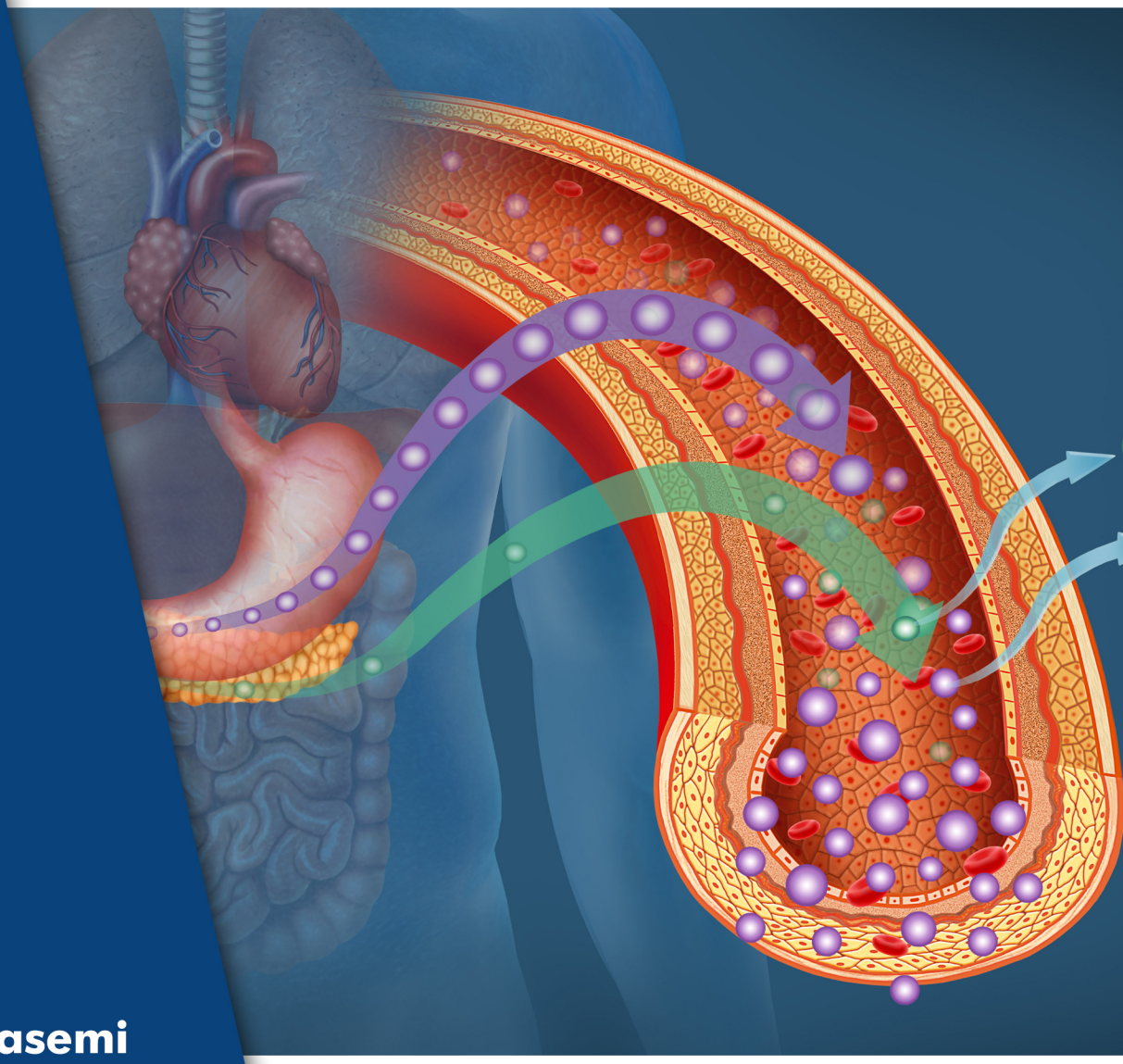
THE ROLE OF NITRIC OXIDE IN TYPE 2 DIABETES



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FOREWORD

The global obesity and overweight pandemic that causes the increasing number of patients with type 2 diabetes (T2D) is a major challenge for healthcare systems worldwide. With its cardiovascular complications, this metabolic disorder is one of the major causes of morbidity and mortality worldwide. On top of lifestyle and dietary recommendations to prevent or control T2D, a tremendous amount of research has been invested in understanding disease mechanisms better and developing novel drugs. Even if new pharmaceuticals have entered the clinical arena in recent years, metformin is still the first-line option, even after more than 60 years. This points to the necessity to develop therapeutic strategies based on biological pathways that have not previously been the center focus of diabetes research. Such an area is nitric oxide research.

Nitric oxide (NO) is one of the universal signaling molecules in mammalian species. When discovered in the 1980s, it portrayed a completely novel principle, where a small, unstable, and reactive free radical gas was involved in cell signaling. Its chemical nature makes it react with other radicals and transition metals; one example of the latter is how NO activates soluble guanylyl cyclase to generate cGMP, a classical form of NO signaling that, e.g., induces vasodilation. Binding to heme in cytochrome c oxidase, leading to inhibition of mitochondrial respiration, is another example. In addition, post-translational nitrosation of many proteins, which regulates their function, is another signaling modality of NO. This pluripotency of NO explains why it is involved in regulating such diverse processes as cardiovascular function, metabolism, inflammation, and nerve signaling. The canonical pathway for NO generation involves the substrates L-arginine and molecular oxygen and specific NO synthases (N.O.S.s), of which there are three isoforms. Two of them are more constitutively expressed (endothelial N.O.S. and neuronal N.O.S.), while an inducible isoform (inducible N.O.S.) is involved during inflammatory conditions. The half-life of NO is within seconds due to binding to heme or to rapid oxidation, which forms the inorganic anions nitrite and nitrate that are widely used both *in vitro* and *in vivo* as more stable surrogate measures of NO.

Interestingly, discoveries in the mid-1990s revealed that these supposedly inert anions could be recycled back to bioactive NO and other reactive nitrogen species. The first step in this nitrate-nitrate-NO pathway involves active uptake of circulating nitrate in the salivary glands, after which nitrate in the saliva is reduced to nitrite by oral commensal bacteria, a function that mammalian cells are poor in performing. Swallowed salivary nitrite is rapidly absorbed in the gut, and then there are several pathways for further reduction to NO. Of interest is that the nitrate-nitrite-NO pathway can be fueled by a diet where certain vegetables contain high levels of nitrate. This pathway can be viewed as a parallel backup system to the L-arginin--NOS-NO pathway, perhaps with more importance during hypoxic and ischemic conditions.

This book, to my knowledge, is the first of its kind, Asghar Ghasemi and collaborators present a comprehensive and detailed overview of our current knowledge on the role of NO in T2D. The rationale for this book is the growing evidence of the involvement of the NO system in diabetes. An impressive amount of research has clarified that NO is deeply involved on many levels to uphold metabolic homeostasis and that NO signaling is negatively affected in T2D. Of interest is that several of the pharmaceuticals used in T2D affect the NO system and perhaps even more so by the drugs we use to treat diabetic cardiovascular complications. Experimental works in animal models of obesity or T2D show promising results with interventions aimed to increase NO signaling. However, translation into human studies has so far been less successful, but larger and more prolonged studies are clearly needed. There is an

intriguing dietary aspect here since NO bioavailability can be boosted by nitrate in our diet, which is supported by epidemiological studies showing that green leafy vegetables, which are high in nitrate, stand out as particularly protective against the development of T2D and cardiovascular disease. Clearly, more research on the role of NO in metabolic regulation and T2D is needed, and in this context, the present book is of great value to anyone interested in this field of research.

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PREFACE

Nitric oxide (NO) is a colorless, odorless, primordial flammable gas that has been present in the earth's atmosphere from the beginning of time. Historically, NO was regarded as an industrial toxin or pollutant generated in many industries; however, it is now well recognized that NO is endogenously produced and has an important biological role in most mammalian tissues. The vital role of NO in human biology was recognized in 1992 when the journal *Science* introduced NO as the "Molecule of the Year" [1] and in 1998 when the Nobel Prize in Physiology and Medicine was awarded to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad for the major discoveries surrounding it and establishing its role as a messenger molecule.

According to the World Health Organization (WHO), the prevalence of obesity across the globe has approximately doubled since 1980. In the U.S., about one-third of the adult population is obese, and an additional one-third is overweight [2]. Obesity is the fastest-growing lethal *disease* in Western and developing countries. People do not die due to obesity itself but from its complications, which shorten the life span [3, 4]. In addition, obesity leads to many other diseases, including type-2 diabetes (T2D) and its complication. T2D, which used to be referred to as adult-onset or non-insulin-dependent diabetes, accounts for over 90–95% of all diabetes; T2D is a complex metabolic disorder essentially characterized by alterations in lipid metabolism, insulin resistance, and pancreatic β-cell dysfunction [5]. Unfortunately, there are no effective treatments available for T2D, although there have been many developments in the therapeutic arena [6]. Hence there is an urgent need to develop new preventative and/or therapeutic strategies to combat T2D.

