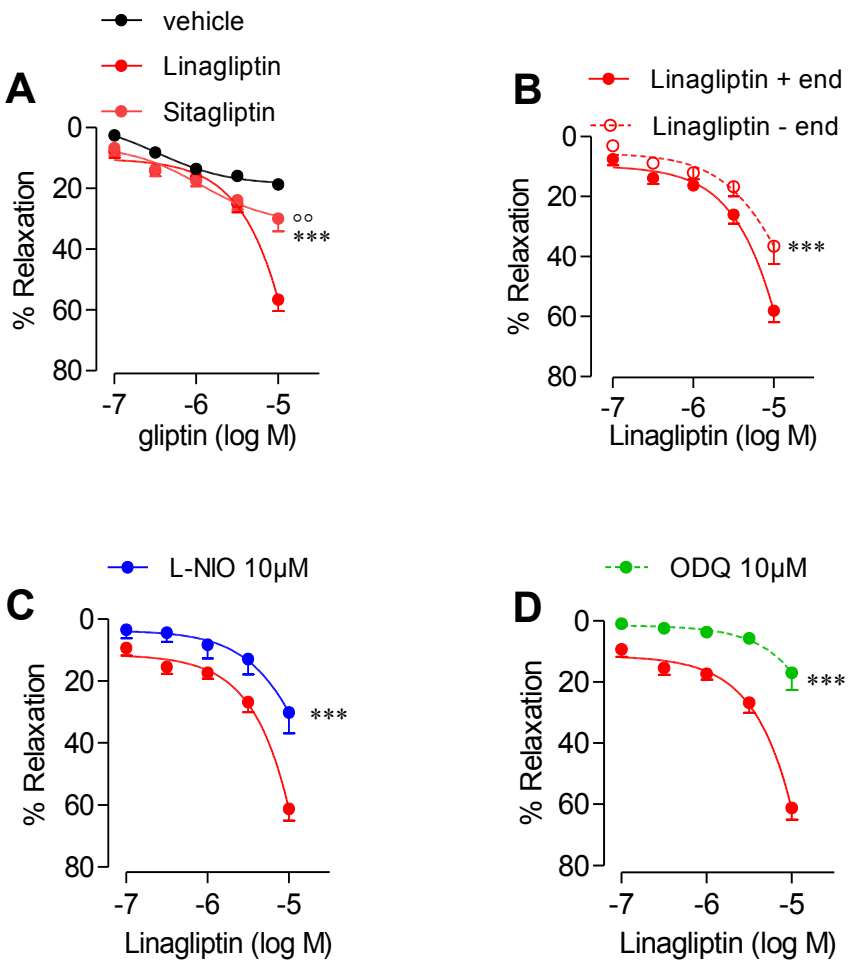
3.5 Linagliptin induces vasodilation involving eNOS/NO/cGMP pathway

Even if several previous studies have already demonstrated that DPP-IV inhibitor are also considered vasorelaxant agent, we have better characterized the vasodilating proprieties of Linagliptin on aorta and carotid rings from CD-1 mice. Our results prove that Linagliptin induces

vasodilation in both vascular tissues in a concentration-dependent manner (100 nM – 10μM), compared the vehicle (DMSO) (Figure 3.5 A. E). This effect is not associated to inhibition of DPP-IV, since Sitagliptin, associated to the same class of drugs, has a less vascular activity (Figure 3.5 A, E).

However, in endothelium denuded vascular rings Linagliptin loses its vasorelaxing proprieties indentifying the endothelium as the main player in its vasodilatation (Figure 3.5 B, F). In this context, NO represent the most important mediator released from the endothelium and involved in endothelium homeostasis regulation. Moreover, our previous studies have shown that Linagliptin treatment ameliorates endothelium dependent vasorelaxation in diabetic mice and in-vitro analysis have proved that it regulates eNOS/NO activity. Therefore in order to better understand the molecular mechanism below Linagliptin action, we have evaluated Linagliptin vascular effect in presence of specific inhibitors of eNOS/NO/cGMP pathway. Incubation of aortic and carotid rings with L-NIO, a selective eNOS inhibitor, significantly reduces Linagliptin-induced vasorelaxation (Figure 3.5 C, G). Similarly, incubation with ODQ, inhibitor of NO-dependent sGC activity, significantly inhibits linagliptin-induced vasorelaxation (Figure 3.5 D, H), even at a major extent compared with the effect achieved with L-NIO, thus confirming a key role for NO/cGMP in linagliptin-induced vasorelaxation.

Isolated aortic rings



Isolated carotid rings

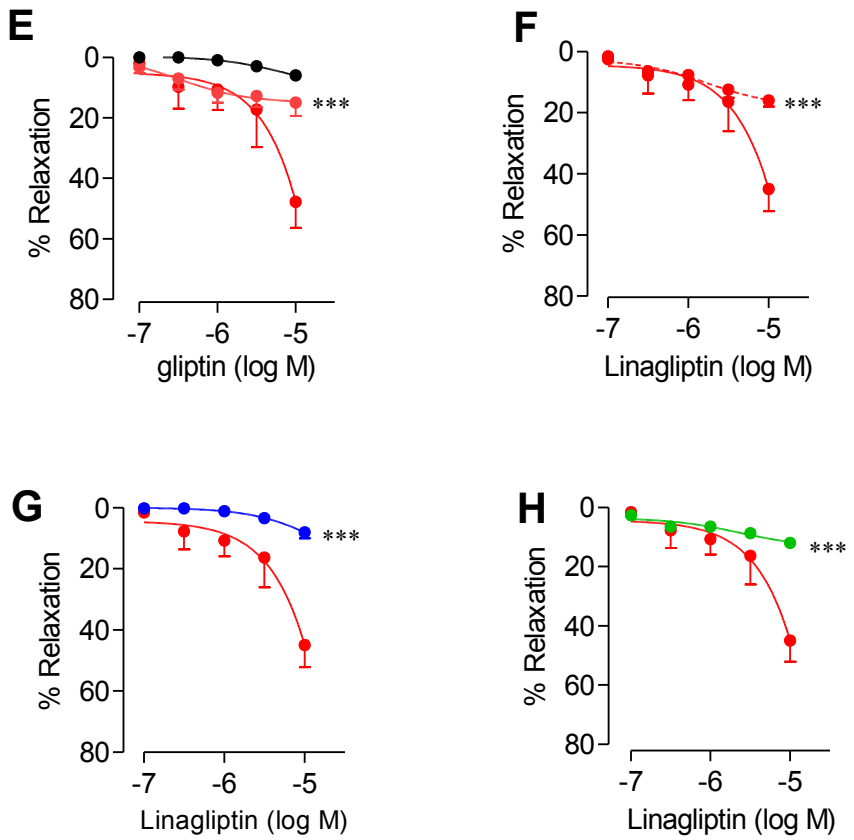


Figure 3.5 ***(A)*** *Linagliptin and Sitagliptin-induced vasodilatation in aortic rings from CD-1 (%Emax:18.8 ±2.53%vs 56.66 ±3.72% as vehicle vs LIN, ***p<0.001, n=10; 56.66 ±3.72% vs 30 ±4.2% as LIN vs SIT, ***p<0.01 n=6). Vasodilatation induced by Lianagliptin* ***(B)*** *in absence of endothelium, after incubation of* ***(C)*** *L-NIO and* ***(D)*** *ODQ in aorta tissue(Emax and* EC_{50}*: 56.66 ±3.72% vs 36.66 ±5.88% as LIN vs LIN w/o endothelium, ***p<0.001, n=10; vs 30.16 ±6.7% as LIN vs LIN +L.NIO, ***p<0.001, n=6; vs 17 ±5.66% as LIN vs LIN +ODQ, p<0.001, n=4).* ***(E)*** *Vascular effect of Linagliptin and Sitagliptin in isolated carotid rings compared the vehicle. Liangliptin induced vasodilatation in* ***(F)*** *endothelium denuded carotid rings, and in presence of* ***(G)*** *L-NIO and* ***(H)*** *ODQ (%Emax:6 ±1.15% vs 45 ±7.19% as vehicle vs LIN, ***p<0.001, n=5; 45 ±7.19 % vs 15 ±4.41% as LIN vs SIT, ***p<0.01 n=5; vs 16 ±1.95% as LIN vs LIN w/o end, ***p<0.001, n=4; vs 78.14 ±1.94% as LIN vs LIN + L-NIO, p<0.001, n=5; vs 12 ±0.7 as LIN vs LIN + ODQ, ***p<0.001, n=4)*

3.6 Endothelium dynsfunction in diabetic mice impairs Linagliptin-induced vasodilatation

The same panel of experiments described above have been performed also on aorta and carotid vessels harvested from NOD III mice with severe glycosuria. As Figure 3.6 A shows, Linagliptin induces vasodilatation in NOD III mice, although to a minor extent compared to control mice. Similarly to what observed in control mice, Sitagliptin- induced vasorelaxation is lower compared to Linagliptin in NODIII mice (Figure 3.6 B). When aortic rings are incubated with L-NIO, Linagliptin-induced vasodilatation results significantly reduced (Figure 3.6 C) confirming the involvement of NO release in Linagliptin vasodilating action. Conversely, in carotid vascular district, Linaglitpin-induced vasodilatation is not different from control mice (Figure 3.6 D), but also in this artery L-NIO significantly inhibits linagliptin-induced vasorelaxation and sitagliptin-induced vasorelaxation is lower. (Figure 3.6 E, F)

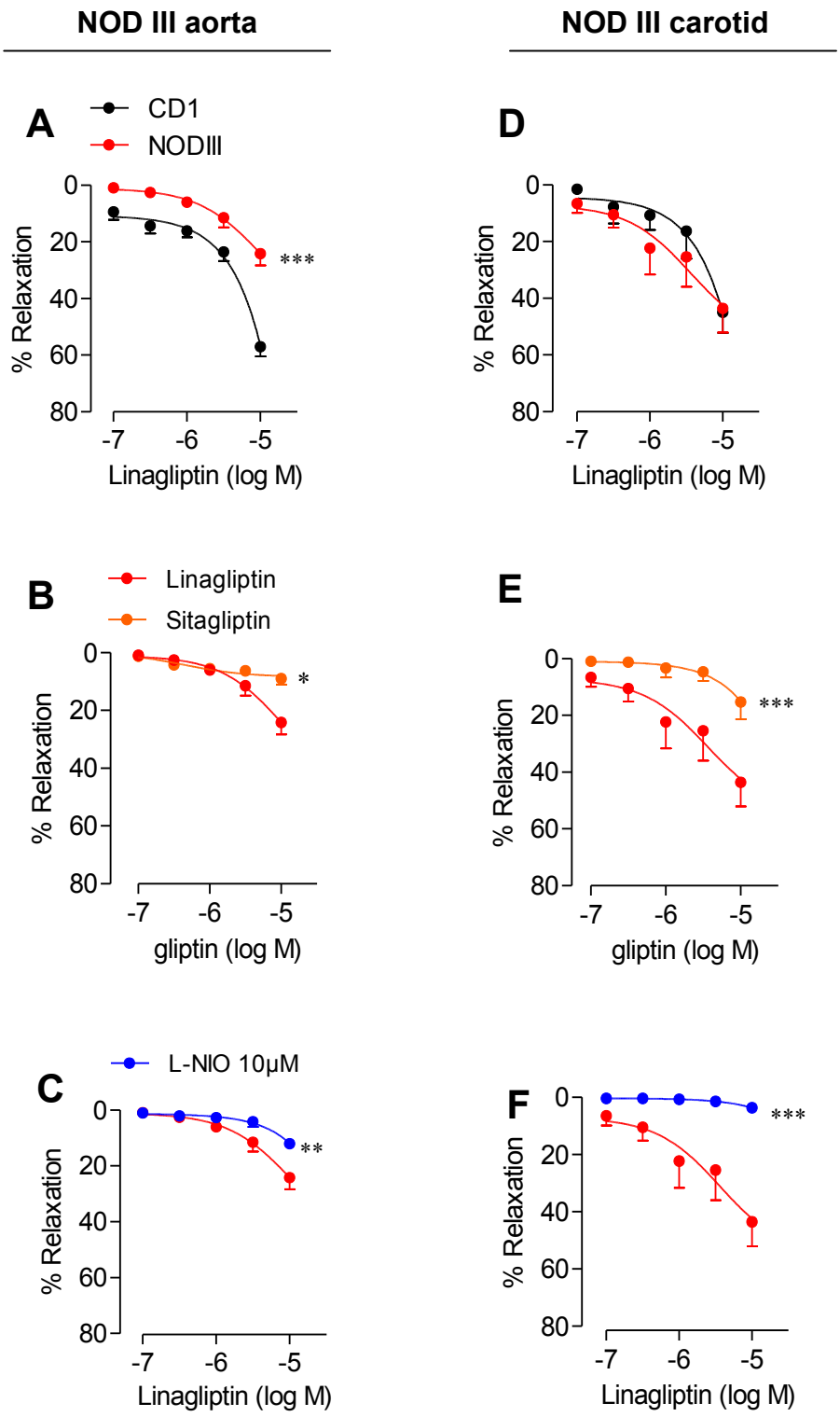


Figure 3.6 ***(A)*** *Concentration-response curve of LIN (0.1-10µM) on isolated aorta rings harvested from NOD III compared to control mice (%E_{max}: 90.27 ±2.94% 53.46 ±5.79% as CD1 vs NOD III, ***p<0.001, n=11).* ***(B)*** *Sitagliptin induced vasodilation and* ***(C)*** *L-NIO effect on Linagliptin vasodilating action in NOD III aorta (%E_{max}: 53.46 ±5.79% vs 12.66 ±4.41% as LIN vs SIT, ***p<0.001, n=4; vs 33.75 ±3.03% as LIN vs LIN+L-NIO, ***p<0.001, n=4).* ***(D)*** *Linagliptin,* ***(E)*** *Sitagliptin on NOD III carotid rings, and* ***(F)*** *effect of L-NIO incubation on Linagliptin vasodilation in this vascular district (%E_{max}: 81.8 ±9.07% 72.7 ±7.34% as CD1 vs*

*NOD III, n=5; 72.7 ±7.34% vs 21.33 ±2.9% as LIN vs SIT, ***p<0.001, n=3; vs 12.5 ±4.8% as LIN vs LIN+L-NIO, ***p<0.001, n=4)*

3.7 Linagliptin-induced vasodilatation does not involve GLP-1 receptor (GLP-1R) activation

It's well know that endothelial cells show, not only DPP-IV enzyme, but also the GLP-1 receptor (GLP-1R) [100] and inhibition of DPP-IV induces a significantly increase of GLP-1 levels promoting its interaction with the GLP-1R expressed in endothelial cells. This interaction leads to an increase of cAMP in endothelial cells and a subsequently activation of K_{ATP} channel, regulating the vascular tone [101]. Since Linagliptin acts as DPP-IV inhibitors and prolongs GLP-1 circulating levels and its vascular activity, we performed in-vitro experiments in aorta rings harvested from GLP1-R -/- mice in order to prove that Linagliptin-induces vasodilatation independently from GLP1R activation.

The same experimental protocol used for the in vitro study described above has been performed on GLP-1R-/- mice and on their respective littermates. Our results show that linagliptin-induced vasodilatation is completely preserved in aortic rings of GLP-1R-/- (Figure 3.7 A, B) and incubation with L-NIO and ODQ further reduces the vascular effect (Figure 3.7 C, D). These evidences mask an directly action of Linagliptin on vascular homeostasis regulation which is completely independent from GLP-1/GLP-1R interaction.

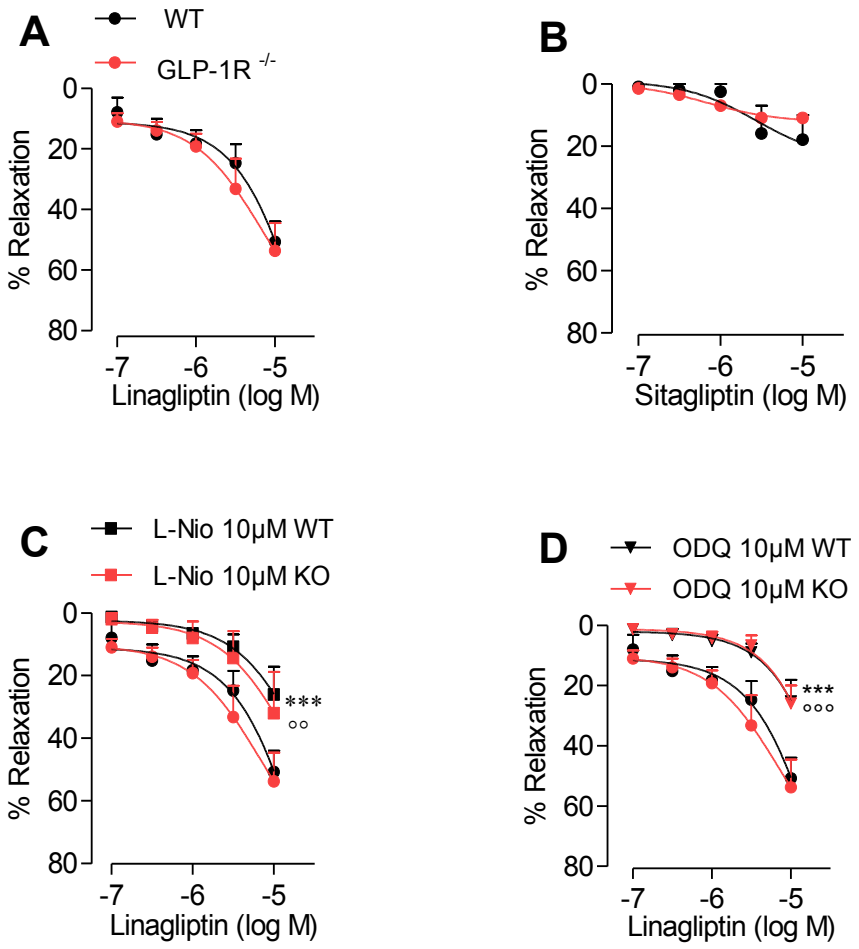


Figure 3.7 ***(A)*** *Linagliptin and* ***(B)*** *Sitagliptin-induced vasodilatation on aorta rings from GLP-1R$^{-/-}$ and their littermates (E_{max} %:50.750 ±6.762% vs 53.750 ±9.214% as LIN in WT vs GLP-1R$^{-/-}$, n=4; 18 ±8% vs 11 ±1% as SIT in WT vs GLP-1R$^{-/-}$, n=4). Linaglitpin-induced vasodilatation in presence of* ***(C)*** *L-NIO and* ***(D)*** *ODQ (E_{max}%: 50.750 ±6.762% vs 26 ±8.8% as LIN WT vs WT + L-Nio, ***p<0.001, n=4; 53.750 ±9.214% vs 32 ±13% as GLP-1R$^{-/-}$ vs GLP-1R$^{-/-}$ + L-Nio, °°p<0.01, n=4; 50.750 ±6.762% vs 25.25 ±7.18% as LIN WT vs WT + ODQ, ***p<0.001, n=4; 53.750 ±9.214% vs 16 ±6.028% as GLP-1R$^{-/-}$ vs GLP-1R$^{-/-}$ + L-Nio, °°°p<0.001, n=4)*

3.8 Absence of Guanilate cyclase abrogates Linagliptin-induced vasodilation

In order to confirm that the direct effect of Linagliptin involves the eNOS/NO/cGMP pathway, we performed the cumulative cpncentration-response curve of the DPP-IV inhibitor on both aorta and carotid rings harvested from $sGC^{-/-}$ mice, where the Guanilate Cyclase enzyme responsible of cGMP production is absent. In this murine model, Linagliptin-induced vasodilatation is completely abrogated confirming the involvement of NO and its downstream pathway in the vasodilating action of Linagliptin (Figure 3.8).

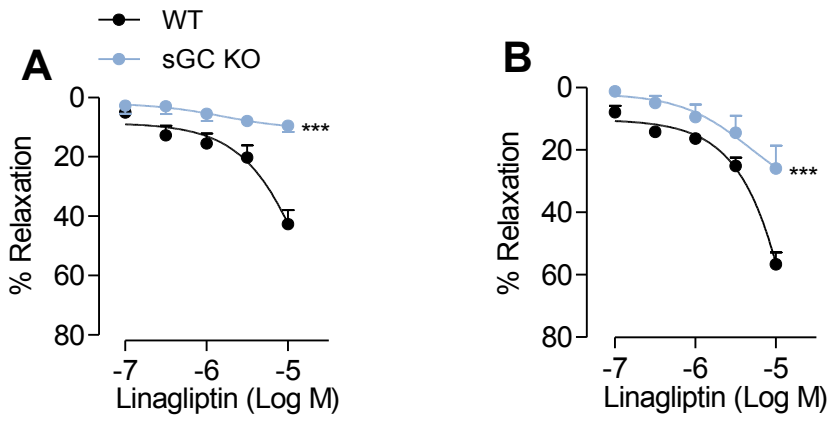
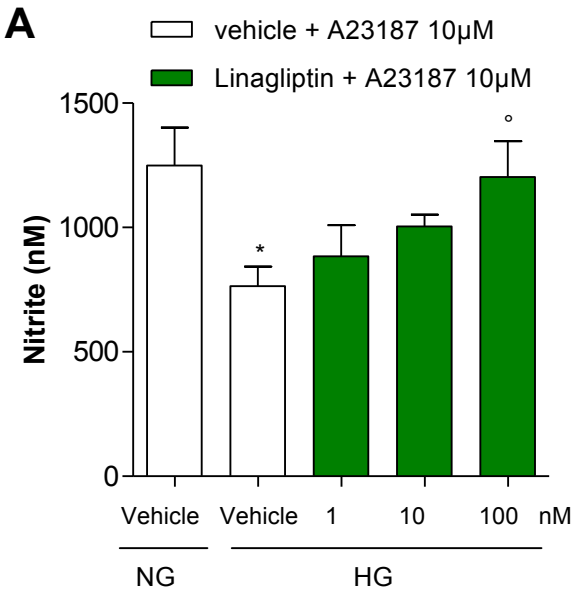


*Figure 3.8 Concentration-response curve of Linagliptin in **(A)** aortic and **(B)** carotid rings harvested from $sGC^{-/-}$ mice compared to WT (E_{max}%:47.750 ±4.7%vs 9.5 ±2.10% as aorta WT vs $sGC^{-/-}$, ***p<0.001, n=4; 55.667 ±3.77% vs 26 ±7.35% as carotid WT vs $sGC^{-/-}$, ***p<0.001, n=7)*

3.9 Linagliptin interferes with eNOS/CAV-1 complex independently from Ca^{2+}

Results obtained above show that eNOS/NO signalling activation is significantly responsible in Linagliptin-induced vasodilation. Since Ca^{2+} intracellular concentration and Cav-1 represent the main regulator of eNOS activation, we performed experiment on BAEC cells, which spontaeously express eNOS. Firstly, following the same experimental protol used for Hek-293 eNOS cells indirectly quantified eNOS activity after treatment with Linagliptin in HG enviroment throught the measurement of NO levels. As happen for Hek-293-eNOS cells, high glucose environment causes a significantly reduction of medium NO levels, compared the normoglycaemic control. However, Linagliptin treatment with 100 nM significantly increases NO production in the medium, restoring the physiological pattern showed in normoglicaemia (Figure 3.9 A). Subsequently, using FLUO-4 AM assay we measured the Ca^{2+} intracellular levels of BAEC after Linagliptin treatment. Compared to positive control such as bradykinin or A24187 stimulation, Linagliptin does not induce Ca^{2+} levels increase (Figure 3.9 B). This findings affirme that eNOS/NO signaling activation is not dependent on an increase of calcium flux inside the cells. immunoprecipitation analysis between eNOS and its negative regulator protein Cav-1, show that in untreated BAEC cells a protein band immunoreactive to anti-eNOS antibody can be detected in the Cav-1 immunoprecipitation. However, treatment with Linagliptin (10 and 100 nM) significantly reduces the eNOS co-immunoprecipitation with Cav-1 in a concentration dependent manner. This last result prove that Linagliptin control eNOS/NO signaling by interfering with eNOS/Cav-1 negative regulator complex (Figure 3.9 C).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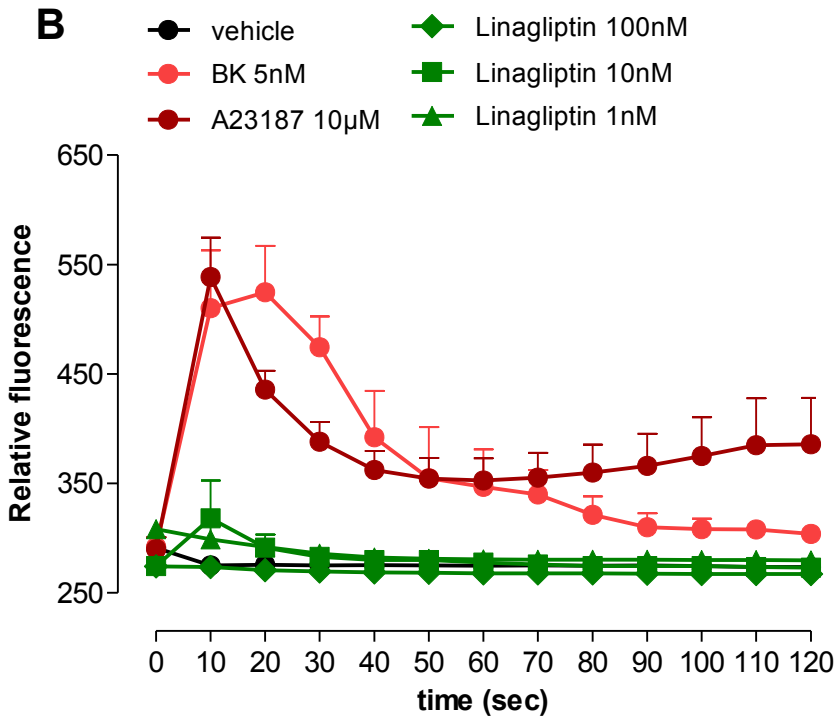
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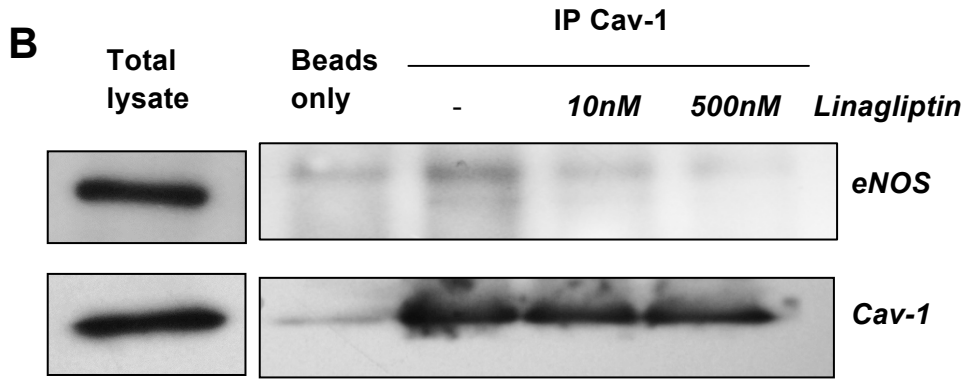

Figure 3.9. ***(A)****Effect of Linagliptin (0.1-10.100 nM) on* Ca^{2+} *ionophpre A23187-induced NOx levels in BAEC placed in normo and hyperglicemic condition (NOx concentration: 1.249 µM ±153.1 vs 764 nM ±78.43 as vehicle in NG vs vehicle in HG, *p<0.1, n=11; 764.6 nM ±78.43 vs 1.204 M ±143 as vehicle in HG vs Lin 100nM in HG, °p<0.1, n=15).* ***(B)*** *Effect of Linagliptin (0.1-10.100 nM) on intracellular* Ca^{2+} *influx in BAEC n=3. Data are compared with BAEC stimulated with vehicle or with BK and Ca2+ Ionophore A23187 as positive control.* ***(C)*** *Immunoprecipitation (IP) of Cav-1 followed Western Blot analysis of eNOS and CAV-1 in BAEC cells treated with vehicle and Linagliptin (10-100 nM).*

4. DISCUSSION

DPP-IV inhibitors represent a class of antidiabetic drugs, which have been shown to improved the vascular function independently to their ability in controlling blood glucose levels. Hyperglycemic condition damages endothelium layer with consequent impairment of eNOS/NO-mediated control on vessels homeostasis leading to vascular dysfunction. Several recent studies have already proved that DPP-IV-inhibitors-treatment in STZ-induced diabetic rats ameliorates endothelial functions increasing Ach-induced vasodilatation in the harvested mesenteric arteries. Even if this observation could be associated to the DPP-IV-mediated reduction of blood glucose levels and consequent decrease of endothelium damage, other articles in this field have demonstrated DPP-IV inhibitors induce vasodilatation in isolated rat mesenteric artery. Thus, these drugs are directly involved in vascular homeostasis regulation, but it has been not yet clarified the molecular mechanism through which DPP-IV inhibitors mediate their beneficial vascular effect.

In our study we have firstly selected a T1DM animal model, as the NOD mice to investigate the vascular effect of DPP-IV inhibitors. DPP-IV are commonly used for T2DM treatment, thus, since NOD mice spontaneously develop an autoimmune diabetes with a remarkable analogy to human T1DM, this approach allowed us to separate the antidiabetic effect of these drugs from their vascular beneficial effect minimizing the confoundable variable that reduction of glycaemia *per se* has a beneficial vascular effect. The number of DPP-IV inhibitors used in therapy is high and it is still increased. Our hypothesis on the involvement of DPP-IV inhibitors in vascular homeostasis control has been developed using

Linagliptin, a DPP-IV inhibitors introduced on 2010 in the T2DM therapy. Isolated aorta and carotid harvested from NOD mice show a drastically reduction of both Acetylcholine and Isoprenaline-induced vasodilatation, associating the development diabetic pathology with an impairment of eNOS/NO/sGC vascular pathway activation. However, *in-vivo* treatment with Linagliptin increases NO-dependent vasodilatation, independently from the development of diabetes, indeed, the spontaneous increase of glycosuria levels in NOD mice are not affected during the all treatment. Subsequently, *in-vitro* experiments prove that Linagliptin has strong vasorelaxing proprieties, which have been assessed on two different vascular districts, aorta and carotid vessels, in both healthy and diabetic conditions. Structurally, Linagliptin is a non-peptidomimetic molecule, and it can be distinguished from other DPP-IV inhibitor, as Sitagliptin characterized by a peptidomimetic structure. In healthy mice, vasodilatation induced by Sitagliptin is less than a half compared to Linagliptin, proving that the vascular effect observed cannot be generalized to all the class of DPP-IV inhibitors.

The integrity of endothelium function is an important requisite for Linagliptin-induced vasodilatation. Diabetic conditions, which are known to be associated to endothelial dysfunction, significantly reduce and this reduction became more marked with the worsening of the disease (NOD I -> NOD II -> NOD III). Moreover, in aorta and carotid vessels from diabetic and healthy mice inhibition of eNOS as well as sGC activity with specific molecular blockers leads to a significantly reduction of Linagliptin-mediated vasodilatation, sustaining the hypothesis that vasodilation involved eNOS/NO/sGC pathway activation, which is further confirmed by the evidence that in aortic and carotid rings from $sGC_{\alpha 1}^{-/-}$ Linagliptin-

mediated vasodilation is strongly impaired. Moreover, our findings demonstrate that this effect is completely independent from the inhibition of DPP-IV enzyme since we have also proved that Linagliptin induced vasodilatation is almost absent in GLP-1R$^{-/-}$ mice isolated aorta.

The hypothesis that DPP-IV inhibitors, in particular Linagliptin, have an important role in the regulation of eNOS/NO vascular signaling activation is supported by previous studies by Salheen et al who have demonstrated that basal contraction induced by NNA as eNOS inhibitor resulted increased in mesenteric artery from STZ-induced diabetic rats treated with *linagliptin* compared the control, confirming that the DPP-IV inhibitor has an important role in improving NO bioavailability and ameliorating endothelial vascular [94]. However, the mechanism underlying this beneficial interaction still needs to be clarified, and for this reason going forward our project and supporting our hypothesis of connection between Linagliptin and eNOS/NO activation, we have investigated the possibility that Linagliptin could interact with eNOS-Cav-1 complex. Since in endothelial cells Cav-1 acts as negative membrane protein modulator of eNOS activity, variation on its expression could easily be responsible for changes in eNOS activity modulation. Moreover, endothelium damage due to diabetic pathology could also affects eNOS-Cav-1 complex altering NO bioavailability [108]. Indeed, in a separate set of *in-vitro* experiments we have collected aorta tissue harvested from NOD III mice after treatment with vehicle and Linagliptin in order to observe variations in eNOS and Cav-1 expression. Molecular analysis has shown that Linagliptin treatment participates in an up-regulation of eNOS activity and simultaneous decrease of Cav-1 expression, suggesting an increase of eNOS activity following the therapy. Based on this results we then focused

our attention on the effect of Linagliptin in a particular cellular *in-vitro* model of hyperglycemia. Firstly, we used Hek-293 cells transfected with eNOS, placed in a normo- and hyper-glycemic (NG and HG) conditions to mimic the physiological diabetic condition. Measurement of NOx levels in the cell medium have reported that, hyperglycemic condition drastically reduces the amount of NOx produced by cells levels, confirming all the previous ex-vivo vascular studies which demonstrated that the diabetic condition impairs eNOS/NO bioavailability. Moreover, these results are associated to a drastically increase of Cav-1 expression coupled with eNOS down-regulation showing a pattern which support the reduction of NO levels. However, in the hyperglycemic environment, Linagliptin treatment restores Cav-1 expression to levels observed in the normoglycemic conditions thus causes a simultaneous increase of NO bioavailability. This kind of pattern has been also confirmed in cells where eNOS enzyme is constitutively expressed, in order to better mimic the physiological condition. BAEC cells have been used for this purpose, and the same previous *in-vitro* experiments confirmed that Linagliptin promotes eNOS activity in high glucose environment increasing NO levels.

At this point, we have developed the hypothesis that eNOS-Cav-1 complex disruption represents the main molecular event responsible for the beneficial vascular effect induced by Linagliptin. Thus, we have addressed our attention to understand how the DPP-IV inhibitor interacts and regulates this endothelial complex. It is well known that one of the mechanism regulating eNOS-Cav-1 interaction is the increase of Ca^{2+} intracellular levels which leads to Ca^{2+}-calmodulin complex and break of eNOS-Cav-1 binding. Thus, we pointed out the hypothesis that Linagliptin

might interact with plasma membrane or intracellular endothelial structures (i.e. membrane receptor or channel for ions cellular movements) acting as a calcium-mobilizing factor which increases eNOS activity by promoting the affinity of calmodulin to the enzyme and breaking the inhibitory interaction of Cav-1. However, this hypothesis has been ruled out by the Fluo-4 in-vitro experiments performed on BAEC cells. Indeed, it has been clearly shown that Linagliptin is not a calcium-mobilizing agonist because has been not highlighted significant rise in intracellular calcium levels after stimulation with the molecule. The second possibility that we have considered is that Linagliptin directly interacts with the eNOS-Cav-1 complex. To assess this hypothesis we have performed Cav-1/eNOS immunoprecipitation study in BAEC cells previously treated with vehicle or with Linagliptin. Our results show that Linagliptin molecularly interacts with Cav-1 causing the break of eNOS-Cav-1 complex and making the enzyme free to acts and increase the NO bioavailability. Thus, these last confirm the involvement of Linagliptin in eNOS/NO signaling regulation, sustaining that the pattern Linagliptin/eNOS/Cav-1 is the main event responsible of the vascular beneficial effect of Linagliptin in diabetic patients.

Our findings conclude that in the pathological condition of hyperglycemia, where vascular homeostasis results impaired, Linagliptin induces a beneficial effect, without affecting hyperglycemia development. Moreover, this effect neither depends on its intrinsic propriety as inhibitor of DPP-IV neither on the increase of GLP-1 produced, while it is due to a direct interaction of Linagliptin on eNOS/Cav-1 complex. In the context of diabetic condition where impaired eNOS/NO pathway leads to vascular dysfunction, Linagliptin is able to replace eNOS from binding the negative

regulator Cav-1. This interaction improves eNOS activity and restores the NO bioavailability. Linagliptin is a drug already used in the therapeutic treatment in T2DM, nevertheless its protective role in vascular dysfunction in diabetic patients could show a relevant importance of this drug in both type 1 and type 2 diabetes in preserving the endothelial impairment; however even if this hypothesis is sustained by powerful studies in the literature, it still needs depth clinical research to be confirmed.

II PART

Department of Pathology and Laboratory Medicine

“Weill Cornell Medical Collage”

New York

WEILL CORNELL MEDICAL COLLAGE

CORSO DI DOTTORATO XXIX CICLO

Second part of my PhD project

Sphingolipid de novo pathway is a novel regulator of vascular homeostasis

Tutors ***Candidate***

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Abbreviations*:*

BP: Blood Pressure

EC: Endothelial cells

VSMC: Vascular smooth muscle cells

SL: Sphingolipids

SPT: Serine Palmitoyl Transferase

S1P:Sphingosine-1-Phosphate

GPCR: G-protein-coupled receptor

TK: Tyrosine Kinase receptor

MA: Mesenteric arteries

INTRODUCTION

The endothelium, considered for long time as passive barrier between the blood stream and the surrounding tissues, is now recognized to play an active role in regulating blood fluidity and pressure, and refraining plasma proteins and leukocytes from extravasation. Blood pressure regulation is a highly dynamic process that is determined by the integration of chemical and rheological stimuli on the vascular wall [109]. Among all the factors released by the endothelium, nitric oxide (NO) is of critical importance to maintain blood pressure homeostasis [110, 111], and alteration of NO bioavailability strongly contributes to increase in vascular resistance [112]. Bradykinin, acetylcholine, vascular endothelial growth factor (VEGF), as well as blood flow, strongly induce eNOS activation and NO-mediated vascular tone regulation.

In addition to NO, the endothelium releases other vasorelaxing factors, such as prostacyclins and endothelial-derived-hyperpolarizing factor (EDHF). Endothelium derived contracturant agents (EDCF), endothelin and prostanoid play an important role particularly when the endothelium is activated, such as during inflammation. The increase of Ca^{2+} influx in endothelial cells leads to activation of eNOS and increase of NO production, stimulation of phospholipase A2 (PLA2) enzyme involved in the production of arachidonic acid as precursor of PGI_2 and opening of potassium channel currents representing the classical EDHF response. In vascular smooth muscle cells (VSMC) EDCF stimulates G-protein coupled receptors (GPCRs) leading to increase in calcium, phosphorlitation of myosin light chain (MLCP) and vessels contraction.

The lack of the endothelium to accomplish any of these tasks is defined endothelial dysfunction [112, 113], which is an early event in the

pathogenesis of multiple cardiovascular diseases including hypertension. In this condition, endothelial-derived contractile factors prevail over the vasorelaxant mediators, resulting in enhanced peripheral resistances and increase in BP [48, 114].

Emerging line of evidence suggest an important role of sphingolipids, a class of lipids, in the regulation of cardiovascular functions, in physiological and pathological conditions.

1.1 Sphingolipids: biosynthesis, sources and transport

Sphingolipids (SL) are important components of plasma membranes and contribute to maintain the integrity of the cells. However, in the past three decades they have come to the foreground as bioactive lipids regulating different cellular processes including apoptosis, growth, differentiation, migration, inflammation, invasion, metabolism and angiogenesis [115, 116]. Sphingosine (Sph), ceramide (Cer) and sphingosine-1-phosphate (S1P) are the best characterized. Sphingosine has a role in regulating the actin cytoskeleton, endocytosis and apoptosis, ceramide is mostly involved in the cell responses to the stress, while S1P has a crucial role in cell survival, cell migration and inflammation [117]. However, other molecules belonging to this family, such as ceramide-1-phosphate, glycosphingolipids (GSL) and sphingomyelins (SM), also play relevant roles in inflammation and intracellular trafficking.

Sphingolipids are composed of a polar head group attached to a non-polar tail, and their core is represented by the long-chain amino alcohol,

sphingosine (Figure 1.1). Modification of this basic structure is what gives rise to the vast family of sphingolipids.