Over the past three decades, NO has emerged as a central regulator of energy metabolism and body composition. NO bioavailability is decreased in animal models of diet-induced obesity and in obese and insulin-resistant patients, and increasing NO output has remarkable effects on obesity and insulin resistance [7]. This volume is a collection of reviews dealing with *The Role of Nitric Oxide in Type 2 Diabetes*". These reviews provide a unique overview of NO signaling, pointing out key areas for more detailed research. We hope that the breadth of the topics covered in this volume will provide new perspectives and help to stimulate research towards unanswered questions.

Chapter 1 is an overview of the pathophysiology of T2D by Drs. Ghasemi and Kashfi entitled, "Pathophysiology of Type 2 Diabetes: A General Overview of Glucose and Insulin Homeostasis". A better understanding of the pathophysiology of T2D provides an opportunity for revising the current therapeutic modalities, from a primary glycemic control to a pathophysiological-based approach. This chapter provides essential information on glucose homeostasis and the pathophysiology of T2D. Chapter 2 by Drs. Ghasemi and Kashfi is entitled "Nitric oxide: A Brief History of Discovery and Timeline of its Research." This chapter highlights the discovery of NO in mammals and its role as a signaling molecule. The overview describes the chronological development of NO, emphasizing the events in the last two decades of the 20th century. Chapter 3 is a review by Drs. Bahadoran, Carlström, Mirmiran, and Ghasemi entitled, "Impaired Nitric Oxide Metabolism in Type 2 Diabetes: At a Glance". Abnormal NO metabolism is associated with the development of insulin resistance and T2D, which in turn can lead to impaired NO homeostasis. The concept of NO deficiency is supported by results from human studies on polymorphisms of endothelial NO synthase (eNOS) gene, animal knockout models for NO synthase isoforms (N.O.S.s), and pharmacological inhibitors of N.O.S. This chapter focuses on the role of impaired NO metabolism in T2D.

Chapter 4 by Drs. Bahadoran, Carlström, Mirmiran, and Ghasemi is entitled "Asymmetrical Dimethyl Arginine, Nitric Oxide, and Type 2 Diabetes". Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthases. Over-production leads to decreased NO bioavailability and diabetes complications, including cardiovascular diseases, nephropathy, and retinopathy, with increased mortality risk. This chapter discusses how disrupted ADMA metabolism contributes to the development of T2D and its complications. Chapter 5 is a contribution by Drs. Bahadoran, González-Muniesa, Mirmiran, and Ghasemi is entitled, "Nitric Oxide-Related Oral Microbiota Dysbiosis in Type 2 Diabetes". This chapter gives an overview of oral microbiota dysbiosis in T2D, focusing on nitrate-reducing bacteria and their metabolic activity.

Chapter 6, entitled "Nitric oxide and Type 2 Diabetes: Lessons from Genetic Studies", is a contribution by Drs. Bahadoran, Mirmiran, Carlström, and Ghasemi. They discuss current genetic data linking NO metabolism to metabolic disorders, especially insulin resistance and T2D. Chapter 7 is a contribution by Dr. Afzali, Miss Ranjbar, and Drs. Kashfi and Ghasemi entitled, "Role of Nitric Oxide in Diabetic Wound Healing." NO deficiency is an important mechanism responsible for poor healing in diabetic wounds. The beneficial effects of NO in wound healing are related to its antibacterial properties, regulation of inflammatory response, stimulation of proliferation, differentiation of keratinocytes and fibroblasts, and promotion of angiogenesis and collagen deposition. In this chapter, the function of NO in diabetic wound healing and the possible therapeutic significance of NO in the treatment of diabetic wounds are discussed.

Chapter 8 is entitled "Role of Nitric Oxide in Type 2 Diabetes-Induced Osteoporosis" by Drs. Yousefzadeh, Jeddi, Kashfi, and Ghasemi. Diabetoporosis, which is osteoporosis in type 2 diabetic patients, contributes to and aggravates osteoporotic fractures. Decreased eNOS-derived NO and higher iNOS-derived NO are some of the critical mechanisms in diabetoporosis. This chapter closely examines the role of NO in diabetoporosis. Chapter 9 by Drs. Bahadoran, Mirmiran, Kashfi, and Ghasemi is entitled, "Hyperuricemia, Type 2 Diabetes and Insulin Resistance: Role of Nitric Oxide". Hyperuricemia is a risk factor for developing hypertension, cardiovascular diseases, chronic kidney disease, and T2D. It leads to the development of systemic insulin resistance, impaired NO and glucose metabolism, with induction of inflammation and oxidative stress. This chapter highlights the mediatory role of NO metabolism on hyperuricemia-induced dysglycemia and insulin resistance. Chapter 10 is entitled "Therapeutic management of type 2 diabetes: The nitric oxide axis," by Ms. Ranjbar, O'Connor, and Dr. Kashfi. Current drugs approved for the management of T2D include biguanides, thiazolidinediones, sulfonylureas, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, alpha-glucosidase inhibitors, and sodium-glucose co-transporter 2 (SGLT2) inhibitors. In this chapter, the authors discuss these drugs, examine their mechanism of action, and present evidence that these drugs directly or indirectly modulate NO metabolism.

In Chapter 11, "Brain Insulin Resistance, Nitric Oxide and Alzheimer's Disease Pathology," Drs. Pei, Lee, Khan, and Wang discuss the role of NO availability in brain insulin resistance in dementia associated with Alzheimer's disease. Chapter 12 by Drs. Mirmiran, Bahadoran, Kashfi, and Ghasemi, and is entitled "Arginine, Nitric Oxide and Type 2 Diabetes". In this chapter, the authors provide an overview of the potential efficacy of L-arginine (Arg) as an NO precursor and its effects on glucose and insulin homeostasis and diabetes-induced cardiovascular complications. Chapter 13 is also by Drs. Mirmiran, Bahadoran, Kashfi, and Ghasemi and is entitled "Citrulline, Nitric Oxide and Type 2 Diabetes". L-Citrulline (Cit) is a precursor of Arg and is involved in NO synthesis. Oral ingestion of Cit effectively elevates

total Arg flux and promotes NO production. In this chapter, the authors discuss the potential use of Cit as an effective anti-diabetic agent.

Recent data suggest the utility of the nitrate-nitrite-nitric oxide (NO_3-NO_2-NO) pathway in treating T2D. Supplementation with inorganic NO_3-NO_2 in animal models of T2D resulted in improved hyperglycemia, insulin sensitivity, and glucose tolerance [8 - 10]. However, the efficacy of NO_3-NO_2 supplementation on glucose and insulin homeostasis in humans is unproven. In chapter 14, entitled "Nitrate, Nitrite, and Type 2 Diabetes', Drs. Bahadoran, Mirmiran, Kashfi, and Ghasemi review the animal experiments and human clinical trials, addressing the potential effects of inorganic NO_3/NO_2 on glucose and insulin homeostasis in T2D. They also provide several plausible scenarios to address the challenge of lost-in-translation of beneficial effects of inorganic NO_3 and NO_2 from bench to bedside.

The final chapter of this book, chapter 15, is a review by Drs. Bahadoran, Mirmiran, Bahmani, and Ghasemi entitled, "Potential Applications of Nitric Oxide Donors in Type 2 Diabetes". NO-donors have increasingly been studied as promising therapeutic agents for insulin resistance and T2D. This chapter reviews the effects of sodium nitroprusside, S-nitrosothiols, and N-diazeniumdiolates on glucose and insulin homeostasis.

We hope that the breadth of topics covered in this volume will provide the readers with new perspectives, give some food for thought, and stimulate more research into major unanswered questions.

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CHAPTER 1

Pathophysiology of Type 2 Diabetes: A General Overview of Glucose and Insulin Homeostasis

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Abstract: The prevalence of diabetes is increasing worldwide, and this disease has a tremendous financial burden on most countries. Major types of diabetes are type 1 diabetes and type 2 diabetes (T2D); T2D accounts for 90-95% of all diabetic cases. For better management of diabetes, we need to have a better understanding of its pathophysiology. This chapter provides an overview of glucose homeostasis and the underlying pathophysiology of T2D.

Keywords: β-Cell Dysfunction, Glucose Homeostasis, Insulin, Impaired Glucose Tolerance, Insulin Resistance, Insulin Signaling Pathways, Impaired Fasting Glycemia, Type 2 Diabetes.

INTRODUCTION

Diabetes is the largest epidemic in human history [1], and there is currently a rapid-growing diabetes pandemic [2]. From 1980 to 2014, the total number of subjects with diabetes has quadrupled [3, 4]. More than 70% of global mortality is attributed to non-communicable diseases, including diabetes [5, 6]. Diabetes is the ninth leading cause of death [7], and in 2017, it caused one death every eight seconds (2.1 and 1.8 million in women and men aged 20–79 years, respectively) [2]. On average, healthcare expenditures for diabetic subjects are two-fold higher than those without diabetes [2]; in addition, approximately 79.4% of people with diabetes live in low- and middle-income countries [8]. Hyperglycemia is the third leading modifiable cause of death after high blood pressure and tobacco use [3]. A better understanding of the pathophysiology of type 2 diabetes (T2D) provides

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an opportunity for revising the current therapeutic modalities in the management of T2D, from a primary glycemic control to a pathophysiological-based approach. This chapter provides essential information on glucose homeostasis and the pathophysiology of T2D.