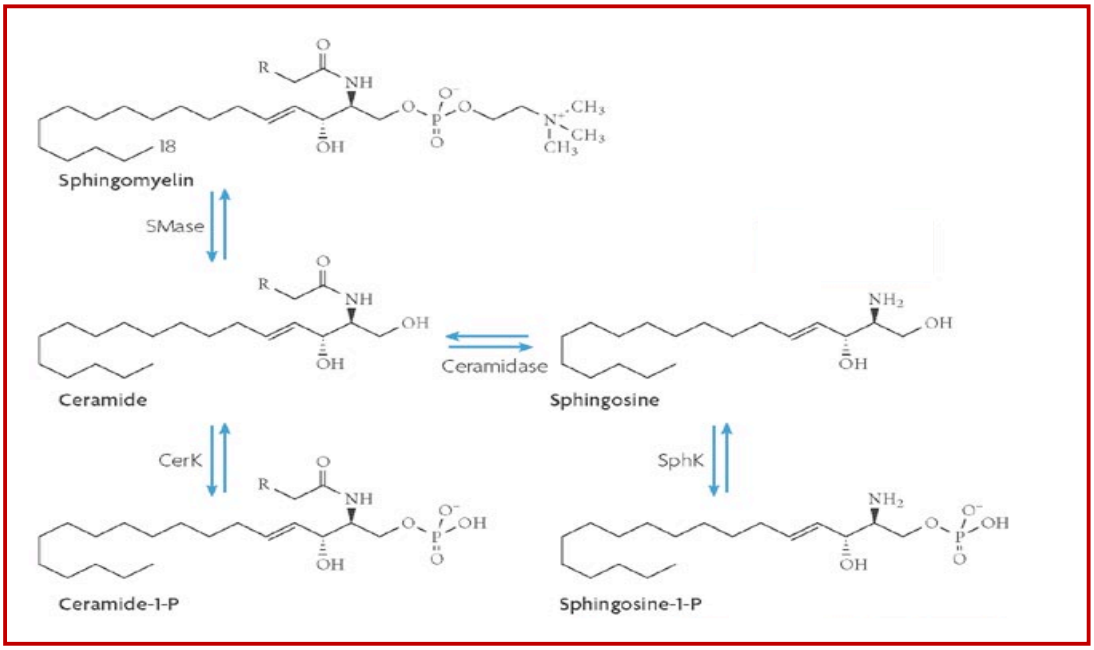


Figure 1.1. ***Sphingosine and related sphingolipids***. Different chemical reactions allow the continuous inter-conversion of sphingolipids, as sphingomyelin, ceramide, ceramide-1-phosphate and sphingosine-1-phospahate which always maintain the structural core of sphingosine.

Sphingolipids are generated through three different pathways: the *de novo* biosynthesis, a direct hydrolytic pathway and the salvage pathway [117]. The *de novo* biosynthesis begins in the cytosolic leaflet of the smooth endoplasmic reticulum (ER), with the condensation of palmitoyl-CoA and serine, catalyzed by the pyridoxal phosphate-dependent enzyme, Serine Palmitoyltransferase (SPT) to generate the 3-keto-dihydrosphingosine. Since this represents the first reaction of the *de novo* sphingolipids biosynthesis, SPT controls the first rate-limiting step of the sphingolipids *de novo* biosynthesis (Figure 1.2).

The highly conserved SPT enzyme belongs to the oxamine synthases family and acts as a membrane-bound heterodimer composed of two subunits, first identified in the yeast as Lcb1/Lcb2 [118-120]. However, these are replaced by the corresponding homologous subunits in mammalian cells; the serine palmitoyltrasnsferase long-chain subunit 1 (Sptlc1) is the only homolog for the Lcb1, whereas Lcb2 can be substitute by the Sptlc2 or Sptlc3 [121, 122]. It has been confirmed that the active catalytic site of the SPT is expressed at the interface between the two subunits, thus, they are both necessary to preserve the SPT activity. Indeed, it has been shown that mice knockout for Sptlc1 or Sptlc2 are embryonically lethal, suggesting the importance of the two subunits during the development; however, while Sptlc1 is present in all catalytically active SPT enzymes, the presence of Sptlc2 or Sptlc3 depends both on the tissues where SPT enzyme is expressed and on the cellular sphingolipids requirement.

Following the formation of the 3-ketosphinganine, a NADPH dependent reductase converts this compound to dihydrosphingosine, also called sphinganine, and this molecule is then N-acylated to produce dihydroceramide. Subsequently, oxidation of the dihydroceramide by dihydroceramide desaturase generates *ceramide*. Central metabolite of this metabolic pathway, ceramide, not only shows its biological role in cell processes, but it represents also the start point for the synthesis of the other bioactive sphingolipids. Modification on the C-1 of the Cer can generate ceramide-1-phosphate (C1P) through phosphorylation, which then is metabolized in *glycosphingolipids* (GSL) and *sphingomyelins* (SM), high order sphingolipids. These kinds of metabolization processes do not take place in the ER anymore. Cer can be transported to the Golgi

through the action of a *ceramide transfer protein CERT* which couples the molecule to the SM synthesis by transferring phosphorylcholine from phosphatidylcholine to ceramide in a reaction catalyzed by sphingomyelin synthases (SMS) [123], moreover ceramide can be transformed in GluCer, and then transported by the protein *FAPP2* in Golgi apparatus where it is metabolized in GSL. At this point, SM and GSL are transported to the plasma membranes of the cells by a vesicular trafficking.

Plasma membrane SM can become sources for ceramide production by sphingomyelinase, which is then hydrolyzed in *sphingosine* (Sph) by a ceramidase. This represents the second important pathway involved in the intracellular sphingolipids production and ending with the phosphorylation of Sph by sphingosine kinases (Sphk1 and Sphk2) in order to obtain *sphingosine-1-phosphate* (S1P), a high bioactive lipid.

Moreover, recycling pathways of SL mainly interconnects with the *de novo* biosynthetic pathway to maintain cellular SL homeostasis at low energy cost. SL from plasma membrane, as well as from extracellular fluids, can be internalized in lysosome and degraded in ceramide, which can be further metabolized as aforementioned. The integration of these two pathways preserves intracellular sphingolipids levels in a physiological range, essential to preserve both the structure and the function of cells.

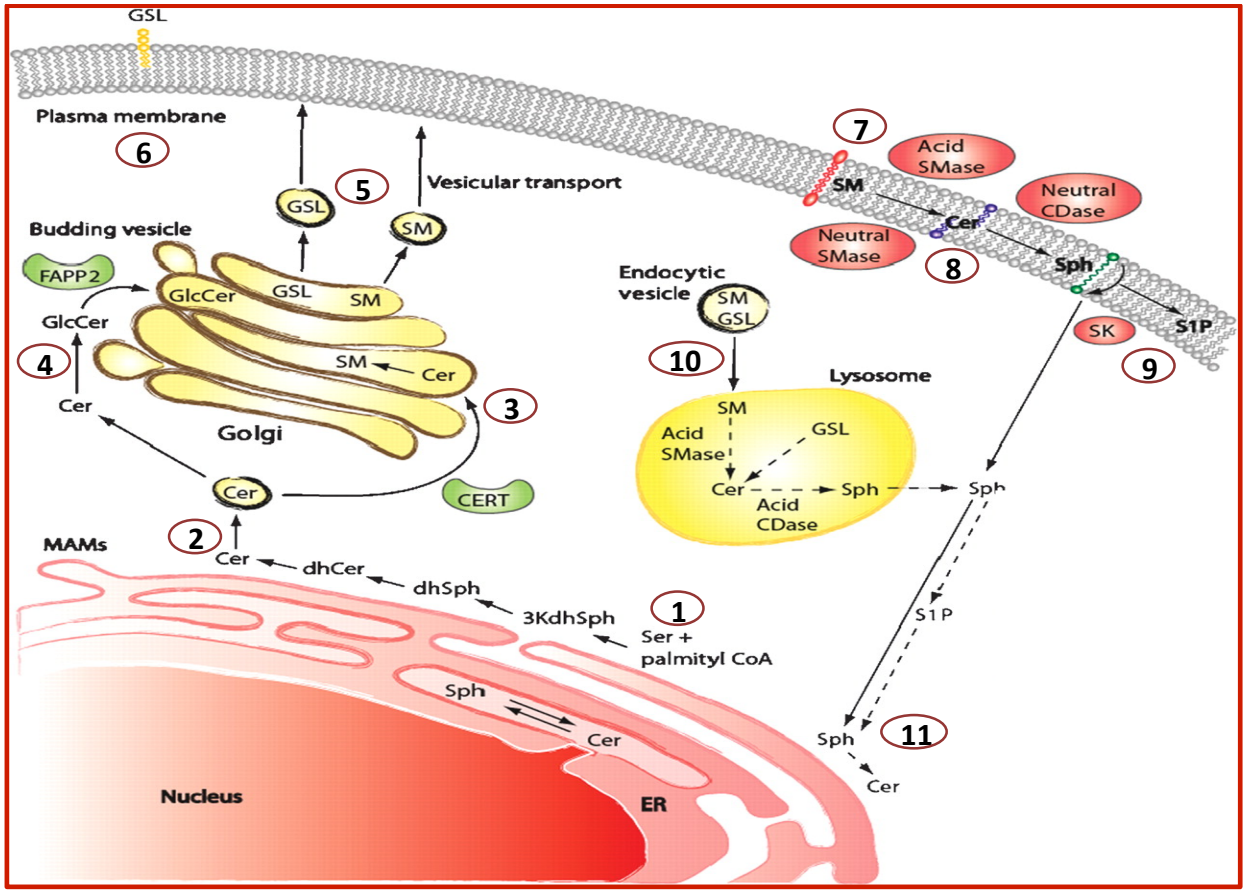


Figure 1.2 ***Sphingolipids metabolism and compartmentalization***. In the ER, Serine Palmitoyl CoA starts the *de novo* sphingolipid biosynthesis by mediated the condensation between Serine and Palmitoyl CoA (1) in order to generate 3-Keto Sphinganine and other sphingolipid, which are subsequently converted until the formation of ceramide (2). Ceramide from the ER membrane is then transported to the Golgi apparatus where is metabolized in high order sphingolipids, as Sphingomyelins (3) and Glycosphingolipids (4). By a vesicular transport SM and GSL move to the cell plasma membrane (5). Here, GSL mainly act as membrane components (6), whereas most of the SM is metabolized to form again ceramide (7). In plasma membrane a ceramidase converts ceramide in Sphingosine (8), which is then phosphorylated b a sphingosine kinases in Sphingosine-1-Phosphate (9). Endocytic vesicles recycle SM and GSL from plasma membranes and from extracellular fluid in order to be degraded in ceramide, which is then converted again in new sphingolipid molecules (GSL, SM and Sphingosine) (10). Sphingosine and S1P are also recycled from the plasma membrane and can be metabolized in ceramide in ER membrane (11).
These are called “sphingolipid salvage pathways”.

Sphingolipids are distinguished by their specifically biophysical proprieties on which depends their subcellular localization. Indeed, in absence of specific mechanisms of transport, such as protein or vesicles,

the site of generation of sphingolipids represents also the site of their action. This represents the reason why most of the enzymes of sphingolipids metabolism are localized where these molecules show their regulatory functions. SL can be separated depending on their biophysical proprieties, for examples: C1P carries ionic charges at neutral pH and also contains two hydrophobic chains, thus for this composition C1P mostly reside in its compartment of generation without flipping across the bilayer; ceramide, instead, for its hydrophobic composition can flip across the membrane; whereas sphingosine, S1P and also sphingomyelin are characterized by sufficient aqueous solubility that allows them to move between the membrane and the cytosol without transporters [117]. However, cells contain particular protein transporters that can deliver sphingolipids across the membrane or between different membranes inside the cytosol. We have previously talked about the protein *CERT* which delivered ceramide for SM production in the Golgi apparatus and the protein *FAPP2* involved in the synthesis of glycosphingolipids, however there are also present in the plasma membrane other two transporter belonging to the *ABC transporter superfamily* [124]. These are particularly involved in the S1P influx and efflux through the membranes. S1P is produced in the inner leaflet of the plasma membrane, but it is well know that its biological function depends on its interaction with the S1PRs expressed on the outer leaflet. The cystic fibrosis transmembrane regulator (CFTR) and the ABCC1 transporters have been identified as the two mainly structure involved in S1P internalization from the extracellular environment, and S1P efflux respectively [125, 126]. Moreover, recently by Fukuhara and colleagues has been discovered another S1P transporter specifically in endothelial

cells, named spinster-2-transporter (Spns-2). They shown that once produced in EC, S1P is transported outside where activate its receptors inducing protective functions and regulating vascular tone [127].

Several studies sustain that a great amount of SLs derive from the circulating system, where many of the enzymes as ceramidase, sphingomyelinase and sphingosinkinase have been detected mostly in association with circulating lipoprotein enriched of sphingomyelin and ceramide [117]. Moreover, erythrocytes, endothelial cells [128, 129] and platelets [130] represent the major sources of S1P in the plasma where it is bound to apolipoprotein M (ApoM) of HDL (≈60%) and albumin (≈30%) reaching a concentration between 0.1uM and 1uM [131]. Plasma circulating sphingolipids can be internalized by the surrounded cells and used for recycle [132]. Exogenous sphingolipids can enter into cells as free molecules, or as lipoprotein complexes. While monomers of sphingolipids can cross the cell plasma membrane, lipoproteins are internalized by endocytosis. In both cases, internalized sphingolipids are catabolized in sphingosine and then recycle for the production of new bioactive molecules [132].

Thus, sphingolipids production and transport inside the cells is precisely organized, and preserves the role of these molecules as essential membrane components as well as their biological function

1.2 Shingolipids: plasma membrane composition

Plasma membrane not only confers a spatial identity to the cells but also represents a barrier between intracellular and extracellular environment.

In the same way, intracellular organelles are also delineated by plasma membrane allowing the separation from the surrounded cytosol. These membranes are generated by interaction between lipids and proteins. Lipids are amphipathic molecules containing a non-polar hydrophobic domain and a polar hydrophilic portion, which rapidly interacts with the water. During the formation of the bilayer, the hydrophobic portions spontaneously interact each other, while the hydrophilic moieties tend to self-associate and interact with the intra or extracellular environment, thus generating two parallel leaflets, one in contact with the cytosol (baso-lateral membrane), and the other with the extracellular fluids (apical membrane). The major lipid components of the eukaryotic plasma membrane include glycerolphospholipids, as phosphatidylcholine (PC), phosphatidylserine (PS) and phosphetanoammina (PE), sphingolipids, like SM and GSL, and cholesterol. Lipids are distributed in the plasma membrane in order to obtain an asymmetric organization between the two leaflets. While PC, PS and PE are mostly distribute in the outer leaflet (the extra cytosolic portion), SM and GLS are predominantly present in the inner leaflet (cytosolic portion) [124]. Lipids can translocate from a leaflet to another by “flip-flop” indicating with “flip” the movement from the extra cytosolic to the cytosolic portion, while “flop” is the opposite direction. However, the polar head of SM and GSL makes difficult this kind of translocations through the hydrophobic interior of the membrane therefore, plasma membrane has three different protein which act as lipid translocases [124]: 1) a sub-family of P-type ATPase, known as amino phospholipids translocases which mediate the flip of PS and PE specifically; 2) ATO-binding cassette, also called ABC transporter which move to the outer leaflet GSL and SM; and the 3) lipid scramblases which

move all glycerophospholipids classes through the trans bilayer [124]. These transporters and the movements of lipids within the bilayer make the membrane a fluid mosaic where hosted functional proteins can differently distribute.

The characteristic asymmetry and fluidity of the plasma membrane are important to preserving the physiological functions of cells. For instance, the loss of SM in the outer leaflet and the trapping of PE on extracellular surface alter the formation of the cleavage furrow during cytokinesis leading to a stopping of the cellular division. Moreover, expression of PS on the cell surface is associated to disruption of cell asymmetry and consequent apoptosis due to inhibition of myotubule formation and consequent cell division arrest [133]. Moreover, PS on the cell membrane surface represents also a trigger for blood coagulation, thus showing a significant pro-coagulant effect [134]. In this context, Annexin V, which have high affinity for PS, could be used as a direct index for plasma membrane alteration.

Membrane is considered not only a barrier for the cells and for the other intracellular organelles, but it is also the site where many cellular machineries carry out their function [135]. For several decades, the research on plasma membrane was dominated by the hypothesis that proteins were the key factors for the function of the membrane while lipids represented only a fluid solvent [124]. However, from the 1997 to date the idea is changed, and plasma membrane is considered a biological structure governed by sphingolipids-cholesterol-proteins interactions, which are essential to maintain the cellular bioactivity.

The dynamic clustering between SL and cholesterol within plasma membrane form particular compartmentalization, or subdomains, called *lipid raft* [136]. In these microdomains, the dynamic assemblies of cholesterol and sphingolipids, in particular glycosphingolipids and sphingomyelins, are mainly concentrated in the exoplasmic leaflet of the bilayer: here, hydrophobic and hydrophilic bounds allow the interaction between the satured hydrocarbon chains as well as the polar heads of the sphingolipids whereas the cholesterol, as a molecular spacer, fills all the voids between the molecules [137]. On the other side, the inner leaflet of rafts is enriched not only of cholesterol but also of phospholipids, which are the mainly components of *non-raft* regions [138, 139]. However, whereas *non-raft* regions are characterized by unsatured phospholipids, rafts microdomains are highly enriched in saturated fatty acids, allowing for the close packing of lipids within rafts, compared with the surrounded *non rafts* membrane regions [138, 139]. Indeed, as a result of the presence of cholesterol and saturated fatty acids, lipid rafts are more ordered and less fluid than the surrounding membrane [140].
The existence of the two different lipid organizations between the two-membrane leaflets implies that rafts could be considered as bilayer structure as well as the whole membrane. They appear to be small in size, with a diameter between 100-200nm, but may constitute a relatively large fraction of plasma membrane [136].

Biosynthesis of these microdomains starts in the ER where cholesterol and ceramide are produced and concentrated in vesicles direct to the Golgi apparatus. Here, vesicles became more enriched of ceramide-derived sphingolipids, as SM and GSL, as well as cholesterol and other synthesized lipids such as PC, PE and PS [141].

Glycosylphosphatidylinositol (GPI)-anchor proteins (GPI-AC) bind these vesicles and mediated their transport from the ER-to-Golgi, and finally the segregation from Golgi-to-plasma membrane [142]. Defects in GPI-anchor synthesis dramatically reduced the total membrane sphingolipid levels leading to a disruption of the membrane rafts compartmentalization. Moreover, cytoplasmic proteins that are covalently modified by saturated fatty acids (palmitoyl or myristoyl moieties) and transmembrane proteins could be attached by GPI-anchor protein and thus can be segregated within the lipid rafts in the cell surface [143].

The tightly packed and the highly order construction as well as the relatively higher lipid/proteins, give rise to define lipid rafts as detergent-resistant low-density microdomains (DRM) [135, 136]. For this reason, lipid rafts have been physically isolated based on their combination of low density and insolubility in detergent. Typically, cells are scraped into cold buffer containing 1% Triton X-100 and then the lysate is homogenized [144]. Rafts are then isolated by flotation in a 5% to 30% sucrose density gradient where they distribute in the top few fractions of the gradient (low density portions) [144]. This procedure yields to a consistent product that is enriched in cholesterol and then using raft marker proteins, such as GPI-anchor proteins or flotillins, raft microdomains can be separated from the surrounded membrane [136].

It has been recognized the existence of two kind of “low density, detergent-resistant membrane”, distinguished in non-*caveolar* lipid rafts and *caveolar* lipid rafts, where formation of caveolae takes place [145]. Caveolae are small plasma-membrane invaginations that can be viewed as a subset of lipid rafts. They are also enriched of cholesterol and

sphingolipids, however they are distinguished from the lipid rafts by the presence of caveolin proteins [146] (Caveolin-1, -2, -3) which bind the cholesterol and appear to be mainly responsible for stabilizing the invaginated structure of the caveolae [147, 148].

As lipid rafts, caveolae also exhibit a resistance to detergent extraction and thus they can co-isolated with the non-caveolar fraction by low-density flotation centrifugation [149-152]. However, it is necessary to separate them in order to perform specific biochemical analysis in their lipid and protein composition. In this regard, Yao Y. and colleagues in 2009 used a modified four-step sucrose density gradient to separate caveolae from other *non-caveolar* lipid microdomains which are recognized by protein marker specifically expressed in one of the two compartments, as Gangliosides (GM), Caveolin-1 (Cav-1), Flotillin-1 (Flot-1) and Thy-1 (Thy) which represents a GPI-anchored protein (Figure 1.3).

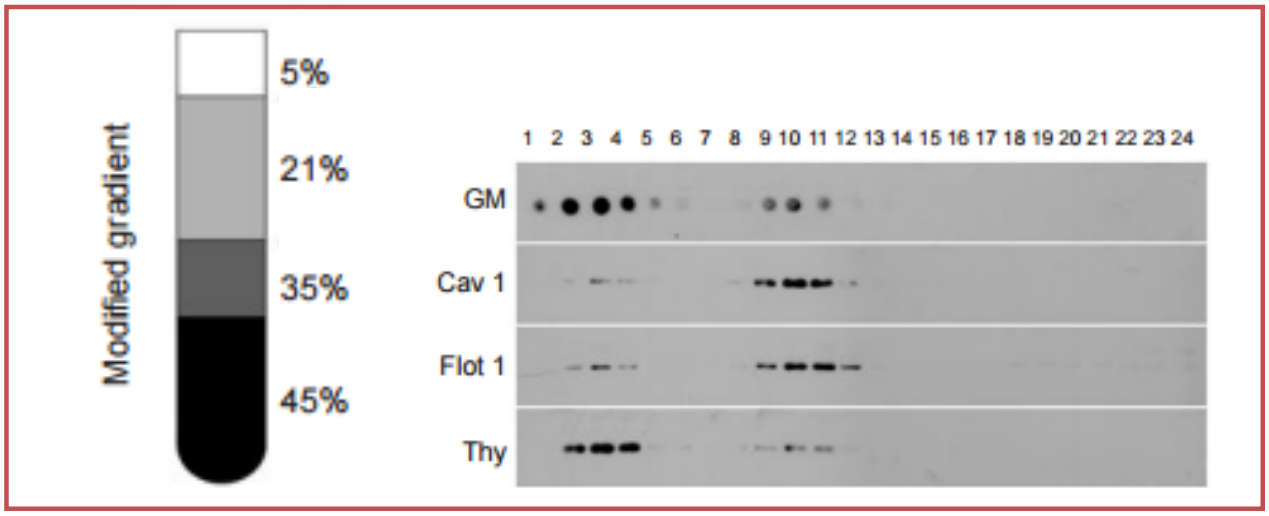


Figure 1.3 *Yao Y. et al. Cell Research, 2009.* ***Separation of caveolae and non-caveolar lipid microdomains from purified plasma membrane of 3T3-L1 cells.*** (A) Modified four steps sucrose density gradient. (B) Analysis of lipid-microdomains associated proteins: Gangliosides (GM) is the major lipids in characterizing the *non-caveolar* lipid microdomains [153], Cav-1 is marker protein for caveolae, Flotillin-1 (Flot-1) represents a integral component of caveolae and also have been suggested to interact with caveolins, then Thy-1 (Thy) which represents a GPI-anchored protein.

It is well known that both caveolae and lipid rafts lipid rafts are composed by cholesterol, sphingolipids, and phospholipids [136], however Yao Y and colleagues demonstrated that the amount of cholesterol and sphingomyelin, as well as PE and PC are higher in *non-caveolar* lipid rafts compare to caveolae subdomains, pointing out possible differences in fluidity and lipid-order between the two plasma membrane microdomains, which could be correlated to a different partitioning as well as function of cytosolic and membrane proteins. However, other studies reported that cholesterol is more enriched in caveolae because the ability of caveolin proteins to binding it [146].

Beside these differences in the composition, a variety of proteins have been found to be enriched in lipids rafts and/or caveolae. These include caveolins, flotillins, GPI-anchor proteins, heterodimeric G-proteins, SRC family kinases, growth factors receptors, as well as MAP kinase, protein kinase C and also eNOS enzyme [144, 154-157]. The mechanisms through which raft associations occur seem to be variable, however the ordered-lipid organization mediates and regulates the portioning of the associated proteins and receptors during their activity or basal conditions.

1.3 Lipid raft and it role in endothelial neurotransmitter signaling

Lipid rafts have been implicated in a variety of cellular processes including protein and lipid trafficking [158], viral infection [159] and signal transduction [137]. Many proteins involved in cell signaling, including receptors, low molecular weight G protein and heterodimeric G protein,

have been shown to be enriched in lipid raft [136, 154]. Depletion of cholesterol from plasma membrane microdomains has been associated to alteration of the signal transduction from the physiological condition, suggesting that cholesterol-rich domains participate in the control of cell signaling [136].

In the simplest case, rafts can represent a signaling platform that spatially localizes all pathways' components facilitating their interactions and promoting the signaling. Receptors, coupling factors, effector enzymes and substrates of a specific signaling pathway are localized in the lipid rafts. This spatial proximity favors a rapid and efficient activation of the signaling transduction pathway following the binding of the receptor by the ligand. [136]. Moreover, receptors can be localized in a particular class of rafts containing all the components necessary for the transduction in order to increase the specificity of signaling [136]. On the contrary, complementary components of a signaling pathway, such as membrane bound enzymes and receptors, are segregated in different lipid rafts, which merge following the ligand-mediated activation of the receptors [136]. Lastly, receptors or signaling transduction molecules can localize in *non-raft* regions, and only upon their activation, localize to the rafts domains together with signaling transduction molecules leading to the activation of the intracellular pathway. In conclusion, rafts can regulate the receptor-activated signaling pathway by compartmentalizing the receptors and their interacting partners in separated membrane rafts [149], preventing an hyperactivation of these pathways [136]. Therefore, the disruption of lipid rafts could lead to the deregulation of signaling pathways [136].

Moreover, in the lipid rafts have been reported regulatory molecules able to modulate the receptor activation. For instance, in endothelial lipid rafts, caveolin-1 (Cav-1) is one of the major regulatory molecules. Cav-1 is a member of a family of homologous molecules localized to cholesterol-enriched low-density membrane domains. Cav-1 is present in the striated coat that is seen surrounding caveolae in the electron microscope [149]. With the exception of the endothelial insulin receptor (IR), Cav-1 binds to and inhibits growth factors tyrosine-kinase receptors, as well as S1PR1, a Gi-protein coupled receptor, and also eNOS enzyme. Indeed, Cav-1 knock-out mice show insulin resistance and decreased insulin receptor expression in adipose tissue, defects in nitric oxide and calcium signaling, as well as an enhancement of basal and S1P and VEGF-induced activation of Akt [160]. In addition, the distribution of tyrosine-kinase receptors, eNOS and G-protein coupled receptors is not affected by knocking out of Cav-1, suggesting that this regulatory molecule is not necessary for the localization of signaling proteins to lipid rafts, but for the modulation of their function [161].

Many raft proteins localized to lipid rafts, also partition to non-raft portions of the membrane, where they interact and activate different subsets of signaling partners. Changes in lipid composition of the rafts, such as the depletion of cholesterol, affect the partitioning of signaling molecules between raft and non-raft compartments, altering the activation signaling pathway.

Rafts may also play a role in signal termination. Rafts are known to be involved in endocytic events [147, 150], and could limit cell signaling by

internalizing specific components, preventing them from further participation in a particular pathway.

Considering the importance of preserving lipid raft composition to maintain proper intracellular signaling mechanisms.

1.3.1 Lipid raft and regulation of G-protein coupled receptors signaling

G-protein coupled receptors are the most abundant class of receptors in the human body. These receptors are transmembrane proteins showing the ligand-binding site on the extracellular surface whereas possess on the intracellular region a unique class of signaling molecules called G protein which regulate the intracellular messengers (G proteins are so named since they bind the guanine nucleotides GTP and GDP). Each transmembrane region consists of a single α helix, and the α helices are arranged in a characteristic structural motif that is similar in all membranes of this receptor class. In the resting state (non-stimulated), the cytoplasmic domain of the receptor is non-covalently linked to a G protein complex formed of α and βγ subunits. Upon activation, the α subunit exchanges GDP for GTP. The α-GTP subunit then dissociates from βγ subunit, and they both cross the inner leaflet of the plasma membrane to interact with a number of different effectors. These effectors include adenylate cyclase, phospholipase C, various ion channels, and other classes of proteins. Signal mediated by G proteins are usually terminated by the hydrolysis of GTP to GDP, which is catalyzed by the inherent GTPase activity of the α subunit.

The intracellular molecular pathways recruited by the GPCRs are strictly dependent on the typology of the α subunit, indeed a large number of Gα

protein isoforms have been identified, with specific effect on their target. The most common are distinguished in $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$):

- $G\alpha_s$ induces the activation of adenilate cyclase;
- $G\alpha_i$ or $G\alpha_o$ have an inhibitory effect on the adenilate cyclase activity;
- $G\alpha_q$ is responsible of the a Ca^{2+} intracellular levels increase;
- $G\alpha_{12/13}$ is involved in the cytoskeleton proteins regulation by a Rho-GTPase.

Moreover, the βγ complex of the G proteins can also act as second messenger molecules, although their actions are not as thoroughly characterized.

Synthesis of GPCRs starts in the ER where they form either homo- or heterodimeric structures. Following ER exit, GPCRs transit through the Golgi apparatus and they undergo through additional modifications, such as oligosaccharide processing, fatty acylation and/or myristoylation in order to allow their final segregation in the lipid rafts. Indeed, at the end of these chemical reactions, GPCRs are packaged in exocytic transport vesicles and enter the endosomal system where they are subsequently targeted to the plasma membrane [162].

A large number of G-protein-coupled receptors are enriched in the lipid rafts or caveolae. This includes β1- and β2-adrenergic receptors, adenosine 1 (A1) receptor, angiotensin II type 1 (AT1) receptor, bradykinin B_1 and B_2 receptors, S1P1 receptor (S1PR1) and also muscarinic cholinergic receptor M_2 [163-171]. Switching from inactive and active state, these receptors differently move between *raft* and *non-raft* region and mediate their intracellular signaling. Indeed, A1 and β2-adrenergic

receptor initially localize in the lipid raft and subsequently translocate into *non-raft* membranes after activation, whereas M_2, AT1, S1PR1 as well as B_1 receptors are targeted to the rafts upon activation by the agonist. Thus, ligand interaction induces changes in the receptor localization between *raft* and *non-raft* compartments. Moreover, ligands can also induce the translocation of the receptors into the lipid rafts, where G proteins and effector enzymes are localized to promote the development of the signaling. However, for some GPCRs, such as muscarinic acetylcholine receptor or AT1, interaction with the substrate leads to desensitization via a mechanism which involves the sequestration of the receptor from the cell surface [172] since both *caveolar* and *non-caveolar* lipid rafts are known to be involved in the endocytosis [137, 147, 150, 154]. Thus, for receptors that are recruited into the lipid rafts following agonist activation, it is possible that recruitment of rafts not only initiates signaling but also represents the first stage of the desensitization of the signal.

Caveolins have been shown to interact with several GPCRs regulating their signaling, as A1 receptor, M_2 receptor, and also B_2 receptor. The interaction with caveolin not only serve for the localization of the receptor, but also profoundly influence signaling from certain neurotransmitter receptors, indeed Cav-1 is known to be a lipid raft regulatory protein [173]. A particular experiment has used the “caveolin scaffolding domain“ as a bait to screen a library of synthetic peptides containing a motif able to bind Cav-1. GPCRs as α-adrenergic receptor, muscarinic receptor, endothelin receptor have been found to contain this peptidic motif but it is still undefined if it is responsible for association with the caveolar-domain [174].

After their segregation in the lipid rafts, localization of G-protein coupled receptor depend on their distribution between planar lipid raft and caveolae microdomains. However, a paper of Phil Oh in 2001 showed that after isolation of caveolae from other *non-caveolar* lipid raft microdomains in endothelial cells, it has been observed that $G\alpha_q$-protein interacts directly with caveolin, targeting the receptor in the caveolae, while $G\alpha_s$, $G\alpha_i$ are targeted to lipid raft that does not contain caveolins [175] [176] (Figure 1.4). It has been reported that AT1 receptor, as a $G\alpha_q$ protein-coupled receptor, even if contains the "caveolin scaffolding domain", it is highly expressed in *non-caveolar* lipid raft. However, mutations of the "caveolin scaffolding domain" causes a reduction of AT1 expression on cell surface, meaning that Cav-1 bound has an important role in mediating the receptor sorting in plasma membrane even if it will be not localize in caveolae domain [177].

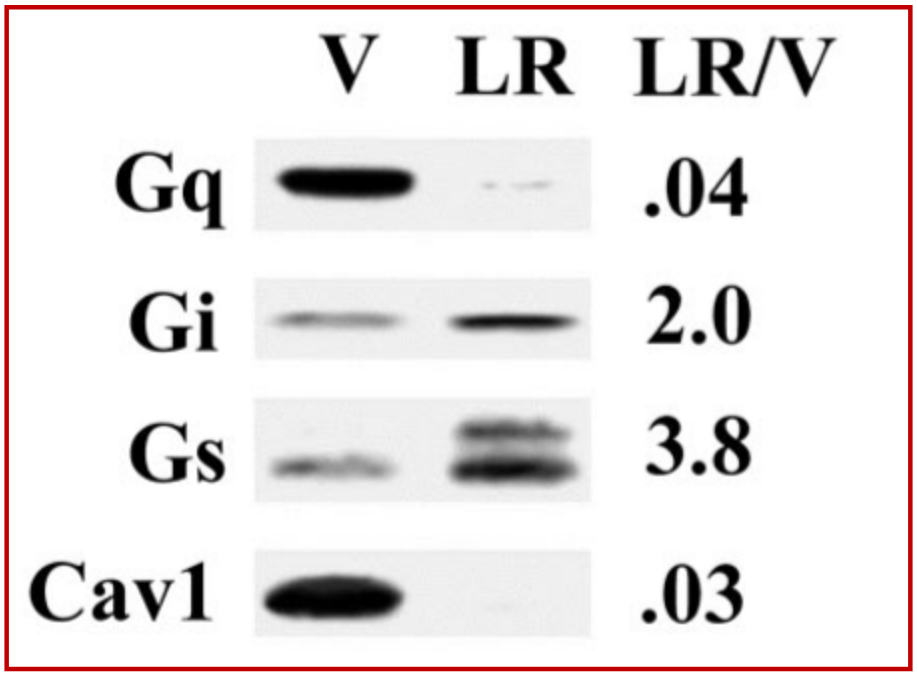


Figure.1.4 *Phil O. et al. Molecular Biology of the Cell. 2001. Receptors localization in caveolae (V) and lipid rafts (LR), isolated from endothelial cell plasma membrane. Gi and Gs appear to be concentrated primarily in the lipid raft, while Gq is preferentially associated to caveolae*

Thus, different G proteins may segregate into different subtype of lipid rafts depending on the presence of other components of the cells. Beside their localization it has been reported that an intact lipid rafts is necessary for the G protein-mediated signaling, indeed alteration of the lipid composition could affect the shift of the receptor from basal to active state and vice-versa. This has been confirmed by in-vitro experiments in cells treated with methyl-β-cyclodextrin, which is known to cause extraction of cholesterol from plasma membrane. Depletion of cholesterol destroys the tight lipid-order of the lipid rafts. This negatively affects the signaling of G protein targeted in the lipid rafts upon their activation, whereas facilitating the pathways mediated by G protein, which translocate in non-raft domains after activation. Indeed, depletion of cholesterol has been associated to impairment of particular G-protein mediated signaling, such as bradykinin-stimulated phosphatydil-inositol turnover [178], as well as thrombin–stimulated phosphatidic acid generation and phosphatidyl-inositol 3,4,5 triphosphate production is inhibited by cholesterol depletion [179]. On the contrary, both myocyte contraction and adenilate cyclase activation mediated by β2-adrenergic and adenosine-1 receptors, respectively, are enhanced by cholesterol depletion [165, 166], whereas supplementation of cholesterol and their fixation in the lipid raft environment inhibit the activity.

These findings suggest the important role that cholesterol and lipid rafts composition play in the regulation of G protein signaling, which is also dependent not only on the localization of the receptors in the lipid rafts or caveolae, but also on the partitioning of these protein between the *rafts* and *non-rafts* domains changing from a basal to an active state and vice-versa [136].

1.3.2 Lipid raft and regulation of Tyrosine kinase signaling

Tyrosine kinases receptors are another prominent example of proteins involved in cell signaling that are enriched in lipid rafts. Epithelial Growth Factor receptor (EGF), Vascular Endothelial Growth Factor (VEGF), Insulin receptor (IR), Nerve Growth Factor receptor (NGF), Platlet Derived Growth Factor receptor (PDGF) and others have been localized to low-density cholesterol-enriched membrane domains and their function is strictly regulated by cholesterol content [158]. TK-associated receptors constitute a diverse family of proteins that, although lacking inherent catalytic activity, recruit active cytosolic signaling proteins in a ligand-dependent manner. They are present in the membrane as monomer and only upon binding of their ligand,they cluster together to generate a dimer, leading to activation of the intracellular tyrosine kinases activity of the receptor [180]. At this point, the receptor auto-phosphorylate in *trans* in the C-terminus generating phosphor-tyrosine residues that serve as binding sites to recruit cytoplasm proteins that are then activate to phosphorylate other proteins on tyrosine residues involved in the process of the signaling transduction [181, 182]. EGF, PDGF, VEGF and others TK receptors follow this activation mechanism since they are segregated as monomer on plasma membrane, on the contrary, IR already appears as dimer. Two different subunits, alpha and beta interacts each other through disulfide-bound forming a monomer where the alpha contains the binding site for the insulin, while beta subunit has the tyrosine kinases domain. IR is a dimer formed by two disulfide-bound alpha-beta units. Interaction of the ligand on the extracellular portion [182] of the alpha subunits modifies the conformation of the receptor within the hetero-tetrameric structure leading to the activation of the tyrosine kinase activity

[183, 184]. Moreover, IR, is also associated to Insulin Receptor Substrate (IRS) which are phosphorylated on tyrosine residues by the respective receptor upon its activation, and serve as adapter proteins for the formation of the protein-protein complexes necessary for signal transduction [185-187].

As the GPCR, also the TK receptor could differentially segregate in caveolar and non-caveolar lipid rafts. When the physical separation of these two lipid rafts subdomains became possible, different studies have been carried out to specifically determine into which of these two classes of low-density membrane domains the receptor actually partitioned. These kind of experiments have been performed mostly using the electron microscopy analysis where TK receptors were labeled with gold in order to determine if the target receptor is present within the caveolar invaginations or whether their presence in localize on the flat lipid rafts. These experiments pointing out the evidence that the distribution of EGF is distinct from that of caveolae and immunoprecipitation analysis also showed that in non-stimulated conditions the EGF receptor is separated from the caveolin-containing membrane domains. However, stimulation of the EGF leads to the loss of this receptor from the lipid rafts domain toward a localization in non-raft membranes. Contrarily, IR largely localizes in caveolae, indeed immunogold electron microscopy demonstrated that insulin receptors are essentially restricted in caveolae domain in both 3T3-L1 [188] and human-derived adipocytes [189]. Likewise the IR, PDGF and VEGF receptors are also associated with caveolar lipid rafts and upon ligand-binding receptors partitioning in the lipid raft rather then non-raft region. However, it has been reported that in

cells lacking of caveolae, IR is localized in non-raft plasma membrane but insulin stimulation enhances the affinity for raft domains.

The observation that membrane receptors move in or out of lipid rafts following hormone stimulation suggest that their association with specific membrane microdomains is not permanent but is subjected to regulation. The association of tyrosine kinases receptors with rafts or caveolae is an equilibrium process that can be shift in one direction or another based on external condition. Indeed, depletion of cholesterol drastically affects TK function as happened for GPCR. Treatment with methy-β-cyclodextrin leads to the flattening of the caveolae, which disappear from the cell surface. Despite this event receptors do not change their membrane distribution, indeed insulin receptor remains associated with the residual part of caveolae but depletion of cholesterol reduces its activity since upon insulin stimulation, phosphorylation of IRS as well as stimulation of glucose uptake inside the cells are decreased [188, 190-192]. Likewise, PDGF-, VEGF-mediated signaling diminishes upon cholesterol depletion, whereas EGF-mediated signaling is up-regulated. Indeed, disruption of lipid rafts was shown to enhance EGF-induced receptor dimer formation and auto-phosphorylation, whereas cholesterol loading is associated to an inhibition of receptor activation [193]. EGF in the only receptor that moves into the non-raft membranes after stimulation, thus disruption of the lipid rafts facilitates its movements outside the microdomains enhancing its activity.

Based on all the findings relating to GPCR and TK receptors, we can affirm that lipid rafts composition affects the localization and the function of the binding receptors, altering the membrane fluidity, thickness and

clustering of the same receptors and other effector proteins both in *caveolar* and *non-caveolar* domains. In this scenario, as cholesterol causes a disruption of the lipid rafts after depletion, it is intuitive that SL levels needs to be tightly regulated in order to preserve the biological function of cells. Recently, the alteration sphingolipid levels has been associated to several disorders such as myocardial infarction, hypertension, stroke, diabetes mellitus type 2 and obesity. However, the specific molecular mechanism controlling SL metabolism and the impact of these changes on the pathogenesis of these diseases are poorly understood.

1.4 Sphingolipids and cardiovascular homeostasis

Sphingolipids biologically participate in several cellular functions. The three-sphingomyelin metabolites, ceramide, sphingosine and the sphingosine-1-phospate (S1P) are the main protagonists, of the sphingolipids class, playing an active role in regulation of the homeostasis of several different cell type. Indeed, sphingolipids give their contribution in the development and the regulation of mitogenesis, cell growth, inflammatory response, and cardiovascular homeostasis acting on different regulatory molecular pathway. In pathological state, where these pathways is altered, regulation of sphingolipids synthesis and production can represent a possible good approach to restore the physiological conditions of the organism.

The role of sphingolipids in the cardiovascular system it has been well studied, however there are several controversial hypothesis that still have to be clarified.