EPIDEMIOLOGY OF DIABETES

Amongst adults aged 20–79 years, the worldwide prevalence of diabetes in 2019, was 9.3% (9.0% in women and 9.6% in men), and unfortunately, this is expected to rise to 10.2% (578.4 million) and 10.9% (700.2 million) in 2030 and 2045, respectively [8]. There is considerable geographical/cultural heterogeneity relating to the incidence of diabetes. For example, the crude incidence of diabetes ranges from 2.9 per 1000 population in France to 23.5 per 1000 population in the Pima Indians of the United States [9]. Also, the incidence of diabetes increases with age because of decreased ability of the β-cells to compensate for insulin resistance [10]. Major types of diabetes are type 1 and type 2. Type 1 diabetes accounts for 5-10% of all diabetes [2], and patients require insulin therapy. Type 2 diabetes, which used to be referred to as adult-onset or non-insulin-dependent diabetes, accounts for over 90–95% of all diabetes [11]; T2D is a complex metabolic disorder essentially characterized by alterations in lipid metabolism, insulin resistance, and pancreatic β-cell dysfunction [12, 13].

Worldwide, the prevalence of prediabetes is also increasing [14]. Prediabetes is defined as a state of higher than normal glycemia that does not meet the established criteria for diabetes diagnosis and includes subjects with impaired fasting glycemia (IFG), impaired glucose tolerance (IGT), or both [11]. Prediabetes can predict the risk of developing diabetes [11, 15], and in some subjects, it can be alleviated by lifestyle modifications or pharmacological interventions, such as metformin administration [16]. Table **1** summarizes some statistical data about diabetes according to the International Diabetes Federation (IDF) report.

Table 1. Diabetes: Global statistics overview*.

	Year		
	2019	**2030**	**2045†**
Prevalence of diabetes % (million)	9.3% (463.0)	10.2% (578.4)	10.9% (700.2)
Prevalence of impaired glucose tolerance % (million)	7.5% (373.9)	8.0% (453.8)	8.6% (548.4)
Attributable all-cause mortality to diabetes (million)	4.2	-	-

(Table 1) cont.....

	Year		
Healthcare expenditure for diabetes (USD billion)	760.3	824.7	845.0

* According to the International Diabetes Federation (IDF) [8]
† Estimated values; total world population was 7.7 billion in 2019 and is estimated to be 8.6 and 9.5 billion in 2030 and 2045, of whom 5.0, 5.7, and 6.4 billion are aged 20-79 years.

DIAGNOSIS OF DIABETES

Diabetes is diagnosed using glucose-based criteria, *i.e*., fasting plasma glucose (FPG) levels or 2-h plasma glucose (2-hPG) levels during a 75-g oral glucose tolerance test; hemoglobin A1c (HbA1C) levels are also used as an indicator [11, 17]. Table **2** provides diagnostic criteria for T2D according to the World Health Organization (WHO) and the American Diabetes Association (ADA).

Table 2. Diagnostic criteria of diabetes*.

		ADA	WHO
Diabetes		FPG ≥ 126 mg/dL or 2-hPG ≥ 200 mg/dL or HbA1C ≥ 6.5%	FPG ≥ 126 mg/dL or 2-hPG ≥ 200 mg/dL or HbA1C ≥ 6.5%
Prediabetes	**IFG**	FPG: 100–125 mg/dL	FPG: 110–125 mg/dL
	IGT	2-hPG: 140–199 mg/dL	2-hPG: 140–199 mg/dL
	HbA1C	5.6–6.5%	–

* According to World Health Organization (WHO) and American Diabetes Association (ADA) criteria [11, 17].
2-hPG, 2-h plasma glucose; FPG, fasting plasma glucose; HbA1C, glycated hemoglobin; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.
To convert glucose concentration from mg/dL multiplied by 0.05551.

GLUCOSE HOMEOSTASIS

Maintaining blood glucose concentrations within a physiologic range, either in a fasted state or excess nutrient availability, is essential for keeping normal bodily functions [18]. This critical homeostasis is achieved through a complex network involving hormones and neuropeptides released mainly from the brain, pancreas, liver, intestine, adipose tissue, and skeletal muscle [19].

Nutrient sensing and hormonal signaling regulate glucose homeostasis, controlling tissue-specific glucose utilization and production [18]. With the use of homeostatic mechanisms, the body protects itself against either hyperglycemia (and its complications, *i.e*., retinopathy, neuropathy, nephropathy, premature atherosclerosis, diabetic ketoacidosis, and hyperosmolar hyperglycemic state) or

CHAPTER 2

Nitric oxide: A Brief History of Discovery and Timeline of its Research

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Abstract: Nitric oxide (NO) plays a critical role in many physiological and pathological functions in the human body. Following the discovery in 1986-1987 that endothelium-derived relaxing factor (EDRF) is NO, the number of NO-based publications within all fields of medicine has increased exponentially. This report provides a brief historical view of NO-based research, emphasizing the events in the last two decades of the 20th century.

Keywords: Cyclic Guanosine Monophosphate, Endothelial Nitric Oxide Synthase, Endothelium-Derived Relaxing Factor, History, Inducible Nitric Oxide Synthase, Nitric Oxide, Neural Nitric Oxide Synthase, Nitric Oxide Deficiency, Nitric Oxide Donating Agents, Wall Saltpeter.

INTRODUCTION

Humans have a long history of using nitrogen-containing substances. About 5000 years ago, "wall saltpeter" [$Ca(NO_3)_2$] or "niter" (KNO_3) were used to preserve foods [1, 2]; in addition, sublingual KNO_3 was used for treating angina about 1000 years ago [3]. Research on nitric oxide (NO) functions began in the late 1970s to the early 1980s. It bloomed in 1998 when the Nobel Prize in Physiology or Medicine was awarded to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad "for their discoveries concerning NO as a signaling molecule in the cardiovascular system" [4].

Nitric oxide is a free radical gas [5]. It has been called an ephemeral substance [6], a pharmacologic smart bomb [5], a powerful queen of communication and

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defense [7], and the most ubiquitous intercellular signaling molecule [8]. Nowadays, we accept that NO is involved in the homeostasis of almost every physiological system in the human body [9]. In addition, active research is ongoing in which NO is a target molecule for disease treatment. Thus, historicizing science acknowledges the pioneers and helps us better conceptualize the issues. This chapter provides a brief historical view of NO-based research.

HISTORY OF NO FIELD

Table **1** presents chronological progress in NO research, and (Fig. **1**) highlights the main events. According to a paper by Ignarro, the NO field study was launched by the Murad group in the 1970s [10]. However, as Furchgott had discussed, the story of NO began in 1953 when it was found that acetylcholine (ACh), which was known to be a vasodilator in the whole animal, caused contraction of aortic strips of the rabbit aorta [11]. However, in an experiment conducted in 1978, it was accidentally realized that ACh partially relaxes norepinephrine-induced precontracted rabbit aorta [11].

In 1980, Furchgott and Zawadzki reported that ACh, *via* its muscarinic receptors, stimulates endothelial cells to release a substance or substances, which cause(s) relaxation of vascular smooth muscle cells [12]. The substance was initially named the Furchgott Factor to distinguish it from other endothelium-dependent relaxing factors [13]. ACh-induced endothelium-dependent relaxation of vascular smooth muscle was also observed in other vessels [12] and considered a principal mechanism of the vasodilatory effect of muscarinic agonists [14]. Cyclooxygenase inhibitors did not affect ACh-induced endothelium-dependent relaxation of the vascular smooth muscles, leading to this hypothesis that activating muscarinic receptors by ACh releases a non-prostanoid substance from the endothelial cells that diffuses to and relaxes vascular smooth muscle cells [11]. Besides, the sandwich procedure, in which endothelium-denuded transverse strips of the aorta and endothelium-intact longitudinal strips mounted together in clips with intimal surface apposed, provides direct evidence for the hypothesis as at this condition, ACh-induced relaxation of the muscle was restored [11, 12].

In 1981, it was found that hydroquinone, a free radical quencher, inhibits ACh-induced relaxation suggesting that the relaxing factor may be a free radical [14]. In the same year (1981), it was found that there are other endothelium-dependent relaxing agents, including a Ca^{2+} ionophore (A23187) [14], which suggested a central role for calcium in the release or synthesis of the relaxing factor [11]. For the details of the first reports of substances that elicited endothelium-dependent relaxation, interested readers are referred to Furchgott and Vanhoutte [15]. Regarding the positive association between increased cyclic guanosine

monophosphate (cGMP) and smooth muscle relaxation, in 1981, Furchgott speculated that the relaxing factor relaxes vascular smooth muscle *via* increasing cGMP; this hypothesis was true [11].