In the field of the cardiac physiology, it has been show that overproduction of SL, such as sphingomyelin and ceramide, causes a severe damage to the cardiac function through both acute and chronic effects [194]. Indeed, increased SL levels in myocardial cells enhances the chronotropic and inotropic action of the heart, following apoptosis and hypertrophy process during the time [195]. Pathological state, such as the ischemic/reperfused heart, shows a decrease of the cardiac contractility and myocardial apoptosis probably due to an increase of TNFα production and inhibition of ceramidase and sphingomyelinase which then leads to an increase of sphingolipids levels and worsening of the cardiac function [196]. Moreover, oxidative stress in myocardial cells is associated to depletion of glutathione, which is involved in the regulation of sphingomyelinase and ceramidase. Activity of these enzymes is down regulated in absence of glutathione with consequent drastically increase of sphingolipids levels to a pathological range [197]. Indeed, glutathione precursor, N-acethyl-cysteine is able to restore ceramide and sphingosine recycle and re-stabilize cardiac function [198]. On the other hand, it has been also discovered that S1P, as other sphingolipids, protects the heart from inflammation, fibrosis and loss of function during a cardiac pressure overload through S1P/S1P1r/eNOS signaling activation [199]. These evaluations are also in line with the evidence that S1P plays also an important role in vascular homeostasis through the activation of eNOS/NO endothelial pathway. Thus, cardiomyocytes-derived sphingolipids are the

necessary to sustain the heart function, however they need to be contained in a physiological window in order to preserve cardiac damage.

Our attention has been more focused on the role of sphingolipids in vascular homeostasis regulation. Ceramide and S1P are the most lipids which show an active role in the new vessels formation (angiogenesis), endothelial barrier integrity maintenance and most important in the regulation of the vascular tone. There are different studies that have shown controversial hypothesis about the role of ceramide in the vascular tone regulation. Igarashi et al in 1999 have demonstrated a vasorelaxant effect of ceramide in isolated vessels as activator of eNOS/NO pathway [200], whereas other hypotheses sustain a role of ceramide in vascular homeostasis by inhibiting eNOS activity [201]. Ceramide, has been also associated to the pathogenesis of the stroke [202, 203]; increased levels of ceramide have been observed in the hippocampus during the middle cerebral artery occlusion (MCAO) [203], and attenuation of SMase activity significantly reduced brain tissue injury in a mouse model of ischemic stroke [204, 205]. Beside all these evidences showed in the literature, the molecular mechanisms underlying the effect of ceramide in vascular tone regulation are still controversial, and they are most likely related to the concentration and further metabolization. Data more consistent and reproducible have been obtained in relation to the S1P. S1P regulates vascular tone acting through G-protein coupled receptors expressed on plasma membrane surface of both endothelial and smooth muscle cells of the vascular bed. Since circulating S1P is transported mainly by HDL [206], it has demonstrated the ability of HDL to induce vasodilation and migration of endothelial cells, as well as to serve a cardioprotective role, and this

beneficial propriety to reduce the risk of cardiovascular disease has been in part associated to the role as an S1P chaperone.

S1P released in the extracellular environment can then interact with the receptor of the cells from where it has been synthesized and released (autocrine action) or on the same type of cells, which are peripheral from its origin site (paracrine). There are 5 types of S1P GPCRs named S1PR1, S1PR2, S1PR3, S1P4R, S1PR5. While S1PR4 is particularly expressed in the lung and lymphoid system and S1PR5 is predominately in brain tissues, S1PR1, S1PR2 and S1PR3 are three main receptors mediating the S1P signaling in the vasculature. However, the localization of these receptors is different between vascular smooth muscle and endothelial cells. Indeed, S1PR2 and S1PR3 mainly regulated the signaling of S1P in smooth muscle cells [207], whereas endothelial cells predominately express S1PR1 [208]. Stimulation of receptors on smooth muscle layer is associated to a vasocontracturant response, however S1P3R and S1PR2 are GPCR coupled to different α subunits, indeed S1PR3 is a $G\alpha_q$ protein thus, upon it activation, increases the intracellular flux of Ca^{2+} leading to contraction [209] whereas S1R2 acts through a different molecular signaling since it is associated to a $G\alpha_{12/13}$-coupled protein which is responsible of RhoA/Rho pathway activation and constriction of vascular smooth muscle cells [210]. On the contrary, endothelial S1PR1 is a $G\alpha_i$-coupled receptor and its interaction activates the intracellular PI3K/Akt pathway. Akt is a kinase, which regulates several proteins through phosphorylation [211]. S1P/S1PR1 is associated to an increased phosphorylation eNOS in EC promoting vasodilatation through the eNOS/NO pathway [211]. Nevertheless the localization of S1P

receptors is not always the same in all the vessels, thus vascular effect of S1P depends on the vascular bed where it acts.

Interaction of S1P with S1Pr has shown a clinical important role in the immunological process, indeed Fingolimod (FTY720), an orally active drug targeting 4 out 5 S1PRs, has been approved by FDA for treatment of multiple sclerosis. Pharmacologically, FTY720 acts as an agonist at four of the five S1P receptors, S1P1-3-4-5R and inhibits lymphocyte egress from secondary lymphoid organs [212]. Nevertheless, it has been observed that chronically treatment with FTY720 leads to S1PR1 internalization and degradation; hence FTY720 was defined “functional antagonist [213]. Moreover, the activation of the receptors S1P1 in the cardiovascular system accounts for the transient effects of FTY720 on heart rate, on the atrio-ventricular conduction and the lasting effect on BP [214-218]

Moreover, the interaction between S1P and its S1P1R appears of a great importance not only for the regulation of the vascular tone, but also because mediates angiogenesis, inhibition of apoptosis, chemotactic response and proliferation [219]. Indeed it has been demonstrated that low levels of plasma S1P, such as in absence of Sphk [220], ApoM [221] cause the break of S1P-S1PR1 interaction leading to an increase of vascular permeability. This confirm the importance of S1P signaling in maintain the physiological vascular homeostasis.

One of the most relevant signaling regulated by S1P is the eNOS/NO pathway in endothelial cells. Several *in-vitro* studies have proved the ability of S1P in directly activate eNOS. Studies on fibroblast-like cell lines derived from monkey kidney tissue (COS-7 cells) transfected with both

eNOS and S1PR1 and then stimulated with the lipid molecule, showed that NO production was increased after stimulation with S1P, while didn't mutated in the absence of S1PR1 [169]. These data have been then confirmed on Bovine Aortic Endothelial Cells (BAEC), where eNOS and S1P1R were physiologically expressed. Subsequently, from the same group of research it has also been discovered that S1PR1 activity is also regulated by Cav-1, as happen for eNOS enzyme. Indeed, in BAEC cells, the overexpression of Cav-1 leads to a decreased activation of eNOS following the stimulation of S1P. Moreover, S1P stimulation progressively caused a translocation of the S1PR1 from the plasma membrane microdomains, to caveolae where the bound with Cav-1 shouted down the signaling, acting as a negative feedback for a prolonged S1P stimulation [169]. Moving then the attention on an in-vivo model, endothelial vascular cells following chemical or mechanical stimuli are continue sources of different vasoactive mediators, such as NO, prostacyclin and also S1P. S1P through an autocrine or paracrine manner acts as a potent activator of eNOS through S1PR1 on vascular endothelial cells [211] of a magnitude comparable to acetylcholine and bradykinin. It is also known that eNOS is activated by mechano-trasduction signal, as the blood flow-induced shear stress which, increase the release of NO from eNOS with consequent vasorelaxation [222]. Moreover recently different evidences have been accumulated regarding the involvement of S1P-S1PR1 interaction in blood flow-induced vasodilation. Absence of S1PR1 in endothelial cells fails to induce vasodilatation in response to flow as well as eNOS phosphorylation [223]. This data has been also confirmed by the evidence that inhibition of S1PR1 with W146 reduced both vasodilation induced by the flow and NO production, suggesting that S1P- S1PR1

interaction plays the major role in the mechano-trasduction signaling regulated by the blood flow [224]

S1P produced by the vascular wall also participates in the regulation of the myogenic tone. In rabbit posterior cerebral arteries it has been show that changes in the intraluminal vascular pressure are followed by an increase of S1P release, which acting of S1PR2 and S1PR3 on the smooth muscle cells, induces a Ca^{2+}/Rho-mediated contraction of the vessels [210]. Moreover, overexpression of Sphk1 [225], and deficiency of S1P phosphohydrolase [226] increase the myogenic tone in resistance arteries, while the overexpression of S1P phosphohydrolase causes the opposite effects, confirming that S1P plays a significant role in the vascular tone regulation. Moreover, it has been reported that S1P is also involved in AngII-induced hypertension. AngII causes an increase of vascular tone and blood pressure through a biphasic Ca^{2+} entry in the vascular smooth muscle cells. Sphk1 participates in the AngII-mediated activation of store-operated calcium channels (SOCs), indeed, $Spk1^{-/-}$ mice show lower blood pressure compare to WT mice after AngII-induced hypertension [227]. Although these findings, it is still difficult to associate the increase of the vascular tone during hypertension to the hyper-activation of S1P-S1PR2 or S1P3R signaling, since it can be due also to a disruption of the vasoprotective S1P-S1PR1 pathway in endothelium.

1.5 Aim of my study

Recently, the research group of Prof Di Lorenzo demonstrated that S1PR1 is a key regulator of blood pressure and flow through endothelial S1P-S1PR1-eNOS stimulated autocrine signaling [224]. Furthermore, they discovered a novel mechanism by which endothelial sphingolipids biosynthesis is regulated identifying NogoB [224].

Nogo-B is a Reticulon (RTN) proteins localized in the tubular ER through two transmembrane domains. The large family of RTN proteins is composed by three major isoforms, RTN-4A, -4B and -4C, also called Nogo-A, -B and -C. While Nogo-A and -C are expressed in the central nervous system, Nogo-B is highly localized in blood vessels [224]. It has been demonstrated that NogoB has an inhibitory activity on SPT, indeed murine and human derived EC depleted of Nogo-B show a 40% increase of SPT activity compare to the control, while the lentiviral- mediated re-expression of the protein restored the physiological activity of SPT in blood vessels [118]. Starting from these evidences, the group of research of Prof. Di Lorenzo demonstrated that Nogo B binds to and inhibits serine palmitoyltransferase (SPT), the rate-limiting enzyme of the de novo sphingolipids biosynthesis, thereby controlling local endothelial S1P production to impact vascular function, blood pressure [224] and heart failure[199] (Figure 1.5). Nogo-B ECKO mice were hypotensive and protected by AngII-induced hypertension; these effects were reverted by *in vivo* administration of myriocin, pharmacological inhibitor of SPT, which restored BP in these mice to WT levels.

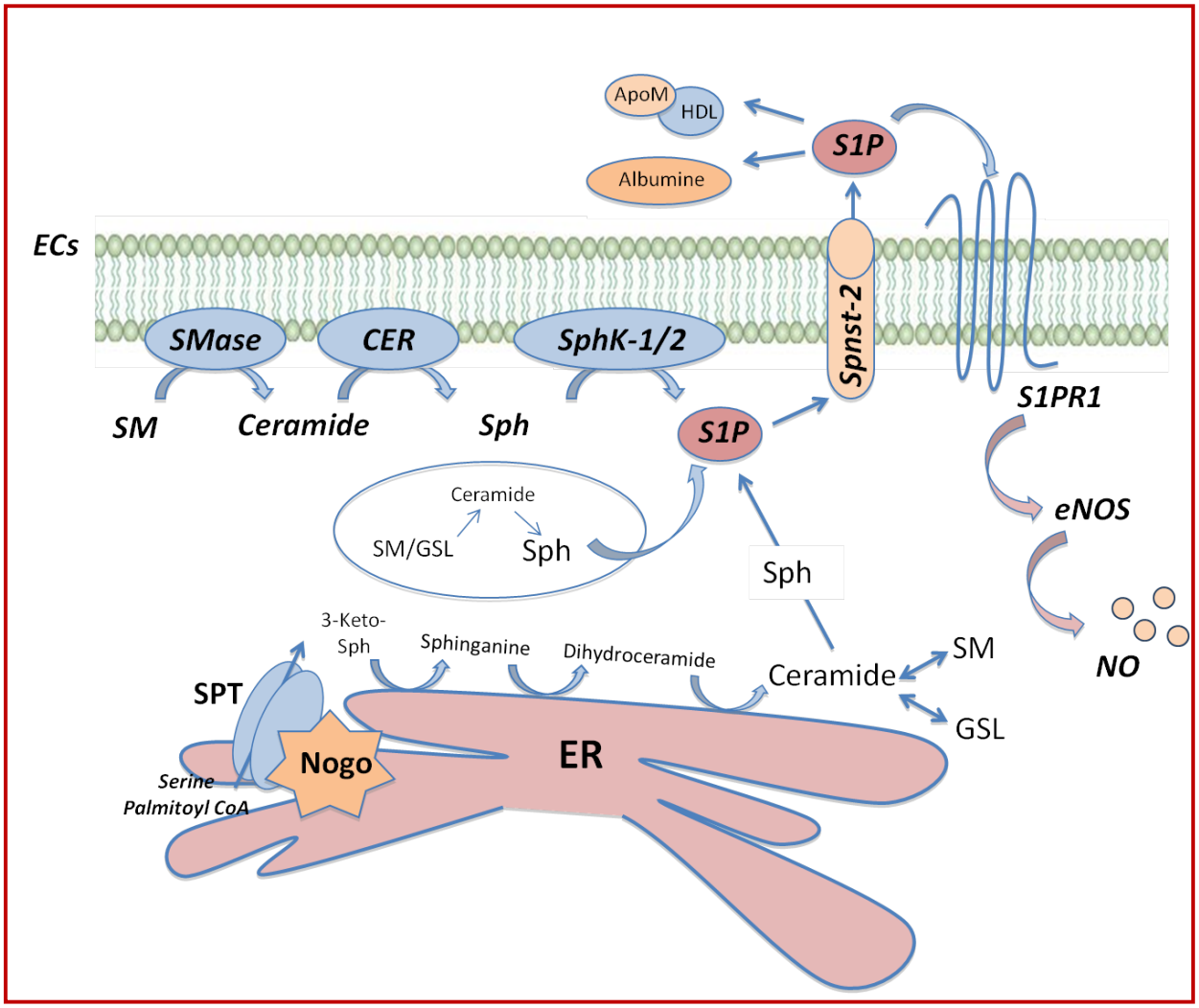


Figure 1.5 ***NogoB down-regulates the de novo sphingolipids biosynthesis by inhibition of SPT enzyme***

These findings established a strong foundation for further research on the role of sphingolipids in cardiovascular pathophysiology.

It has been well established that endothelium is bathed in SL-enriched plasma, and it can recycle SL up-taken from plasma, as well as deriving from intracellular catabolism process. Thus, during my last year of PhD in Professor Di Lorenzo Lab, I have been interested in the role of endothelial *de novo* biosynthesis in preserving vascular homeostasis. In particular we focused our attention in the involvement of this pathway in vascular tone

and blood pressure regulation by using a novel conditional genetic mouse model lacking of *Sptlc2* subunit in EC.

2. MATERIALS AND METHODS

2.1 Animals

We carry out experiments on conditional mouse model lacking of Sptlc2 subunit, or both Sptlc2 and NogoB protein, specifically in the endothelium, named **ECKO-*Sptlc2*** and **ECKO-*NogoB-Sptlc2***

Mice lacking *Sptlc2* specifically in endothelial cells (ECKO-*Sptlc2*) were obtained by crossing Sptlc2$^{f/f}$ mice with transgenic mice in which the VE-cadherin promoter drives expression of tamoxifen-responsive Cre (VE-Cad-CreERT2) [228] such that tamoxifen treatment selectively deletes the *lox*P-flanked ('floxed') region of *Sptlc2* in endothelial cells. Mice from this strains were born with Mendelian frequencies. To induce *Sptc2* deficiency in endothelial cells, male Sptlc2$^{f/f}$-VE-Cad-CreERT2-positive and Sptlc2$^{f/f}$-VE-Cad-CreERT2-negative littermates were injected intraperitoneally (i.p.) with 15 mg/kg of tamoxifen daily for 5 d at the age of 7-8 weeks. The same protocol has been applied to generate mice lacking of Nogo and Sptlc2 specifically in endothelial cells using *Nogo$^{f/f}$-Sptlc2$^{f/f}$.* In both cases, the extent of Cre-mediated excision of exon 2 and 3 of *Sptlc2* and *Nogo$^{f/f}$-Sptlc2$^{f/f}$* was confirmed by real-time PCR on endothelial cells isolated from mouse lungs and on endothelial cell and VSMC mRNA isolated from the thoracic aorta of *Sptlc2$^{f/f,}$ Nogo$^{f/f}$-Sptlc2$^{f/f}$* mice and EC-deficient *Sptlc2* and *Nogo-Sptlc2* animals.

In another set of experiments we used **Sptlc2$^{+/-}$**mice, since Sptlc2$^{-/-}$ mice are embryonically lethal [229].

The Weill Cornell Institutional Animal Care and Use Committee approved all animal experiments.

2.2 Sphingolipid analysis by LC-MS/MS

Plasma from ECKO-*Sptlc2* mice and their littermates controls has been used for quantification of sphingolipids by LC-MS/MS. The levels of ceramide (Cer) species, sphingosine (Sph) and S1P were analyzed by the Lipidomics Analytical Core at the Medical University of South Carolina as previously described [230].

2.3 Blood pressure measurement

Systolic blood pressure was measured in conscious ECKO-*Nogo*-*Sptlc2*, ECKO-*Sptlc2* male mice and their littermates control after treatment with tamoxifen using the pneumatic tail-cuff method (MRBP System, Life Science, Woodland Hills, California). Animals were placed in a plastic chamber maintained at 34 °C and a cuff, with a pneumatic pulse sensor, was attached to the tail (Figure. 2.1 A). As the cuff is inflated of air, the circulating flow is progressively reduced until it is completely blocked (thin signal - Figure. 2.1 B). Then, as the air goes outside the cuff, blood starts again to flow and the first pressure felt by vessel's wall represents the Systolic Blood pressure (SBP) (thick signal - Figure 2.1 B), the highest pressure of the artery corresponding to the contraction of the left ventricle during the systole to pump the blood in the peripheral circulation. Mice were trained for three days, then pressure was monitored for one week. Each day, three consecutive measurements were performed per mouse, and the values were averaged.

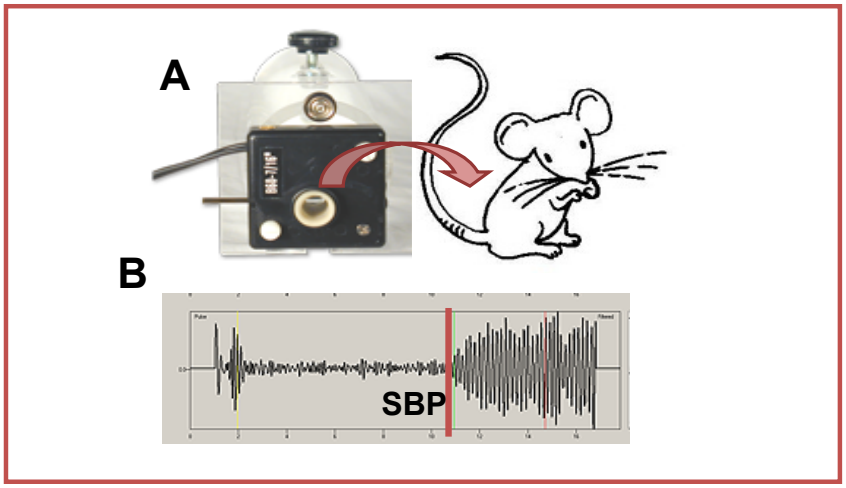


Figure 2.1 ***Tail-cuff system***

2.4 Vascular reactivity assessment through pressure myograph

WT and Sptlc2$^{+/-}$, as well as *Sptlc2*$^{f/f}$ and ECKO-*Sptlc2* mice after tamoxifen treatment were sacrificed. Second order mesenteric arteries (MA) from ECKO-*Sptlc2* were harvested, carefully cleaned from adhering tissue and mounted on micropipettes in a pressure myograph chamber (Danish MyoTechnology, Aarhus, Denmark); the orientation of the vessel in relation to the flow *in vivo* was maintained. Viability of the vessels was maintained using Krebs solution (in mM: NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 10.1), at 37 °C and oxygenated (95% O_2 and 5% CO_2). Micropipettes were connected to a pressure interface, which regulated intraluminal pressure and flow. The vessel diameter was monitored in real time using a microscope connected to a digital video camera (IC Capture) and computer software with edge detection capability (Figure 2.2).