In 1982, the non-prostanoid substance involved in vascular smooth muscle relaxation was referred to as endothelium-derived relaxing factor (EDRF) by the Furchgott group [11, 16, 17]. In 1987, Ignarro and colleagues reported that based on direct chemical and indirect pharmacological evidence, EDRF closely resembles NO [18]. According to Furchgott, based on several works, the concept that EDRF is NO was independently proposed by him and Ignarro in 1986 in a symposium; papers of this symposium were published in 1988 [11]. One year after the 1986 symposium, in 1987, three groups, including Ignarro *et al*. [19], Moncada *et al*. [20], and Furchgott *et al*. [11], reported that EDRF is NO. According to a recent review by Lancaster, investigations in parallel lines of cancer/immune, cardiovascular, and the nervous system converged in 1986-1988, which led to NO's discovery [21].

In 1988, *L*-arginine was identified as the endogenous substrate for NO synthesis by the Moncada group [22] and others [23]. This discovery was based on previous reports highlighting that activated macrophages generate nitrite and nitrate from *L*-arginine [6]. In 1990, Bredt and Snyder isolated and purified NO synthase (they named it NO synthetase) from rat cerebellum as a calmodulin-requiring enzyme [24]; following neuronal NOS (nNOS) isolation, inducible NOS (iNOS), and endothelial NOS (eNOS) were also isolated in 1991 [25, 26]. NOS isoforms originally were named after the tissues in which they were first identified [6, 27]. However, because both eNOS and nNOS have inducible forms and iNOS is also constitutively expressed in some tissues, nNOS, iNOS and eNOS are currently referred to as NOS-I, NOS-II, and NOS-III, respectively, according to the order in which they were first cloned [28]. NO production *via* the *L*-arginine-NO pathway occurs in almost every cell in the human body [29].

In 1992, Science magazine selected NO as the molecule of the year [30], and it was discovered that S-nitrosylation is a cellular regulatory mechanism of NO action [31]. In 1994, NOS-independent NO generation was reported in the stomach [32, 33]; this suggested an alternative or backup system besides the classical *L*-arginine pathway for sufficient NO generation, particularly during hypoxia [34]. In 1998, Pfizer company introduced sildenafil (Viagra®) for managing erectile dysfunction in men [10, 35]; sildenafil inhibits phosphodiesterase 5A (PDE5A) and increases intracellular cGMP [28]. In 1999, inhaled NO was approved by the U.S. Food and Drug Administration (FDA) for treating infants with persistent pulmonary hypertension [36, 37]. In 2001, the first report of enzymatic denitrosation and nitrosothiols' regulation by NO was

CHAPTER 3

Impaired Nitric Oxide Metabolism in Type 2 Diabetes: At a Glance

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Abstract: Abnormal nitric oxide (NO) metabolism has been associated with the development of insulin resistance and type 2 diabetes (T2D). The concept of NO deficiency is supported by human studies on polymorphisms of endothelial NO synthase (eNOS) gene, animal knockout models for NO synthase isoforms (NOSs), and pharmacological evidence, showing detrimental effects of NOS inhibitors and salutary effects of NO donors on carbohydrate metabolism. On the other hand, T2D and insulin resistance may impair NO homeostasis due to hyperglycemia, oxidative stress, and inflammation. Reduced production of NO [*i.e.*, impaired *L*-arginine-NOS pathway and function of the nitrate (NO_3)-nitrite (NO_2)-NO pathway], impaired NO transport within the circulation and delivery to target cells, as well as disrupted NO signaling (*e.g.*, *via* oxidative-induced NO quenching, and impaired NO-cGMP signaling pathway) can all lead to a reduced NO bioactivity in T2D. This chapter focuses on the role of impaired NO metabolism in T2D.

Keywords: Dysglycemia, Endothelial Nitric Oxide Synthase, Glucose Metabolism, Insulin Resistance, Inducible Nitric Oxide Synthase, Neural Nitric Oxide Synthase, Nitric Oxide, Nitric Oxide Deficiency, Type 2 Diabetes.

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INTRODUCTION

Nitric oxide (NO), commonly known as a unique biological free radical and signaling molecule with multiple functions [1, 2], is now considered an endocrine hormone [3] critically involved in whole-body glucose and insulin metabolism [4]. Fig. (**1**) illustrates two main pathways of NO production [the *L*-arginine-NO synthase (NOS) pathway and the nitrate (NO_3)-nitrite (NO_2)-NO pathway] and its metabolism.

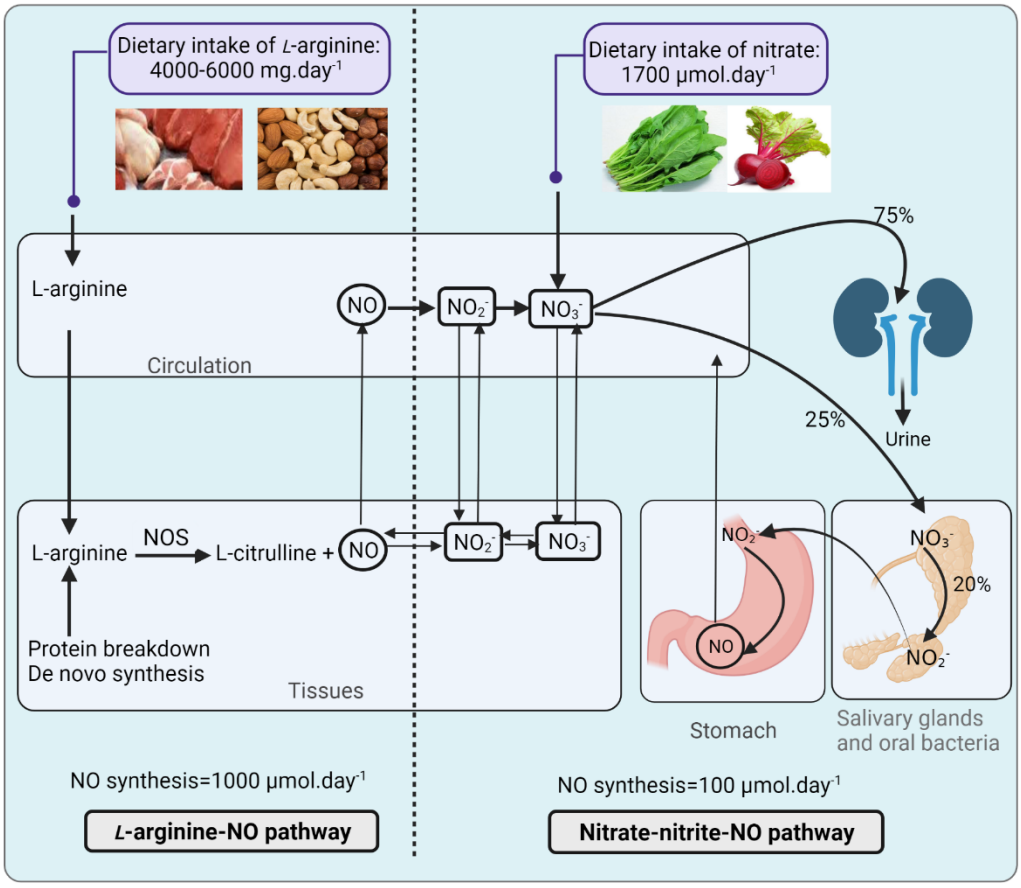

Fig. (1). Pathways of nitric oxide (NO) production and metabolism. Two main pathways of NO production are the *L*-arginine-NO synthase (NOS) pathway and the nitrate (NO_3)-nitrite (NO_2)-NO pathway. *L*-arginine is provided by either exogenous (dietary intake, about 4000-6000 mg/day) or endogenous (de novo synthesis from citrulline and turnover of proteins) sources. This pathway is responsible for producing about 1000 µmol/day NO. Inorganic NO_3 and NO_2, provided either from dietary sources or endogenous NO metabolism, are used as substrates for non-enzymatic endogenous NO generation. This pathway is estimated to produce about 100 µmol/day NO. The source of the Fig. is **(5)**, Quantitative aspects of nitric oxide production from nitrate and nitrite, EXCLI Journal, 2022, 21: 470-486.

Impaired NO bioavailability is a common state in metabolic disorders, including type 2 diabetes (T2D), that is mainly attributed to decreased endothelial NO synthase (eNOS) expression or activity or by NO scavenging in conditions with excessive production of reactive oxygen species (ROS) [6, 7]. A bilateral cause-and-consequence relation exists between impaired NO metabolism and the development and progression of T2D (Fig. **2**).