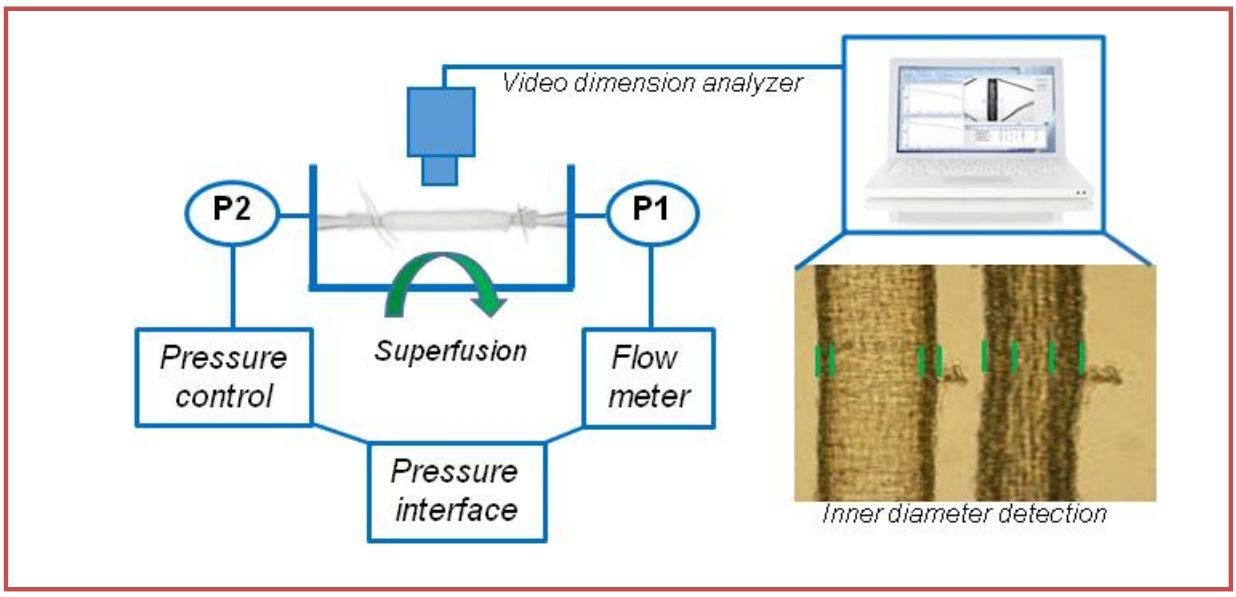


Figure 2.2 ***Pressure Myograph system***

MA were equilibrated for 30 min at 80 mm Hg, pre-constricted with PE (1 μM) and a cumulative concentration-response curve of Ach (0.1nM-30uM) was performed to evaluate the integrity and the function of the endothelium. Normotensive vessels with less than 80% vasodilatory response to Ach were discarded. In order to evaluate the endothelium-dependent vasodilation mediated by particular receptors expressed on the surface of endothelial cells, PE pre-constricted MA have been stimulated by:

- Gαi-coupled receptor ligands, as S1P (1pM-30μM) and Thrombin (1μU/ml-30mU/ml);
- Gαq-coupled receptor ligands, as Ach and Histamine (10nM-30μM);
- Tyrosine-kinase receptors ligands, as VEGF (1μg/ml-30mg/ml) and Insulin (1pU/ml-3μU/ml).

Moreover, endothelium-independent vasodilatation of ECKO-*Sptlc2* MA has been assessed through concentration-response curve of

Isoprotenerol (10nM-30uM) and Sodium Nitroprussiate (10nM-30uM) on PE pre-constricted vessels while vasoconstriction has been evaluated through cumulative concentration-response curve of PE (1nM-30uM).

Flow-mediated vasodilation on mesenteric arteries was measured using pre-contracted vessels arteries with a concentration of PE inducing 60% of the maximum vasoconstriction achieved in the concentration-response curve of PE, followed by a step wise increase of 25 µl/min of the intraluminal flow from 0 to 125 µl/min. Flow was maintained for 5 min at each step.

In another set of experiments ECKO-*Sptlc2* MA were incubated with N5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-Nio) as eNOS inhibitor 10uM for 15 minutes, and then vascular response to Ach and increase of flow has been assessed

At the end of each experiment, a myogenic curve was performed in Ca^{2+}-free Krebs buffer supplemented with 1 mM EGTA. Myogenic tone (%) was expressed as $[(D_1 - D_2)/D_1] \times 100$, where D_1 is the passive diameter in Ca^{2+}-free Krebs buffer and D_2 is the active diameter in complete Krebs buffer, at the same intraluminal pressure.

Carotid vessels from *Nogo*$^{f/f}$-*Sptlc2*$^{f/f}$ and ECKO-*Nogo-Sptlc2* mice were also mounted in Pressure Myograph system following the same standardization protocol. Cumulative concentration response curves of Ach (0.1nM-30uM) have been performed on PE 1uM pre-constricted vessels. Moreover, vasodilation in response to increase of intraluminal flow has been evaluated on pre-contracted vessels arteries with a concentration of PE inducing 60% of the maximum vasoconstriction

achieved in the concentration-response curve of PE. A step wise increase of 25 µl/min of flow has been performed from 0 to 800 µl/min. Flow was maintained for 5 min at each step.

2.5 Bleeding assay

Bleeding assay has been performed as previously demonstrated [231]. Coagulation time has been assessed on male and female ECKO-*Sptlc2* and their littermate control after 4/5 weeks and 6/7 weeks post tamoxifen treatment. Mice were anesthetized and placed on a heating plate in prone position (Figure 2.3 A). The tail was cut (≈ 1mm), immediately immersed in a 50ml Falcon tube filled with PBS 37° and the time of coagulation started to be monitored (Figure 2.3 B). Bleeding time is determined using a stop clock and monitoring each animal for 20 minutes. In order to detect any re-bleeding during the experiment, if bleeding on/off cycles occur, the sum of the bleeding times within the 20 minutes period is calculated and used for the analysis (Figure 2.3 C).

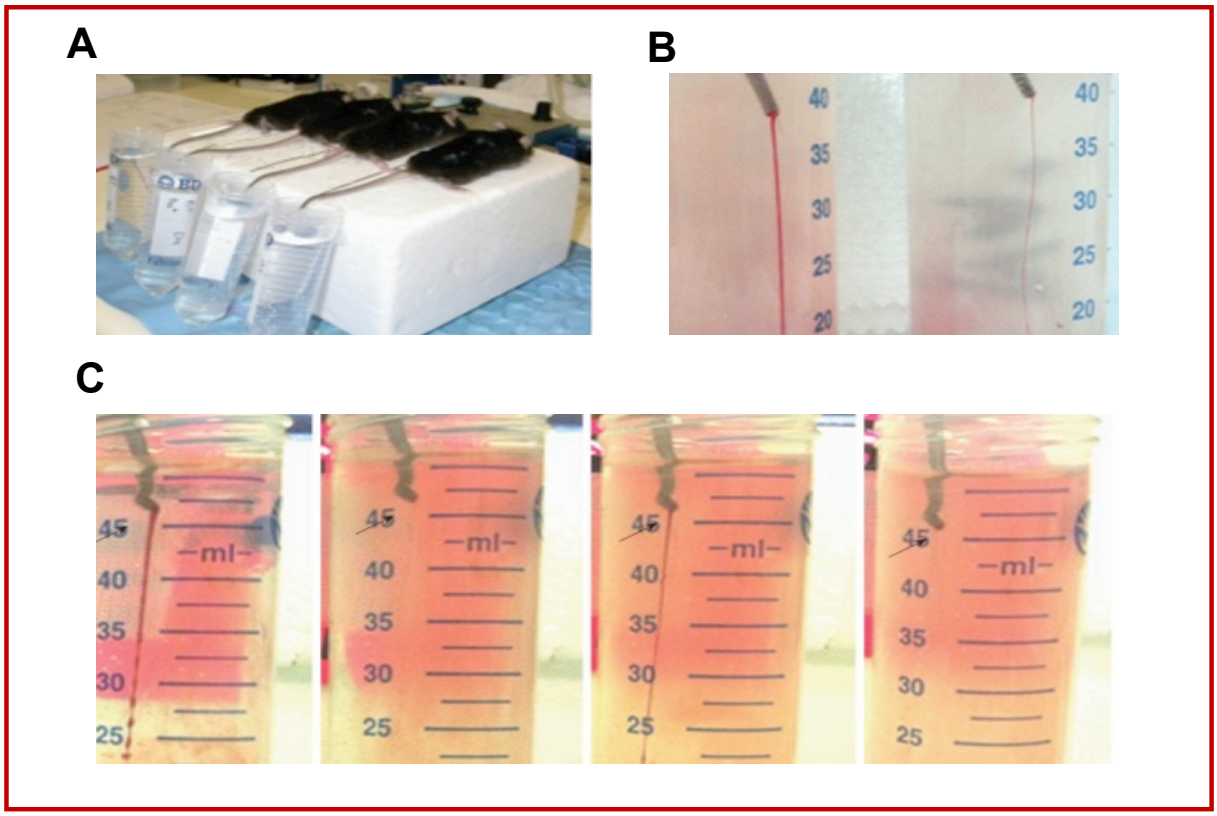


Figure 2.3 ***Bleeding assay***

2.6 Electron Mycroscopy analyses

Males *Sptlc2*$^{f/f}$ and ECKO-*Sptlc2* mice were deeply anesthetized with sodium pentobarbital (150 mg/kg, I.P.). Mice were placed chest up onto the silicon pad and pin the paws to check if it was completely anesthetized. Then, whiteout injuring the underlying organs, the chest was opened by a cut until the heart was visible. Heart was grabbed with the hemostatic forceps and a needle was inserted in the left ventricle without puncturing through in order to perfuse the heart using a pump to a speed of about 9ml/min. Perfusing solution was composed by: (1) 10–15 ml 0.9% saline containing 2% heparin; (2) 50 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4); and (3) 200 ml of 2% paraformaldehyde in PB [232]. After the perfusion, carotid arteries

were collected, cleaned from the adherent tissue and post-fixed in 2% paraformaldehyde in PB for 30 min, and then transferred into PB. Sections were stored in cryoprotectant solution (30% sucrose and 30% ethylene glycol in PB) at 20 °C. Carotid sections were then analyzed through Electron Microscopy (EM) to see caveolae distribution in the endothelial layer.

2.8 Statistical analysis

Data are expressed as mean ± s.e.m. One-way or two-way ANOVA with *post hoc* Bonferroni's test was run for all statistical analyses except where a Student's *t*-test analysis was used. Differences were considered statistically significant when $P < 0.05$. All tests were two-sided. GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA) was used for all statistical analysis. The s.d. considered for the calculation was estimated on the basis of previous published studies using the tail-cuff system to measure blood pressure in mice and by preliminary experiments of vascular reactivity using mesenteric arteries from three mice.

3. RESULTS

3.1 The lack of endothelial SPT increases BP

Mice lacking of Sptlc2 expression in endothelial cells have increased BP then control mice at baseline (Figure 3.1 A), suggesting that endothelial-derived sphingolipids play an important role in preserving vascular homeostasis.

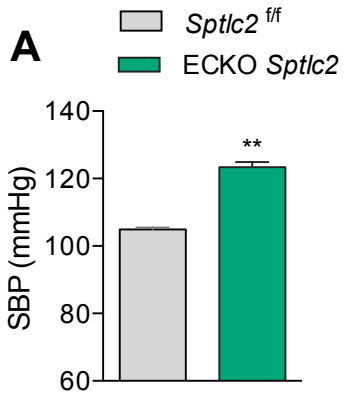


Figure 3.1 *Systolic blood pressure of* ***(A)*** *ECKO Sptlc2 104.9±0.62 vs 123.4±1.53, control vs ECKO Sptlc2, n=5mouse/group, **p<0. 01. Statistical significance was determined by unpaired T-test*.

3.2 The loss of endothelial SPT reduces plasma levels of sphingosine and S1P

The lack of Sptlc2 subunit in EC induces a reduction of plasma levels of different ceramide species as C16-, C22- and C24-ceramide (Figure 3.2 A, B). Moreover, plasma from ECKO-*Sptlc2* mice presents a significantly decrease of dihydrosphingosine-1 phosphate (dhSph-1P), S1P as well as Sphingosine (Sph) levels compared to the control (Figure 3.2 A, B), suggesting that reduction of endothelial SPT activity decreases sphingolipids production and their concentration in the circulating blood.

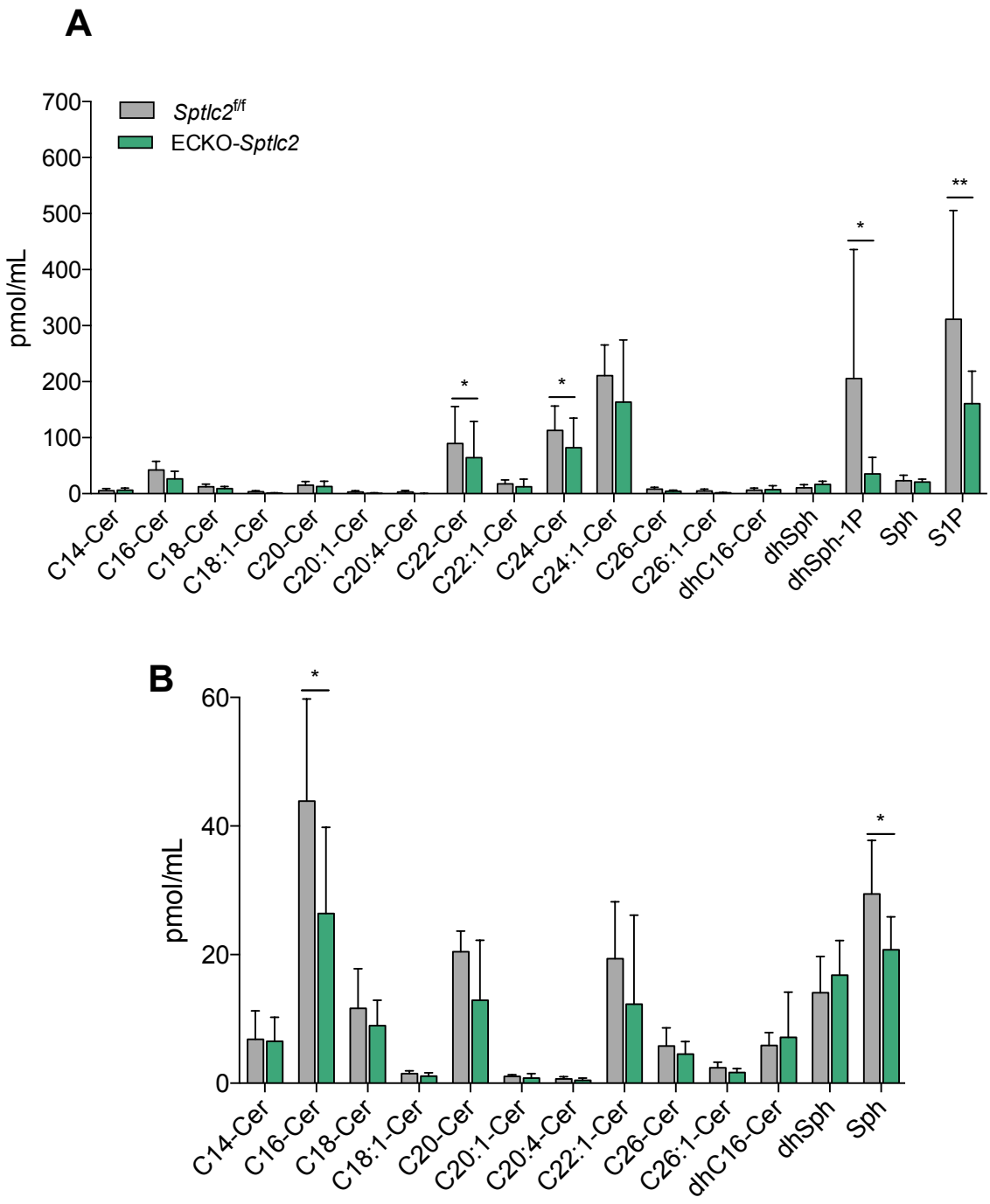
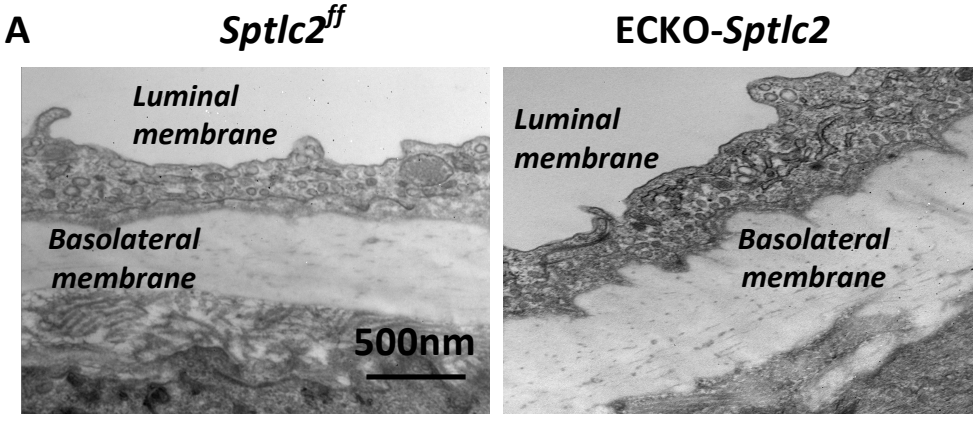


Figure 3.2. ***(A-B)*** *Total ceramide species, sphingosine and S1P levels in plasma from ECKO-Sptlc2 mice and their littermates control measured by LC-MS (n≥9 mouse/group, *p<0.1, p<0.01). Statistical significance was determined by unpaired T-test.*

3.3 The reduction of sphingolipids production affects the number and the size of caveolae in carotid arteries.

It is well known that sphingolipids are important components of plasma membrane microdomains, distinguished in caveolar and *non caveolar* lipid rafts. In order to understand whether the reduction of endothelial sphingolipids production could leads to morphological alterations of plasma membrane, we performed EM analysis of carotid arteries (Figure 3.3 A-B). The size of caveolae in the basolateral and luminal membranes of the endothelium of ECKO *Sptlc2* is reduced compared to the control (Figure 3.3 A-B-C). On the contrary, the number of caveolae is significantly increased (Figure 3.3 D-E-F), probably due to a compensatory mechanism. The lack of SPT activity in endothelial cells could reduce sphingolipids in plasma membrane and impair their relative abundance with cholesterol, altering lipid raft microdomains



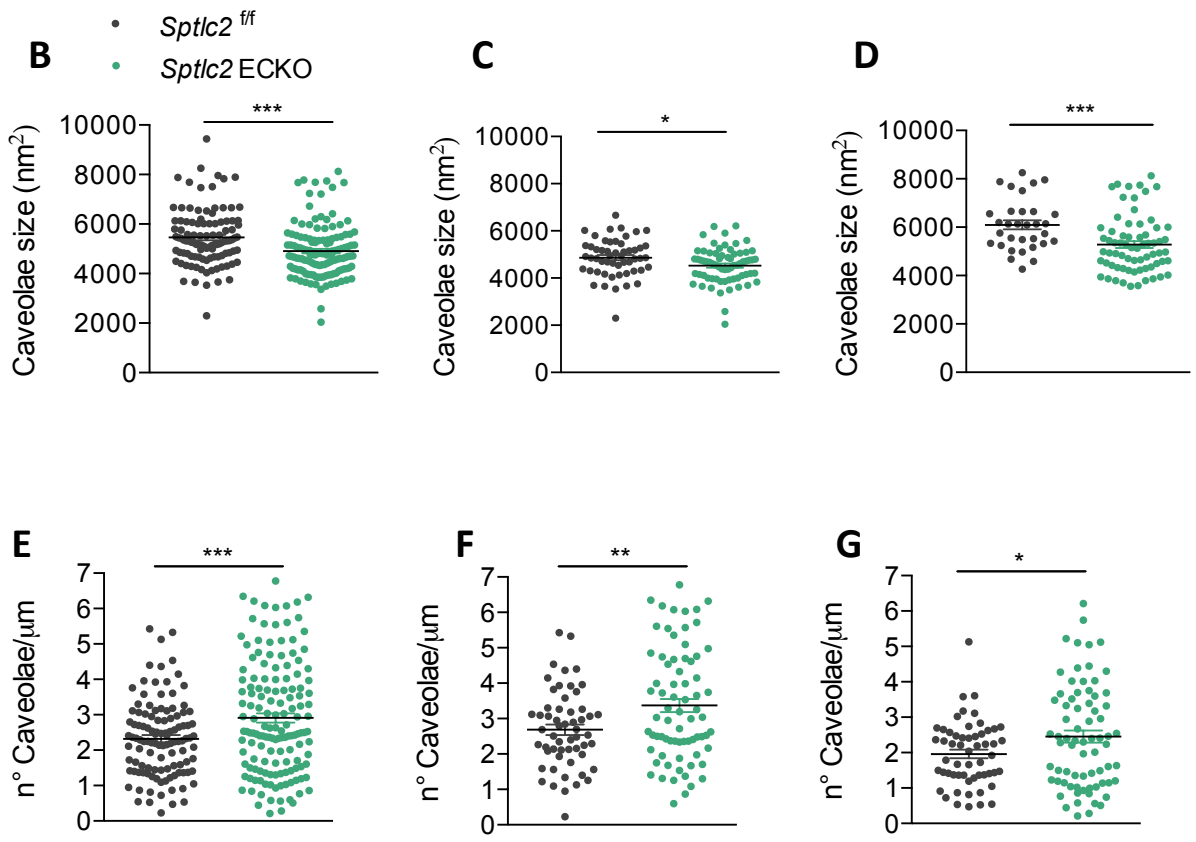


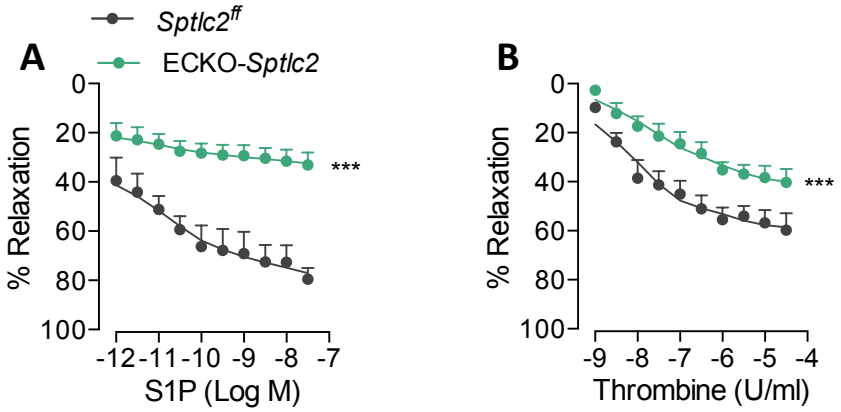
Figure 3.3 ***(A)*** *Electron microscopy analysis of caveolae expressed on carotid arteries endothelial layer from Sptlc2^{ff} and ECKO-Sptlc2 mice.* ***(B)*** *Size of caveolae in* ***(C)*** *basolateral and* ***(D)*** *luminal endothelial cells plasma membrane.* ***(E)*** *Number of caveolae in* ***(F)*** *basolateral and* ***(G)*** *luminal endothelial cells plasma. Data are expressed as mean± SEM.* ***(B-F)*** *Statistical significance was determined by Unpaired t-test, *P<0.1, **P<0.01, ***P<0.001 ECKO Sptlc2 vs. Sptlc2$^{f/f}$.*

3.4 Endothelial-dependent vasodilatation is impaired in ECKO-Sptlc2 resistance arteries.

Ligand-mediated activation of GPCR and TK receptors in EC mainly induce vasodilation via eNOS-derived NO [233]. GPCR and TK receptors are localized in lipid rafts, specialized sphingolipid-enriched microdomains of the membrane functioning as platform for transduction signaling [136, 137, 158]. In order to understand whether the loss of SPT in EC affects

specific microdomains-linked signaling pathways, we assessed the endothelial-dependent vasodilation induced by different GPCRs and TK receptors agonists on ECKO-*Sptlc2* and *Sptlc2*$^{f/f}$ MA. We demonstrate that S1P- and thrombin-mediated vasodilatation, through $G\alpha_i$-coupled S1PR1 and PAR1 respectively, are significantly reduced in ECKO-*Sptlc2* compared the control MA (Figure 3.4 A-B). We also showed that vasodilation of MA in response to insulin and VEGF through IR and VEGRF2, TK receptors, was markedly reduced in absence of endothelial Sptlc2 (Figure 3.4 C-D). On the contrary, Ach and histamine $G\alpha_q$-mediated vascular response were not affected in the two groups (Figure 3.4 E-F). Thus, reduction of SPT activity in EC causes a decrease of $G\alpha_i$-coupled and TK receptors-mediated vasodilatation, whereas vascular response via $G\alpha_q$-coupled receptors remains unaltered.

It has been reported that depletion of membrane cholesterol with Methyl-β-cyclodextrin decreases lipid order and fluidity, blunting the intracellular signaling in response to shear stress [234]. We demonstrated that absence of endothelial *Sptlc2* decreases flow-mediated vasodilatation in MA (Figure 3.4 G), suggesting that lipid order of endothelial membrane is associated to shear-stress-sensing response [234, 235].



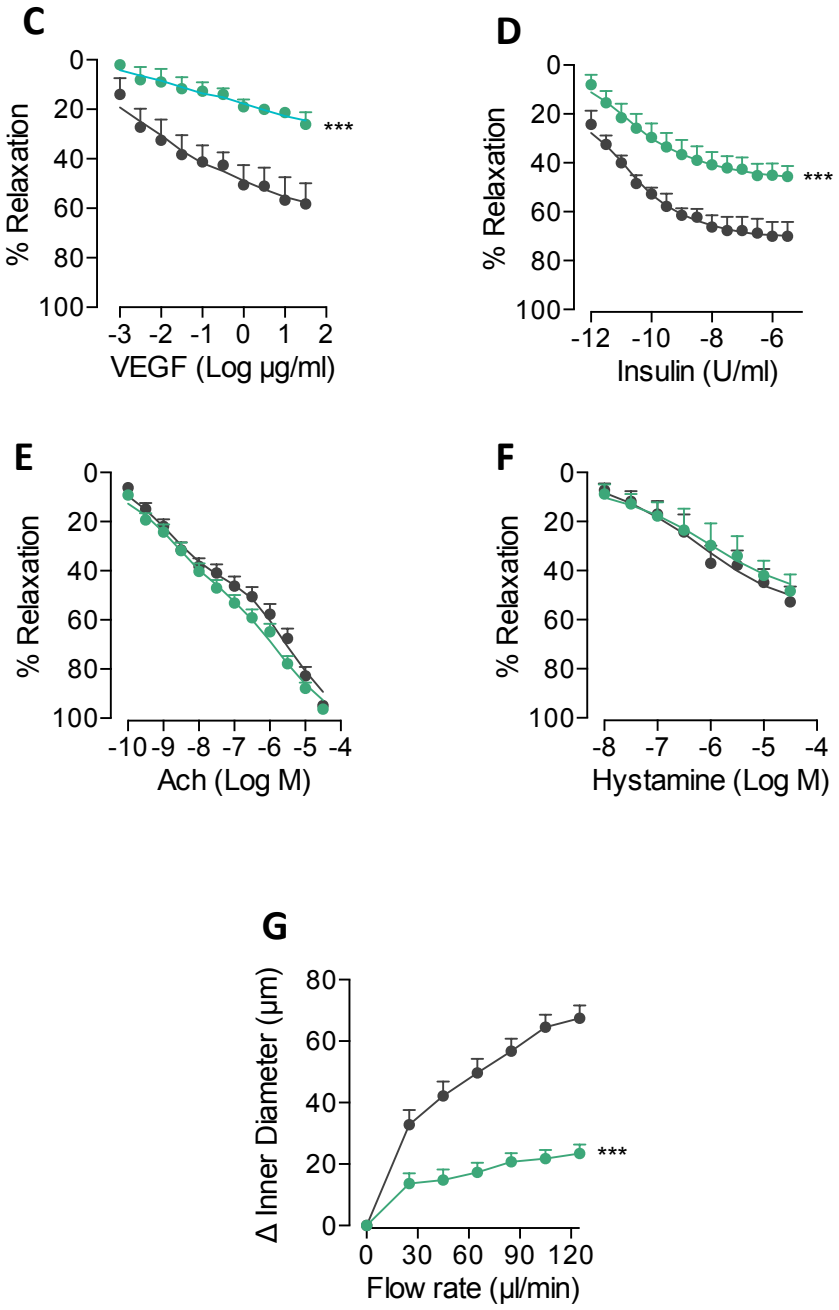


Figure 3.4 ***(A-B)*** *Cumulative concentration-response curve of Gαi-coupled receptor on mesenteric arteries:* ***(A)*** *S1P-induced vasodilatation (n≥5 MA/group, n≥5 mouse/group, ***p<0.001) and vascular response of* ***(B)*** *Thrombin (n≥7 MA/group, n=≥4 mouse/group, ***p<0.001).* ***(C-D)*** *Tyrosine-kinases induced vasodilatation: vascular response of (C) VEGF (n≥5 MA/group, n≥3 mouse/group, ***p<0.001) and Insulin (n≥4 MA/group, n≥4 mouse/group, ***p<0.001). Endothelial-dependent vasodilatation mediated by Gαs-coupled receptor;* ***(E)*** *Ach (n=12) and* ***(F)*** *Histamine (n≥4 MA/group, n≥3 mouse/group, ***p<0.001) vascular response* ***(G)*** *Flow-induced endothelial-dependent vasodilatation in ECKO-Sptlc2 mice vs Sptlc2$^{f/f}$. Data were expressed as the mean ± s.e.m. ***P<0.001 compared to control mice. Statistical significance was determined Two-way ANOVA followed by Bonferroni's post-test.*

3.5 eNOS-NO pathway is preserved in ECKO *Sptlc2* MA

We used L-Nio as pharmacological inhibitor of eNOS enzyme to evaluate the contribution of NO in the endothelial-dependent vasodilatation to Ach and flow in ECKO-*Sptlc2* mice. Ach-induced vasodilatation is significantly decreased following L-Nio, in both ECKO and control MA to the same extent (Fig 3.5 A). Likewise, L-Nio equally reduces flow-mediated vasodilatation in both ECKO *Sptlc2* and control MA (Fig 3.5 B), suggesting that in absence of SPT eNOS-NO pathway still gives a contribution in the vasodilatation.

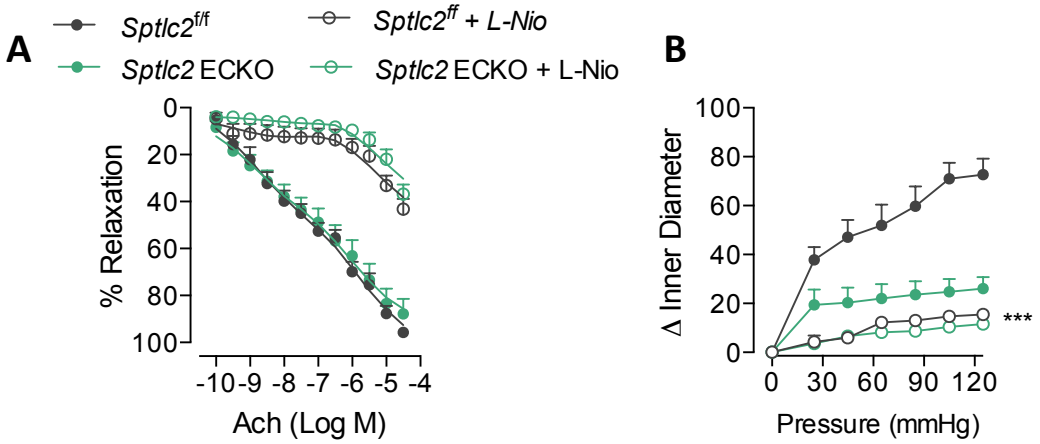


Figure 3.5 ***(A)*** *Cumulative concentration-response curve of Ach (n≥9 MA/group, (n≥8 mouse/group, ***p<0.001) and* ***(B)*** *flow-mediated vasodilatation (n≥9 MA/group, n≥8 mouse/group, ***p<0.001) after incubation of L-Nio 10uM. Data were expressed as the mean ± SEM ***P<0.001 compared to control mice. Statistical significance was determined by (A-B) Two-way ANOVA followed by Bonferroni's post-test*

3.6 Vascular smooth muscle cell-dependent vasodilatation is retained in absence of endothelial SPT

To assess that guanylyl cyclase-dependent cGMP production is preserved in absence of endothelial SPT activity, we employed the Sodium

Nitroprussiate (SNP), a NO-donor. SNP-induced vasodilatation is not impaired in absence of endothelial SPT (Figure 3.6 A).

Next, we evaluated β2-receptor mediated VSMC relaxation. As shown in Figure 3.6 B the vascular response to Isoprotenerol was not altered by the loss of endothelial SPT (Figure 3.6 B) suggesting that cGMP production and its vasorelaxing signaling in VSMC are preserved.

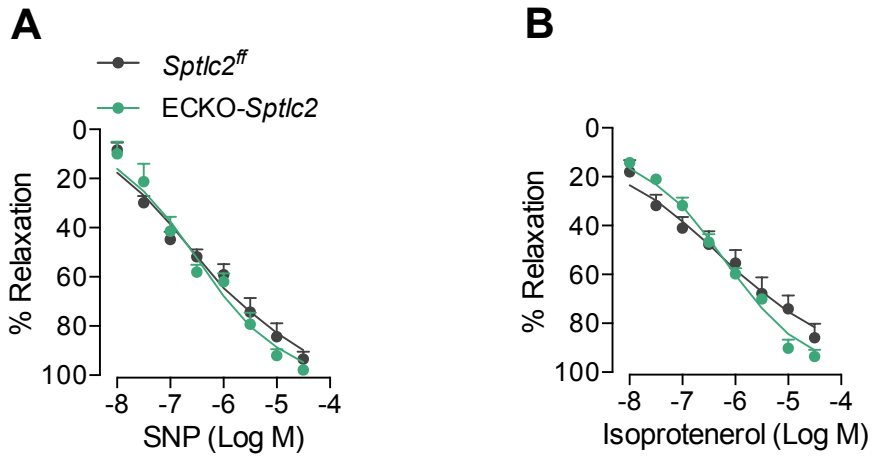


Figure 3.6 *Cumulative concentration response curves of* ***(A)*** *Isoprotenerol and* ***(B)*** *SNP - induced vasodilation (n≥5 MA/group, n≥4 mice group). Data were expressed as the mean ± s.e.m. ***P<0.001 compared to control mice. Statistical significance was determined Two-way ANOVA followed by Bonferroni's post-test*

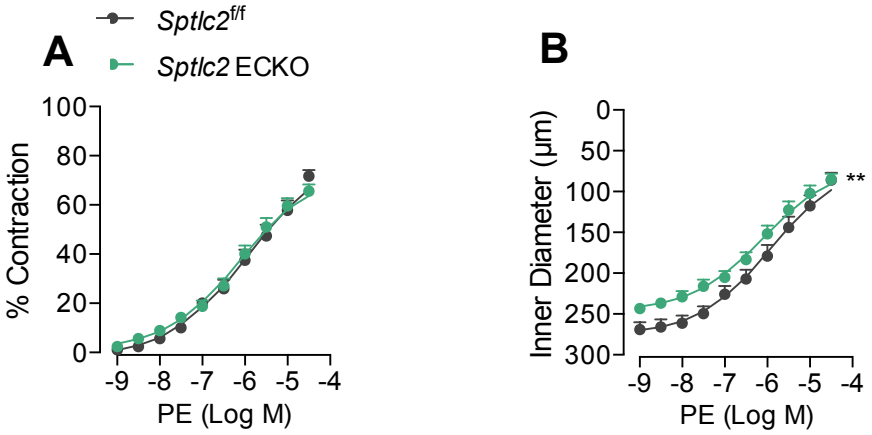
3.7 The lack of SPT increases vasoconstriction in response to mechanical, but not, pharmacological stimuli.

We assessed the vasoconstriction to pressure and pharmacological stimuli in MA from ECKO-*Sptlc2* and *Sptlc2*$^{f/f}$ mice. MA vasoconstriction in response to PE is preserved in absence of endothelial SPT (Figure 3.7 A).

Next, we assessed the myogenic response of MA, which represents the spontaneous contraction of the vessel to the increase of intraluminal

pressure to maintain constant the flow. First, we evaluated the response of the vessels to the increase of intraluminal pressure in complete (active tone) and Ca^{2+}-free Krebs buffer (passive tone). Physiologically, the increase of intraluminal pressure induces vascular contraction, whereas in Ca^{2+}-depletion conditions this response is abrogated and vessels diameter progressively increases. ECKO-*Sptlc2* show a significantly increase of the active tone in response to pressure compared to control MA (Figure 3.7 C), whereas there are no differences in the passive inner diameter (Figure 3.7 D), suggesting that myogenic tone is significantly higher in absence of endothelial SPT (Figure 3.7 E).

Altogether these data suggest that endothelial-derived sphingolipids are essential to maintain a physiological vascular tone in response to pressure, but dispensable to preserve vascular structure.



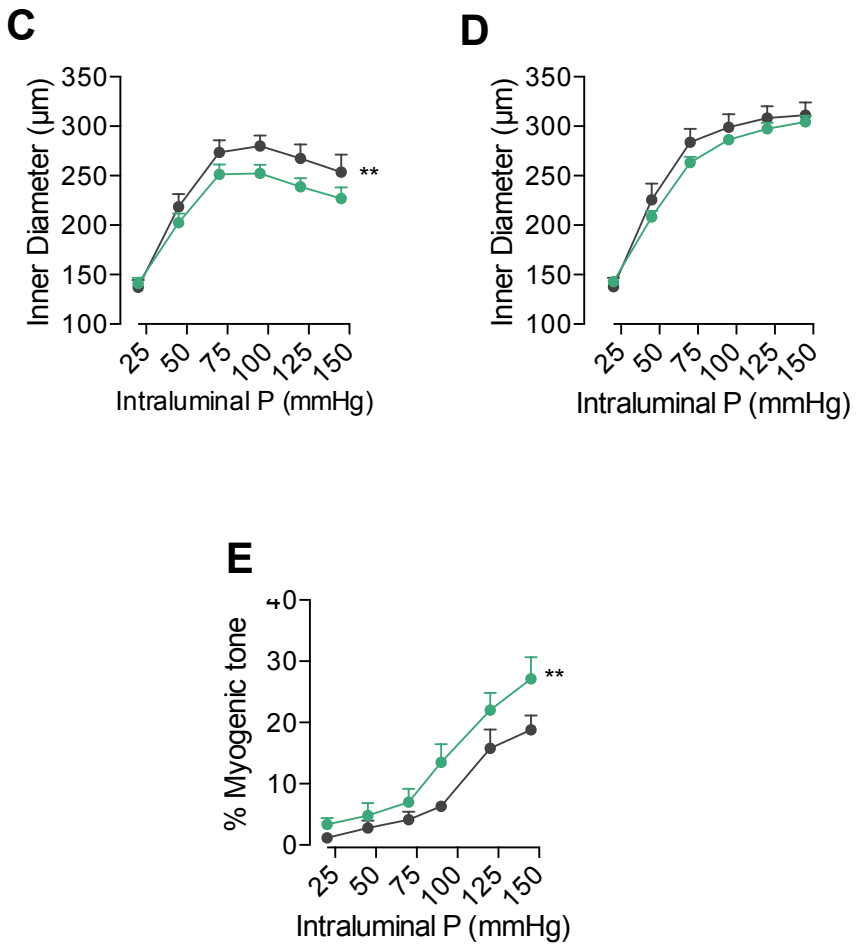


Figure 3.7 *Cumulative concentration-response curve of PE expressed as* ***(A)*** *% Contraction and* ***(B)*** *Inner Diameter (n=23 MA/group, n=13 mouse/group, **p<0.01).* ***(C)*** *Active and* ***(D)*** *Passive diameter and* ***(E)*** *Myogenic tone (%) in response to a stepwise increase of intraluminal pressure (n=8 MA/group, n=5 mice/group, , **p<0.1). Statistical significance was determined Two-way ANOVA followed by Bonferroni's post-test.*

3.8 Heterozygous mice for *Sptlc2* (*Sptlc2*$^{+/-}$) show endothelial dysfunction and increase in BP.

SBP was significantly higher in Sptlc2$^{+/-}$ mice compared to WT (Figure 3.8 A). Flow- and Ach-mediated vasodilation was reduced in MA from Sptlc2$^{+/-}$ mice vs. control (Figure 3.8 B-C). Moreover, systemic reduction of sphingolipids *de novo* biosynthesis increased the myogenic tone of MA, whereas vasoconstriction in response to PE was not altered (Figure 3.8 D-

E). These results recapitulate the phenotype observed in ECKO-Sptlc2 mice suggesting that most likely is the endothelial SPT activity to play a major role in blood pressure homeostasis.