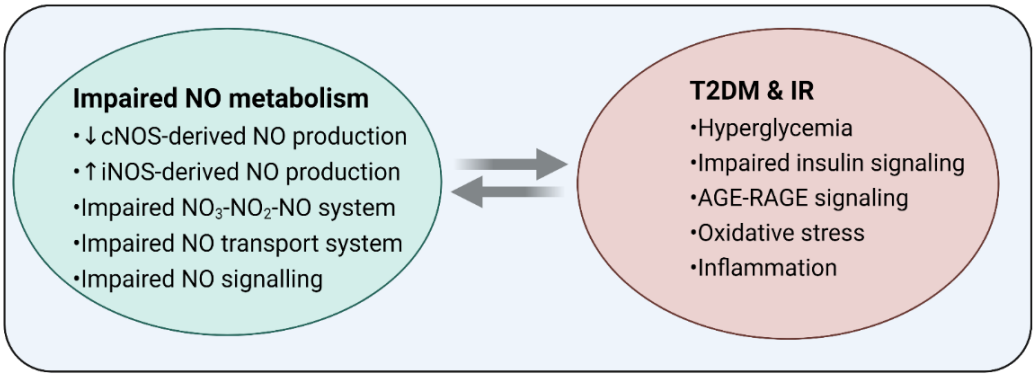

Fig. (2). A proposed cause-and-effect and bilateral relationship between impaired nitric oxide (NO) metabolism and development of insulin resistance and type 2 diabetes (T2D). AGE, advanced glycation end product; cNOS, constitutive nitric oxide synthase; NO_3, nitrate; NO_2, nitrite; RAGE, receptor for AGE. Created with Biorender.com.

The critical role of NO in regulating glucose and insulin homeostasis is supported by experimental studies using genetically modified mice, either eNOS knockouts or eNOS over-expressed mice. Impaired glucose and insulin metabolism, as evident from hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance, occurs in homozygous [8 - 11] and heterozygous eNOS knockout animals [12], whereas overexpression of eNOS prevents diet-induced hyperinsulinemia [13, 14]. In contrast, overexpression of inducible NOS (iNOS) and hence the production of very high levels of NO is associated with the development of skeletal muscle insulin resistance [15], hepatic insulin resistance, hyperglycemia, and hyperinsulinemia [16]. iNOS-deficient mice compared to wild type, did not show metabolic dysfunctions induced by the high-fat diet (HFD) and displayed normal glucose tolerance, insulin sensitivity, and insulin-stimulated glucose uptake in the skeletal muscle [17]. HFD increased iNOS mRNA expression by 2 to 4-fold in skeletal muscle [type I (soleus), type II-a (extensor digitorum longus, EDL), and type II-b (tibialis) fibers] and adipose tissue of wild-type mice. In contrast, obese *Nos2*$^{-/-}$ mice had normal insulin levels, glucose tolerance, and normal skeletal muscle glucose uptake than lean *Nos2*$^{-/-}$ or wild-type mice under a standard diet [17]. This evidence indicates that targeted

CHAPTER 4

Asymmetrical Dimethyl Arginine, Nitric Oxide, and Type 2 Diabetes

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Abstract: Asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of nitric oxide (NO) synthase (NOS) isoenzymes, can substantially inhibit vascular NO production at concentrations that are observed in pathophysiological conditions. Over-production of ADMA (*via* overexpression and/or activity of class 1 of the protein arginine methyltransferases, PRMT-1) alongside decreased catabolism (due to decreased expression and/or activity of dimethylarginine dimethyloaminohydrolase, DDAH) in type 2 diabetes (T2D) and insulin resistance results in increased circulatory and intracellular ADMA levels. Such pathological elevated ADMA levels lead to a decreased NO bioavailability and the development of diabetes complications, including cardiovascular diseases, nephropathy, and retinopathy; elevated ADMA levels also increase the mortality risk in these patients. Here, we discuss current documents indicating how disrupted ADMA metabolism contributes to the development of T2D and its complications. The role of other endogenous methylarginines, *i.e*., NG-monomethyl-*L*-arginine (*L*-NMMA) and NG, NG′-dimethyl-*L*-arginine (SDMA) on NO production and T2D are also discussed.

Keywords: Asymmetric Dimethylarginine, Cationic Amino Acid Transporter, *L*-Citrulline, Dimethylarginine, Dimethylaminohydrolase, Endothelial Nitric Oxide Synthase, *L*-Arginine, Nitric Oxide, N^G-Monomethyl-*L*-Arginine, N^G, $N^{G'}$-Dimethyl-*L*-Arginine, Protein Arginine Methyltransferases, Type 2 Diabetes.

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INTRODUCTION

Asymmetric dimethylarginine (N^G, N^G-dimethyl-*L*-arginine, ADMA), a naturally occurring amino acid, is synthesized upon hydrolysis of methylated cellular proteins and circulates within the plasma [1, 2]. ADMA and other methylarginines were isolated from human urine in 1970 by Kakimoto *et al*. [3]. ADMA is an endogenous inhibitor of nitric oxide (NO) synthase (NOS) isoenzymes [1, 2]. The first report introducing ADMA as an endogenous inhibitor of NOS goes back to 1992, when Vallance *et al*. described that accumulation of intracellular ADMA decreases NO availability and contributes to the development of hypertension and renal dysfunction [4]. Beyond inhibition of NOS activity, ADMA may reduce NOS expression and cause uncoupling of NOS in endothelial cells leading to increased superoxide generation and subsequent oxidative stress [5, 6].

In healthy humans, the mean plasma level of ADMA is ~0.5-0.7 μM [7, 8], with a range of 0.22 to 0.79 μM [9]. Several human studies reported increased plasma ADMA levels in pathologic conditions, including hypertension, kidney diseases, hypercholesterolemia, atherosclerosis, type 2 diabetes (T2D), and chronic heart failure [10 - 13]. ADMA has also received more clinical attention since its elevated level is an independent risk factor for cardiometabolic diseases [14, 15]. Elevated ADMA level is an independent predictor of cardiovascular disease [relative risk (RR)=1.42, 95% CI=1.29-1.56], coronary heart disease (RR=1.39, 95% CI=1.19-1.62), stroke (RR=1.60, 95% CI=1.33-1.91) [15], and all-cause mortality (RR=1.31, 95% CI=1.13-1.53) [16].

Here, we summarize ADMA metabolism and the importance of the ADMA-dimethylarginine dimethylaminohydrolase (DDAH) pathway on whole-body NO synthesis and bioavailability. Then we discuss current reports regarding how the disrupted ADMA-DDAH pathway can contribute to the development of T2D and its complications. We also focused on the potential role of other endogenous methylarginines, *i.e*., N^G-monomethyl-*L*-arginine (*L*-NMMA) and N^G, $N^{G'}$-dimethyl-*L*-arginine or symmetric dimethylarginine (SDMA) on NO production and development of T2D and its complications.

ADMA BIOSYNTHESIS AND METABOLISM

ADMA is released upon hydrolysis of post-translationally methylated intracellular proteins by protein arginine methyltransferases (PRMTs). Methylation of *L*-arginine (Arg) residues in cellular proteins is a post-translational modification, transferring 1 or 2 methyl groups to the guanidine nitrogens of Arg [1, 2, 17]. In mammals, two PRMT isoenzymes are identified, type I (PRMT 1, 3, 4, 6, and 8) and type II (PRMT 5, 7, and F-box only protein 11, FBXO11) [18]. The PRMT type I is responsible for about 85% of total protein Arg methylation activity [19].

Each day about 300 μmol ADMA is constitutively synthesized during the turnover of methylated Arg residues in proteins [1, 20]. About 80-90% of ADMA is metabolized by the dimethylarginine dimethylaminohydrolase (DDAH) into *L*-citrulline (Cit) and dimethylamine, and a small part (10-20%) is metabolized by aminotransferase and excreted by the kidneys [1, 2, 21].

About 250 μmol of ADMA is daily degraded by DDAH [22], and urinary excretion of ADMA over a 24-h period is estimated to be 13.5±3.1 mg (~65 μmol per 24-h) in healthy adults [4]. In humans, red blood cells (RBCs) play a critical role in the synthesis and storage of ADMA; there is fast bidirectional traffic of ADMA across the plasma membrane of the RBCs that leads to equilibrium between intra- and extracellular ADMA [23].

In humans, two isoforms of DDAH are found: DDAH-1 and DDAH-2; DDAH-1 is the predominant form in the liver and kidney tubules [24] as well as in the brain at sites of neural NOS (nNOS) expression [25]. About 70% of ADMA is metabolized in the kidney and liver *via* DDAH-1 [26]. DDAH-1 activity in the kidney and liver is responsible for the metabolism of excessive circulating ADMA [27]. Although DDAH-1 regulates the degradation of ADMA in neuronal tissues, recent data indicate that it is the major isomer regulating systemic ADMA and cardiovascular NO bioavailability [28, 29]. DDAH-2 predominates in tissues expressing endothelial NOS (eNOS) (*e.g.*, heart, vascular endothelium, and smooth muscle cells) and inducible NOS (iNOS) (*e.g.*, immune tissues) [24, 30, 31]. DDAH-2 is a major player in regulating ADMA levels in the heart and vessels [28, 29].