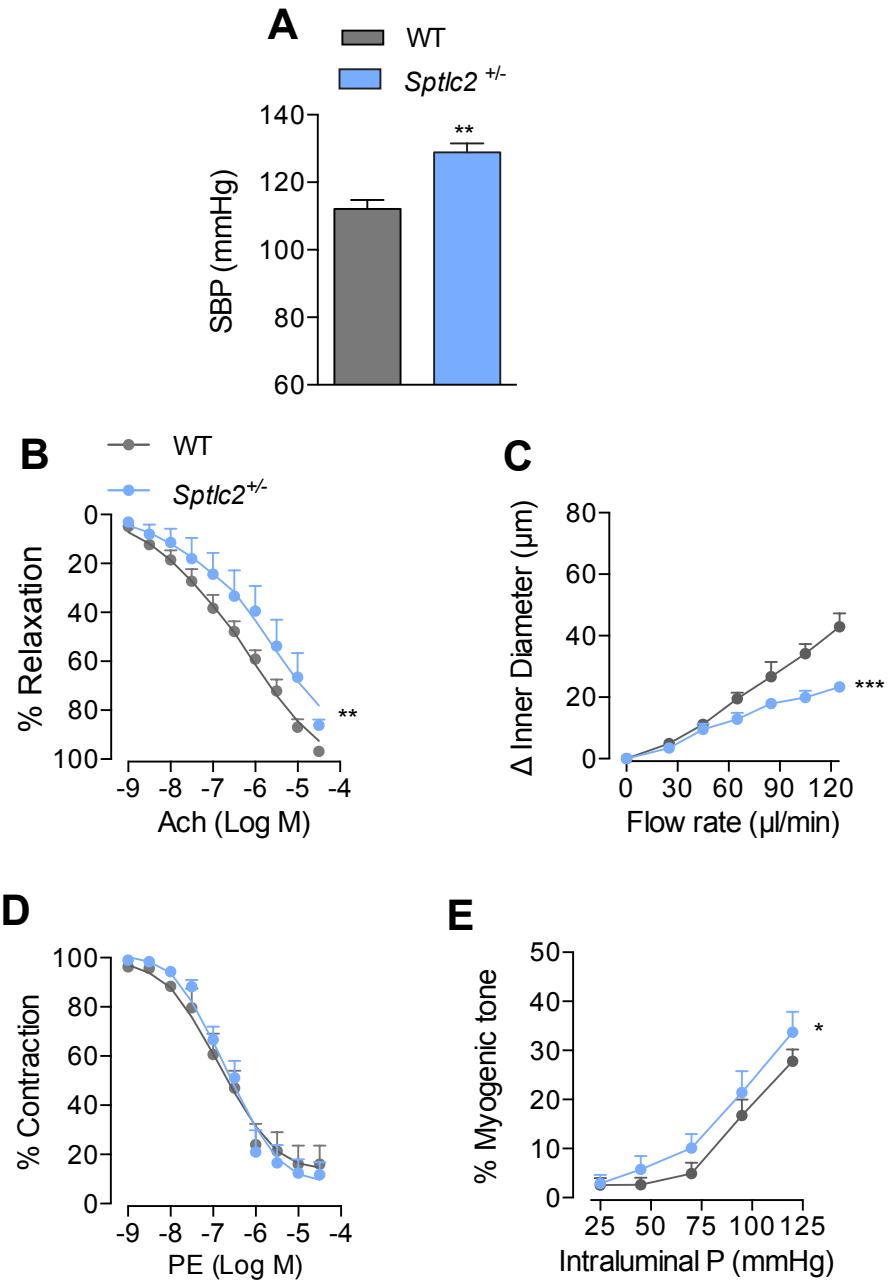


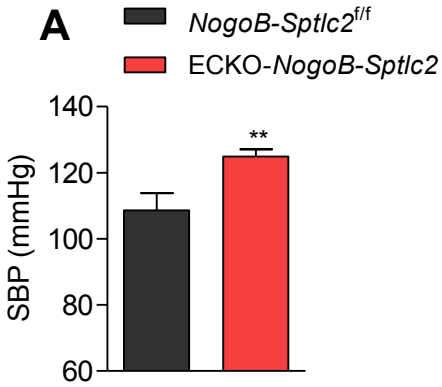
Figure 3.8 ***(A)*** *Systolic Blood pressure of Sptlc2$^{+/-}$ mice compare to the WT (Mean value: 112.133±2.59 vs 128.9±2.58, WT vs Sptlc2$^{+/-}$, n=3mouse/group, **p<0.01). Vasodilation of MA induced by* ***(B)*** *cumulative concentration response curve of Ach (n≥6 MA/group, n≥4 mouse/group, **p<0.01), and* ***(C)*** *flow expressed as inner diameter (n≥4 MA/group, n≥3 mouse/group, ***p<0.001). Vasoconstriction in response to* ***(D)*** *cumulative concentration curve*

*of PE (n≥6 MA/group, n≥4 mouse/group) and **(E)** increase of intraluminal pressure (n≥6 MA/group, n≥3 mouse/group, *p<0.1). Statistical significance was determined by **(A)** unpaired T-test and **(B-E)** Two-way ANOVA followed by Bonferroni's post-test.*

3.9 Nogo-B deletion does not restore endothelial function in ECKO-*Sptlc2* mice

Professor Di Lorenzo's Lab identified Nogo B as negative regulator of the *de novo* sphingolipids biosynthesis. They demonstrated that endothelial absence of Nogo B in the ER up-regulates SPT and thus local endothelial sphingolipids, leading to protection against hypertension [224] and heart failure. In order to demonstrate the main role of SPT in this vascular phenotype, we performed experiments on ECKO-*NogoB*-*Sptlc2* mice.

Our data show that ECKO-*NogoB*-*Sptlc2* mice have increased SBP compare to control (Figure 3.9 A). Moreover, assessment of vascular reactivity in carotid arteries shows that flow-induced vasodilatation (Figure 3.9 B), but not Ach (Figure 3.9 C), is significantly reduced following endothelial *Nogo* and *Sptlc2 deletion* suggesting that the vascular protective effect related to endothelial absence of Nogo is directly due to SPT activation.



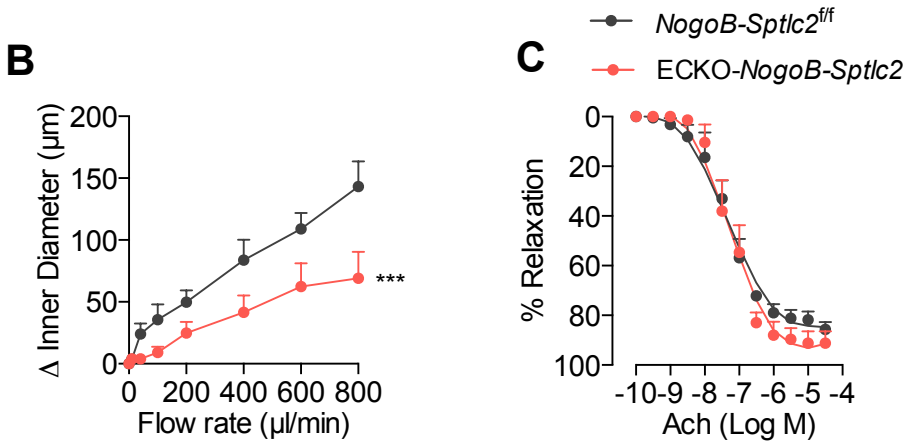


Figure 3.9 ***(A)*** *Systolic blood pressure of ECKO Nogo-Sptlc2, compared to their littermates control (Mean value 108.66±5.186 vs 124.930±2.17, control vs ECKO Nogo-Sptlc2, n=5 mouse/group, ** p<0.01). Vasodilatation induced by* ***(B)*** *flow (n=5 vessels/group, n=5 mouse/group, ***p>0.001) and* ***(C)*** *cumulative concentration-response curve of Ach (n=5 vessels/group, n=5 mouse/group) assessed on carotid vessels from ECKO NogoB-Sptlc2 mice. Data were expressed as the mean ± SEM. Statistical significance was determined by* ***(A)*** *unpaired T-tes and* ***(B-C)*** *Two-way ANOVA followed by Bonferroni's post-test.*

3.10 Absence of SPT activity in endothelial cells accelerates the physiological coagulation process

Endothelial layer plays an important role in the hemostasis. Since our collected data suggest that reduction of endothelium-derived sphingolipids affects ECs function, we assessed whether the absence of SPT affects the coagulation process. Bleeding assay performed on males and females ECKO-*Sptlc2* and *Sptlc2*^{ff} showed that endothelial loss of SPT activity progressively reduces the coagulation time after tail amputation (of ≈ 0.3mm) (Fig. 3.10 A-B), suggesting that reduction of endothelial-sphingolipids production causes an alteration of endothelial function towards a pro-thrombotic effect.

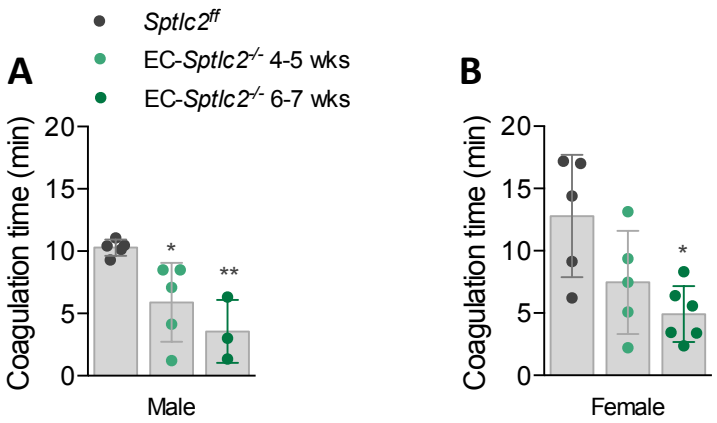


Figure 3.10 *Time of coagulation in* ***(A)*** *males (n≥3 mouse/group) and* ***(B)*** *females (n≥5 mouse/group) Sptlc2^{ff} and ECKO-Sptlc2 at different time point from tamoxifen treatment. Data are expressed as mean± SD.* ***(A-B)*** *Statistical significance was determined by One way Anova vs Sptc2^{ff}, *p<0.1*

4. DISCUSSION

The pathogenesis of hypertension, the leading cause for myocardial infarction, heart failure and stroke [236], is multifactorial and highly complex. Despite current therapies, BP management does not reach a satisfactory outcome in all the patients, suggesting the need for alternative therapeutic targets and a better understanding of underlying mechanisms. The results of this study reveal sphingolipid *de novo* biosynthesis as a necessary pathway to preserve endothelial cell-dependent regulation of vascular tone and BP homeostasis. Our data identify endothelial-derived sphingolipids as key regulatory molecules of endothelial cell function. Specifically, we identified an important role of locally produced SL in endothelial-mediated vasodilation following the activation of $G\alpha_i$-coupled and TK receptors, as well as by flow, leading to a significant increase of systolic BP in ECKO Sptlc2 mice.

SLs are important component of the eukaryotic plasma membrane, and contribute to preserve the integrity and fluidity of membranes. Every cell of our body can synthetize “de novo” sphingolipids. Furthermore, SL can be uptaken by the cells and via recycling pathway can be transformed in higher order SL such as SM and GSL. However, this pathway is not sufficient to compensate the *de novo* biosynthesis as endothelial cells lacking SPT failed to maintain a proper control of vascular tone and BP homeostasis.

The role of SL in BP homeostasis has been the subject of intense studies. S1P, ceramide, as well as sphingosine, represent the best characterized molecules of this class, and have been implicated in pathogenesis of

hypertension, however specific molecular mechanisms are poorly understood.

Recently, Di Lorenzo' group demonstrated that endothelial-derived S1P is a key regulator of blood flow and pressure, through S1PR1-eNOS-NO autocrine signaling [224]. Furthermore, they discovered that a novel regulatory endothelial sphingolipids *de novo* production, mediated by a membrane protein of the endoplasmic reticulum called Nogo, appears to control local endothelial S1P production and impacts vascular function, blood pressure (BP) [224] and also heart function [199]. Considering that the endothelium is bathed in SL-enriched plasma, and it can recycle SL uptaken from plasma, as well as deriving from intracellular catabolism processes, it was surprising the critical role of *de novo* SL production in preserving vascular homeostasis. These findings suggest also that down regulation of endothelial SL biosynthesis contribute or exacerbates the onset of a cardiovascular disease, such as hypertension. Thus, we hypothesized that ablation of endothelial SPT activity by genetic approach would trigger endothelial dysfunction and hypertension.

To test this hypothesis, we deleted *Sptlc2* subunit specifically in EC. In ECKO-*Sptlc2* mice, plasma Sph and S1P levels were significantly reduced, an effect that correlated with increased blood pressure at baseline. Vasodilation of MA in response to flow and Ach was impaired, with the latter manifesting only at later time point post-tamoxifen treatment. Furthermore, endothelial *Sptlc2* deletion led to an increase of vasoconstriction in response to intraluminal pressure. These entire patterns have been also confirmed in heterozygous *Sptlc2*$^{+/-}$ mice. These findings are complimentary to the up regulation of SPT activity in the endothelium, which preserves endothelial dysfunction and blood pressure

homeostasis. Furthermore, deletion of endothelial NogoB in ECKO-*Sptlc2* mice does not restore SBP to a physiological range, neither ameliorates the flow-mediated vasodilatation which is drastically reduced in ECKO-*NogoB*-*Sptlc2* carotid arteries, suggesting that the essential role of Nogo-B is inhibits SPT to impact vascular function and BP.

The loss of SPT also leads to a prothrombotic endothelium. Endothelium regulates the coagulation exerting both an antithrombotic and pro-coagulant activity, and the balance between these two processes is a critical in determining the formation of thrombus, its propagation and its dissolution. Mice ECKO Sptlc2 showed a significant increase in the coagulation time in the tail bleeding assay, suggesting an important role of SL *de novo* biosynthesis in preserving an anti-thrombotic function of the endothelium.

In the plasma membrane, sphingolipids dynamically interact with cholesterol to form *lipid raft* microdomains [135], playing an important role in endocytosis, cells trafficking [145, 237] and signaling [137]. Endothelial cells are characterized by membrane invaginations called caveolae, enriched in SL and cholesterol and hosting multiple receptors [136, 145, 238]. Firstly, electron microscopy analysis showed an increase in the number of caveolae and decrease in their size in EC of ECKO-*Sptlc2* carotid arteries vs. control. However, the changes were moderate, suggesting that the loss of SPT did not induced profound morphological changes in the endothelium, but most likely altered functions associated to the plasma membrane.

Caveolae defined *caveolar lipid rafts*, and are distinct from *non caveolar lipid rafts*, which are enriched in SLs [238]. It has been reported that $G\alpha_q$-

coupled and TK receptors are predominantly expressed in endothelial cells caveolae [158, 238-240], whereas others, such as $G\alpha_i$- and $G\alpha_s$-coupled receptor are more abundant in the *non-caveolar* compartment of lipid raft [238]. Since guanylyl cyclase-cGMP pathway was preserved in the VSMC of ECKO-Sptlc2, we assessed whether the reduction of endothelial-produced SL alters lipid rafts composition, hence receptor-mediated signaling. Thus, we tested the vascular endothelial response to the activation of different GPCRs as well as tyrosine receptors known to be located/distributed in the lipid rafts [136], as readout of the function of the receptors, assuming no differences in the expression levels. Indeed, our findings demonstrated that only the activation of $G\alpha_i$-associated receptors (S1PR1 and PAR1) and tyrosine kinase receptor (VEGFR2, IR) is impaired in ECKO-*Sptlc2*, whereas $G\alpha_q$-mediated effect is unaffected. A study of Igarashi and colleagues reported that following S1P stimulation, S1PR1 translocases in caveolae, where is inhibited by Cav-1 [241], a membrane protein localized in the caveolae and regulating the activation of different signaling pathways. Knocking down of Cav-1 in BAEC led to an up-regulation of S1P-S1PR1 signaling. We do not know whether the loss of SPT alters the expression of Cav-1 in the EC, however, Cav-1 also inhibits eNOS and we do not find any alteration in basal and stimulated NO, suggesting that this molecular mechanism is not accountable for the phenotype.

An alternative molecular mechanism could be that SPT ablation changes membrane lipid composition, altering the dynamic distribution of the receptors among different plasma membrane microdomains, necessary to control the switch from inactive to active states of the receptors, and vice-versa. For instance, the binding of VEGF to VEGFR2 induced the

dimerization and activation of the receptors by auto-phosphorylation of their cytosolic domains [242]. It is possible that the fluidity of lipid bilayer can affects the rates of lateral diffusion and mobility of the receptors in the membrane [243, 244]. Insulin receptor (IR), on the contrary, is expressed in the caveolae as dimer [245]; upon insulin binding, IR undergoes a conformational change that allows *trans*-phosphorylation of the catalytic sites on multiple tyrosine residues, which is essential for the receptor activation and development of intracellular signaling [246]. Changes in cholesterol and sphingolipid levels, particularly GSL, impair the localization of the IR in caveolar lipid rafts [190]. Moreover, mechanical stimuli, such as shear stress, pressure and circumferential stretch, modulate EC morphology and functions by activating mechano-sensors on plasma membrane and developing intracellular signaling [247, 248]. Thus, it is reasonable to speculate that reduction in flow-induced vasodilation in ECKO-*Sptlc2* MA could be associated to possible changes in plasma membrane composition rather than impairment in eNOS/NO pathway, since we did not find any differences in Ach-vascular response, in presence and absence of L-Nio.

In conclusion, our findings suggest that sphingolipids are important in the regulation of endothelial-dependent BP homeostasis. Alterations of their levels below or above a physiological range leads to alteration in vascular tone and BP regulation [249] (Figure 4.1). There are many studies that associated high levels of plasma sphingolipids to a hypertensive state [250]. However, our data demonstrate that down regulation of endothelial *de novo* sphingolipid biosynthesis, by deletion of SPT, increases BP and impairs endothelial cell functions. Endothelial-derived SL are of critical

importance to maintain blood pressure homeostasis and to preserve endothelial cell functions, including vascular tone and blood fluidity regulation. However, further investigations are needed in order to dissect the underlying molecular mechanisms of the phenotype observed in mice lacking endothelial SPT.

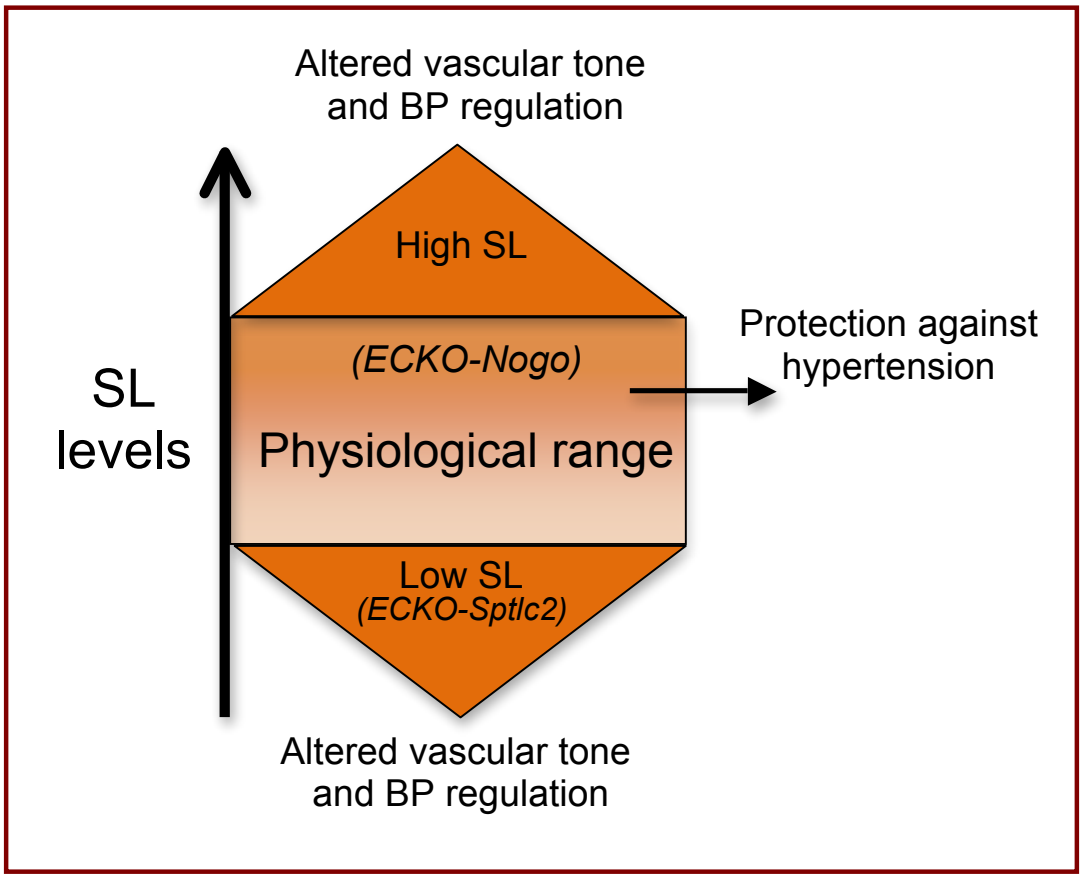


Figure 4.1 **Alteration of SL levels below or above the physiological range alters vascular tone and BP regulation**

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