The K_m values of DDAH-1 and DDAH-2 for ADMA are higher than their intracellular concentrations [31]. K_m value of DDAH-1 for ADMA is 170 μM in humans, and K_{cat} is 1.66 μmol/L/min [32]. K_m value of DDAH-2 for ADMA is 16 μM and V_{max} value is 4.8 nmol/mg/min [33]. The apparent rate of ADMA metabolism for DDAH-2 is ~70 times less than that of DDAH-1 [33]. Fig. (**1**) summarizes ADMA biosynthesis and metabolism. A wide distribution of two DDAH isoforms in various tissues indicates that the regulation of methylarginines level has critical biological importance [31]. Both intra- and extracellular levels of ADMA may be substantially elevated upon increased protein methylation or proteolysis, decreased DDAH activity, or decreased cationic amino acid transporter (CAT) activity [34]. DDAHs' expression and activity also contribute to the pathogenesis of endothelial dysfunction [10]. DDAH-2 critically determines NO availability in endothelial cells [33], and its gene silencing reduces endothelial-dependent relaxation by 40% [35]. Regulation of plasma ADMA level is highly dependent on factors that affect the expression and activity of DDAH

CHAPTER 5

Nitric Oxide-Related Oral Microbiota Dysbiosis in Type 2 Diabetes

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Abstract: The nitrate (NO_3)-nitrite (NO_2)-nitric oxide (NO) pathway, as a storage reservoir for endogenous NO production, is dependent on the oral bacteria with NO_3-reducing capacity. Undesirable changes of oral microbiota towards a decreased load of health-related NO_3-reducing bacteria and an overgrowth of pathogenic species, leading to subsequent decreased NO_2 production in the oral cavity and decreased systemic NO availability, are now considered risk factors for the development of insulin resistance and type 2 diabetes (T2D). This chapter discusses available evidence focusing on oral microbiota dysbiosis in T2D, especially NO_3-reducing bacteria and their metabolic activity (including NO_3-reductase and NO_2-reductase activity), affecting net oral NO_2 accumulation and the NO_3-NO_2-NO pathway.

Keywords: Nitrate, Nitrite, Nitric oxide, Nitrate reductase, Nitrite reductase, Oral microbiota, Type 2 diabetes.

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Asghar Ghasemi, Khosrow Kashfi, Zahra Bahadoran (Eds.)

INTRODUCTION

Dysbiosis in the oral microbiota is associated with increased cardiovascular and metabolic disorders [1, 2]. It has been suggested that the link between oral microbiota dysbiosis and cardiometabolic disorders is in part due to decreased abundance of species with nitrate (NO_3)-reducing capacity and concurrent increase of pathogenic bacterial species in the oral cavity [3, 4].

In the oral cavity, the commensal facultative and obligate anaerobic bacteria with NO_3-reducing capacity reduce salivary NO_3 to nitrite (NO_2) *via* a two-electron reduction, using anaerobic respiration by the action of NO_3 reductases (NaRs) [5]. Although mammalian NaRs activity may contribute to mammalian NO_3 reduction and regulation of NO_2 and nitric oxide (NO) metabolism in the human body [6], the role of oral microbiota is of great importance.

Although changes in the composition of oral microbiota in type 2 diabetes (T2D) have been reported [7, 8], this issue remains a matter of debate [9, 10]; T2D is associated with increased loading of diseases-associated oral bacteria and reduced community of health-related bacteria [11]. Since the recycling process of inorganic NO_3 to NO, a ubiquitous endocrine hormone involved in glucose and insulin homeostasis [12], is critically dependent upon oral commensal NO_3-reducing bacteria [13, 14], oral dysbiosis of these communities has been suggested to contribute to the development of insulin resistance T2D [15]. In this chapter, we focus on NO-related oral microbiota dysbiosis in T2D.

AN OVERVIEW OF ORAL MICROBIOTA

The term microbiota describes the ecological community of symbiotic, commensal, and pathogenic microorganisms [16]. Oral microbiota is the second largest, second most diverse, complex, and dynamic microbial community in the human body, including about 775 species of bacteria (12 phyla and 185 genera) [17]. The commensal bacteria resident in the oral cavity interacts with the host in a balanced manner, whereas potentially pathogenic bacteria emerge following an imbalanced oral resident microbiota [18]. The concept of a fluid continuum now replaces the concept of commensalism *vs.* pathogenicity as a fixed duality since labeling bacteria as strict commensals *vs.* pathogens was a too restrictive approach [19].

About 96% of the oral microbiota comprises the six broad phyla, *Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Bacteroidetes*, and *Spirochaetes* [17, 20]. The *Saccharibacteria*, *Synergistetes*, *SR1*, *Gracilibacteria*, *Chlamydia*, *Chloroflexi*, *Tenericutes, Euryarchaeota, TM7,* and *Chlorobi* comprise 4% of the remaining taxa [21]. The human oral microbiota database (HOMD) provides a

comprehensive database of oral bacterial taxa, a 16S rRNA identification tool, and genome sequences (www.homd.org).

Although the rat tongue microbiota is less diverse than the human [99±40 *vs.* 249±30 operational taxonomic units (OUT, a pragmatic proxy for species at different taxonomic levels, and used to categorize bacteria based on sequence similarity); 2.92±0.53 *vs.* 5.54±0.33 Shannon diversity index], the physiological activity of oral microbiota is comparable in both species [4]. The *Firmicutes* and *Proteobacteria* comprised 40-80% of the tongue communities in both humans and rats; however, the *Actinobacteria* is the predominant phyla in rat tongues, whereas human tongues contained more *Bacteroidetes* [4]. About 70% of human tongue bacterial composition comprises the gram-positive species (mainly *Staphylococcus, Micrococcus*, and *Streptococcus*), and 30% are gram-negative [22].

The oral microbiota has both pro- and anti-inflammatory activities that maintain oral homeostasis [23]. The balance between the resident species in the oral cavity determines how the microbiota changes toward a health-related (symbiosis) state or a disease-associated (dysbiosis) state [16]. Factors including poor oral hygiene, gingival inflammation, change in saliva flow rate or composition, aging, medications, diseases, dietary habits, physical activity, smoking, and a poor immune system drive the oral microbiota composition to dysbiosis and overgrowth of pathogenic species [24, 25].

From the metabolic function point of view, oral bacteria contribute to 4 major metabolic pathways: 1) saccharolytic bacteria, including *Streptococcus*, *Actinomyces*, and *Lactobacillus* species that metabolize carbohydrates into organic acids; 2) proteolytic and amino acid-degrading bacteria, including *Fusobacterium, Prevotella*, and *Porphyromonas* species that metabolize proteins and peptides into amino acids, and further into short-chain fatty acids, ammonia, sulfur compounds, and indole/skatole) [26]; 3) bacteria like *Streptococcus, Actinomyces*, *and Lactobacillus* that contribute to alkalinization and acid neutralization *via* arginine/agmatine deiminase system; 4) species including *Actinomyces* and *Veillonella* with NO_3-reducing capacity that convert NO_3 to NO_2 and inhibits bacterial acid production [26].

The metabolic activities of oral bacteria are closely related to systemic metabolic health and diseases [16, 26]. Although it is difficult to make a clear distinction, carbohydrate metabolizers and acid-producing bacteria, leading to the accumulation of organic acids, are related to oral dysbiosis. In contrast, those involving accumulating ammonia and NO_2 and inhibiting acid production are considered health-associated species [16, 26].

CHAPTER 6

Nitric Oxide and Type 2 Diabetes: Lessons from Genetic Studies

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Abstract: Nitric oxide (NO), a multifunctional gasotransmitter, is now considered an endocrine hormone that essentially contributes to the regulation of glucose and insulin homeostasis. Here, we discuss current genetic data linking NO metabolism to metabolic disorders, especially insulin resistance and type 2 diabetes (T2D). Although several gene variants of NO synthases [NOSs, *i.e.*, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)] isoforms have been identified in humans that affect NO bioactivity and metabolism, only the eNOS polymorphisms are reported to be associated with insulin resistance and T2D. Among the functional eNOS gene polymorphisms, the single nucleotide polymorphisms (SNPs) rs2070744 (T786C), rs1799983 (G894T), and rs869109213 (eNOS 4b/4a) are related to the risk of developing insulin resistance and T2D.

Keywords: Endothelial Nitric Oxide Synthase, Gene Polymorphisms, Inducible Nitric Oxide Synthase, Insulin Resistance, Neuronal Nitric Oxide Synthase, Nitric Oxide, Type 2 Diabetes.

INTRODUCTION

Emerging data suggest that impaired nitric oxide (NO) homeostasis is involved in the development of insulin resistance and type 2 diabetes (T2D) [1, 2]. The

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critical role of NO on glucose and insulin homeostasis is greatly supported by findings of the genetically-modified animal models, including NO synthase (NOS) gene knockout models or over-expressed NOSs [3 - 5]. Extrapolation of the results obtained in the genetically-modified NOS isoforms in the animal models to humans is not straightforward; however, studies that investigated gene polymorphisms of human NOSs confirm the relation between NO metabolism and the development of insulin resistance and T2D.

Among several reports on NOS gene polymorphisms, endothelial NOS (eNOS) gene variants have gained enormous attention due to their association with hypertension and cardiovascular diseases; a meta-analysis of 155 published studies showed that the most common eNOS polymorphisms, *i.e*., rs1799983 (G894T), rs2070744 (T786C), and rs869109213 (eNOS 4b/4a) are significantly associated with coronary artery disease in the general populations [6]. Another meta-analysis of 74 studies showed that the eNOS polymorphisms G894T and T786C increase the risk of hypertension by about 16-40% among different populations [7].

The genetic variants of eNOS that have formerly been reported for individual susceptibility to vascular dysfunction and hypertension [8] are now considered the risk factors for insulin resistance and T2D [9 - 12]. These functional eNOS polymorphisms affect gene expression or protein structure of eNOS, resulting in inadequate eNOS-derived NO production and impaired glucose and insulin homeostasis [9]. Here, we review genetic evidence linking NO metabolism to metabolic disorders, especially insulin resistance and T2D.

A BRIEF OVERVIEW OF NOS ENZYMES: GENE STRUCTURE AND CHROMOSOMAL LOCALIZATION

The enzymatic NO synthesis pathway is mediated by NO synthase (NOS), in which *L*-arginine is converted to NO by constitutive or inducible isoforms of NOSs, including eNOS (EC 1.14.13.39), neuronal NOS (nNOS, EC 1.14.13.39), and inducible NOS (iNOS, EC 1.14.13.39) [13, 14].

The NOSs are flavoheme homodimer enzymes (with two functional domains in each monomer: oxygenase and reductase domains), which catalyze the formation of NO through two consecutive monooxygenation reactions [14, 15]; calmodulin (CaM)-binding region in the middle of the molecule, has been called the third NOSs domain in some papers [16]. Both nNOS and eNOS are activated by the reversible binding of CaM at elevated intracellular Ca^{2+} levels; CaM binds to the linker region between the reductase and oxygenase domains and activates the enzyme by inducing intramolecular electron transfer [17]. The main characteristics of NOS isoforms are summarized in Table **1**. In addition, a number

of splice variants (nNOSμ, nNOSβ, and nNOSδ) are translated from the nNOS gene [18].

The human eNOS gene is located in the 7q36.1 region of chromosome 7 and comprises 28 exons (https://www.ncbi.nlm.nih.gov/gene/4846). The human eNOS gene has a length of 21-22 kb and encodes an mRNA of 4052 nucleotides [19]. The eNOS promoter region is TATA-less, includes Sp1 and GATA motifs, and has several binding sites for transcription factors, *i.e*., activator proteins (AP-1 and AP-2), nuclear factors [nuclear factor-1 (NF-1), interleukin-6 (IL-6), nuclear factor κB (NF-κB)], Ets protein polyomavirus enhancer activator 3 (PEA3), 1 myc-associated zine-finger protein (MAZ), 1 Ying Yang (YY1), acute-phase response elements, shear-stress response elements, and sterol regulatory elements [20 - 22].

The eNOS promoter sequence also contains several half-sites of the estrogen- and glucocorticoid-responsive elements [21]. Mutation of the Sp1 site decreased human eNOS promoter activity by about 85%, whereas deletion of the GATA binding site completely inactive eNOS promoter [23].

Table 1. Characteristics of NOS isoforms.

	NOS Isoforms		
	Constitutive		**Inducible**
	nNOS (NOS1)	**eNOS (NOS3)**	**iNOS (NOS2)**
Main sites of expression [24]	Brain	Endothelial cells	Macrophages in the liver, kidney, and lung
Other sites of expression [24 - 26]	Spinal cord, sympathetic ganglia, and adrenal glands, peripheral nitrergic nerves, epithelial cells, pancreatic islet cells, cardiac and skeletal myocytes, smooth muscle cells, renal macula densa cells	Red blood cells, alveolar macrophages, placenta, kidney tubular epithelial cells	Cytokine-induced cells
Physiological functions [27]	Learning and memory processes, regulation of systemic blood pressure, renal autoregulation, and local cerebral blood flow	Regulation of vascular tone	Implicated in immune defense, antitumor and antimicrobial activities
Cellular localization [27]	Cytoplasm, mitochondria, and plasma membrane	Plasma membrane caveolae and cytoplasm	Cytoplasm
Human locus (Gene ID) [25]	4842	4846	4843

CHAPTER 7

Role of Nitric Oxide in Diabetic Wound Healing

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Abstract: Nitric oxide (NO), a gaseous free radical, is a key signaling molecule in the different phases of the normal wound healing process. The beneficial effects of NO in wound healing are related to its antibacterial properties, regulation of inflammatory response, stimulation of proliferation and differentiation of keratinocytes and fibroblasts, and promotion of angiogenesis and collagen deposition. NO deficiency is an important mechanism responsible for poor healing in diabetic wounds. In this chapter, the function of NO in diabetic wound healing and the possible therapeutic significance of NO in the treatment of diabetic wounds are discussed. Current knowledge supports this notion that NO-based intervention is a promising therapeutic approach for diabetic wound healing.

Keywords: Advanced glycated end products, Coagulation, Diabetic foot ulcer, Endothelial nitric oxide synthase, Hexosamine pathway, Hyperglycemia, Neuropathy, Nitric oxide, Peripheral arterial disease, Polyol pathway, Protein kinase C, Reactive oxygen species, Superoxide anion, Wound healing.

INTRODUCTION

The worldwide prevalence of diabetes is increasing [1]. Delay in wound healing is one of the most disabling problems in diabetes [2]. Results of a systematic review and meta-analysis indicate that the global prevalence of diabetic foot ulcer (DFU) is 6.3%, and this prevalence is higher in men (4.5%) than in women (3.5%) and type 2 (6.4%) than in type 1 (5.5%) diabetic patients [3]. The lifetime risk for developing DFU in a diabetic patient is ~25% [4]. Compared to diabetic patients without ulcers, those with DFU have a 50% higher risk of mortality [5] and a 10-20 times more chance of lower limb amputation [6]. In addition, the yearly cost of

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care for people with diabetes and foot ulcers is 5.4 times higher than for people with diabetes but without foot ulcers [7]. Diabetic patients with DFU have a lower quality of life [8] since chronic wounds decrease a patient's ability to do simple tasks and social functions and are among the leading causes of hospitalization [2, 8]. Current standard care for a diabetic wound is debridement, pressure offloading, appropriate dressing, and infection and glycemic control [9, 10]. However, the current treatment for DFU seems insufficient since a meta-analysis of randomized clinical trials reported that healing rates are 24.2% and 30.9% at 12 and 20 weeks among patients with neuropathic DFU who received standard care [11]. In addition, a recent meta-analysis demonstrated that the global recurrence rate of DFU was high, with 22.1% per person-year [12]. The increasing prevalence of diabetes, adverse effects of DFU on a patient's quality of life, and the high economic burden of DFU warrant further research for finding new therapeutic strategies for more effective wound management in diabetic patients. Understanding pathophysiological mechanisms that interrupt the normal wound healing process in diabetic patients helps in finding new treatments.

Nitric oxide (NO) plays a significant role in skin pathophysiological responses, such as vasodilation, response to ultraviolet (UV) irradiation, inflammation, and apoptosis, and therefore affects several distinct aspects of wound healing [13]. NO regulates inflammatory response during the wound healing process, stimulates proliferation and differentiation of keratinocytes and fibroblasts, promotes angiogenesis and collagen deposition, and has antibacterial properties [14 - 16].

Decreased NO bioavailability in diabetes is an important factor in poor ulcer healing [17], and, therefore, NO-based therapies for DFU have received increasing attention in recent years. NO's role as a mediator in normal wound healing has been reviewed extensively [14 - 16]. This chapter summarizes NO's role in diabetic wound healing and discusses the potential applications of NO-based treatment strategies in diabetic wounds.

Types of Wound

A wound is created following disruption of skin integrity, mucosal surfaces, or organ tissues [18]. According to the healing time, wounds are classified as acute and chronic types [19]. Acute wounds (*e.g.*, surgical incisions) heal quickly through an orderly and timely process [18] in 5-10 days [19] or within 30 days from injury [19, 20]. Chronic wounds (*e.g.*, diabetic ulcers, pressure ulcers, and venous ulcers) are not repaired after 12 weeks of initial insult [18, 20]. These wounds cannot produce anatomical and functional integration of the injury site through an orderly and timely process [21].

Chronic wounds, which make up to ~70% of all skin wounds, are characterized by prolonged pathological inflammation, persistent infections, the inability of epidermal and/or dermal cells to respond to repair stimuli, and the formation of drug-resistant microbial biofilms [22]. Chronic ulcers are rarely seen in healthy people [21] and are more common in people with diabetes, obesity, or spinal cord injury [21].

Pathophysiology of Diabetic Foot Ulcer

Peripheral neuropathy and peripheral arterial disease (PAD) are the two main risk factors that lead to DFU [23]. More than 60% of DFUs result from underlying peripheral neuropathy [23]. PAD incidence is 2–4 times more common in patients with diabetes compared to non-diabetic individuals [24].

Peripheral Neuropathy

Increased levels of intracellular advanced glycated end products, increased hexosamine pathway flux and polyol pathway, activation of protein kinase C (PKC), and reduction of endothelial nitric oxide synthase (eNOS) expression are the main mechanisms causing hyperglycemic nerve damage [23, 25]. Neuropathy in diabetic patients is reflected in sensory, motor, and autonomic nervous system divisions [23].

Sensory neuropathy is the most common predictor of DFU in diabetes [4] and causes damage to sensation, which protects against stimuli, such as heat, pressure, and pain [4]. As a result, these patients are less sensitive to pressure-related trauma or other minor skin injuries, increasing their susceptibility to injury [4]. Damage to the motor nerves in the motor neuropathy causes deformities in the foot, abnormal foot pressure, and subsequent callus formation. It will gradually cause skin damage and create a wound [23]. In autonomic neuropathy, the foot becomes dry due to decreased secretory functions of the sebaceous and sweat glands [4]. Dry skin is prone to fissures and thus creates a site vulnerable to microbial infection [4].

Peripheral Arterial Disease (PAD)

PAD is characterized by stenosis or occlusion of the lower limb's arteries [26]. It commonly affects the tibial and peroneal arteries of the calf. It leads to acute or chronic ischemia, and in combination with digital artery disease, it also impairs wound healing by affecting circulation and blood flow of the lower limbs [23].

Hyperglycemia, smoking, age, hypertension, and hyperlipidemia are the most important risk factors for PAD [26]. Endothelial dysfunction decreases NO

CHAPTER 8

Role of Nitric Oxide in Type 2 Diabetes-Induced Osteoporosis

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Abstract: Osteoporosis affects 200 million people worldwide. Osteoporosis in subjects with diabetes is called diabetoporosis, and type 2 diabetes (T2D) contributes to and aggravates osteoporotic fractures. Hyperglycemia, insulin resistance, bone vasculature impairment, increased inflammation, oxidative stress, and bone marrow adiposity contribute to a higher incidence of osteoporotic fractures in T2D. Decreased nitric oxide (NO) bioavailability due to lower endothelial NO synthase (eNOS)-derived NO and higher inducible NOS (iNOS)-derived NO is one of the main mechanisms of the diabetoporosis. Available data indicates that T2D increases osteoclast-mediated bone resorption and decreases osteoblast-mediated bone formation, mediated in part by reducing eNOS-derived NO and increasing iNOS-derived NO. NO donors delay osteoporosis and decrease osteoporotic fractures in subjects with T2D, suggesting the potential therapeutic implication of NO-based interventions for diabetoporosis.

Keywords: Bone mineral density, Bone marrow stromal cells, Diabetoporosis, Endothelial nitric oxide synthase, Inducible nitric oxide synthase, Nitric oxide, Osteoporosis, Type 2 diabetes.

INTRODUCTION

Osteoporosis is a silent illness that affects 200 million people worldwide [1 - 3] and is generally diagnosed after an osteoporotic fracture [4, 5]. Osteoporosis is characterized by low bone mineral density (BMD) and deterioration of the bone microarchitecture [6, 7]. Osteoporosis causes about 9 million fractures annually, severely impacting patients' quality of life and imposing high healthcare costs [1, 8]. In addition, ~40% of older women will experience an osteoporotic fracture during their lifetime [5, 9], increasing their mortality rate by 15-20% [10 - 13].

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Osteoporosis is generally associated with senescence and estrogen deficiency; however, other factors, including diabetes, contribute to and deteriorate osteoporotic fracture [14]. Osteoporosis in subjects with diabetes was firstly reported by Albright and Reifenste in 1948 and named diabetoporosis by Ferrari S. in 2015 [15].

Diabetoporosis is one of the leading causes of osteoporotic fractures [14] and is characterized by decreased bone quality and quantity [16]. According to population-based studies conducted from 1980 to 2016, the risk of osteoporotic fractures has been increased in subjects with both type 1 diabetes (T1D) and type 2 diabetes (T2D) [17 - 21]. The risk of osteoporotic fractures is 200-700% higher in subjects with T1D [17, 22, 23] and 40-80% higher in subjects with T2D [22, 24]. In addition, fracture healing is delayed in subjects with T1D and T2D; a study of 5966 cases of hip fracture in patients with T1D and T2D showed that these patients required longer in-hospital stay [25]. These data emphasize the need for developing new strategies against osteoporotic fractures in patients with diabetes, especially in subjects with T2D, which accounts for ~90% of diabetic subjects [26].

Nitric oxide (NO) bioavailability is decreased in the bones of humans and animals with T2D and can be considered one of the primary mechanisms underlying diabetoporosis [27]. NO is produced in bone cells by the three isoforms of NO synthase (NOS) enzymes, including endothelial, neural, and inducible NOS (eNOS, nNOS, and iNOS, respectively) [28, 29].

NO in the bone acts as a double-edged sword; a low level of NO increases osteoblast-mediated bone formation [30, 31], decreases osteoclast-mediated bone resorption [32, 33], has protective effects against osteoporotic fractures, and increases the rate of bone healing [30, 34] in postmenopausal women and ovariectomized rats. A high NO level has adverse effects on bone cells' activity [28, 35]. $eNOS^{-/-}$ rodents have decreased osteoblast [36] and increased osteoclast activities [37 - 39] that are associated with lower trabecular bone volume and cortical thickness as well as lower BMD [40, 41]. These changes increase the risk of osteoporotic fractures [42] and decrease the rate of the bone healing process [43] in $eNOS^{-/-}$ rodents. In addition, $iNOS^{-/-}$ rodents show reduced bone growth and bone length [44] only in pre-natal but not in adult bones [44]. Reduced osteoclasts and osteoblasts in $nNOS^{-/-}$ rodents are associated with lower bone remodeling [45, 46]. $nNOS^{-/-}$ rodents have lower bone remodeling and lower numbers of osteoclasts and osteoblasts. The effect of NO on bone function in healthy subjects has been previously reviewed [47, 48]. This chapter deals with the role of NO in diabetoporosis.

NITRIC OXIDE AND BONE: A BRIEF OVERVIEW

The effect of NO on bone function is dependent on its concentration; a low level of NO is beneficial for several physiological functions of bone, including normal bone formation [49, 50], development [28, 51], remodeling [52], and fracture healing [53], whereas, a high NO level has detrimental effects on all of these functions [28]. In support of this notion, low and middle doses of nitroglycerin, as a NO donor, stimulate osteoblast activity and bone formation [35], but its high doses decrease osteoblast activity [35] and thus decrease the rate of bone turnover [35].

Regarding the effects of NO on osteoclast activity and bone resorption, it has been reported that a low level of NO may be necessary for normal osteoclast activity; this proposition rests on observations that NOS inhibitors inhibit the activity and motility of isolated osteoclasts [34, 54]. However, a high NO level inhibits osteoclast formation and activity and promotes apoptosis in osteoclasts [35].

There is a substantial body of evidence showing that the cyclic guanosine monophosphate (cGMP)-dependent signaling is vital for normal bone formation [50], and cGMP has been proposed as the primary mediator of NO on bone function [52]. The stimulatory effects of NO on osteoblasts are mediated by cGMP since it is abolished by guanylate cyclase (GC) inhibitors [55]. NO, *via* activation of soluble GC (sGC), increases osteoblastic bone formation [56], whereas the effect of NO on osteoclasts is mainly inhibitory and, in part, cGMP-independent [47, 56]. NO *via* activation of sGC is also a key regulator of angiogenesis in the bone that plays an essential role in bone repair [57]. In addition, NO, *via* core-binding factor alpha 1 (a critical transcription factor for osteoblastic differentiation and osteogenesis)/cGMP/PKG pathway, increases matrix metalloproteinase-13 (MMP-13) expression in osteoblasts, which is an essential factor for bone development [28, 51]; therapeutic effects of estrogen in bone are also in part mediated by the eNOS/cGMP pathway [55, 58, 59].

NOS Expression in the Bone Cells

NO is produced in the bone cells by eNOS, nNOS, and iNOS [55, 60 - 62]. eNOS is mainly expressed in osteoblasts and osteocytes [63, 64], but its expression has also been documented in osteoclasts and bone marrow stromal cells [55, 65]. eNOS is also expressed in chondrocytes of the epiphyseal growth plate, mainly in the first four weeks of the neonatal period, and after that, it declines with age [66]. iNOS is expressed in osteoclasts in neonatal rats under physiological conditions [66]; in adults, it is expressed in response to pro-inflammatory cytokines under pathological conditions in all bone cells [54, 67, 68].

CHAPTER 9

Hyperuricemia, Type 2 Diabetes and Insulin Resistance: Role of Nitric Oxide

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Abstract: Uric acid (UA) is the end product of purine catabolism in humans. Hyperuricemia, defined as elevated plasma concentrations of UA above 7 mg/dL, is a risk factor for developing hypertension, cardiovascular diseases, chronic kidney disease, and type 2 diabetes. Hyperuricemia can induce pancreatic β-cell death and impaired insulin secretion. It can also disrupt insulin-induced glucose disposal and insulin signaling in different insulin-sensitive tissues, including cardiomyocytes, skeletal muscle cells, adipocytes, hepatocytes, and endothelial cells. These events lead to the development of systemic insulin resistance and impaired glucose metabolism. Induction of inflammation, oxidative stress, and impairment of nitric oxide (NO) metabolism mediate hyperuricemia-induced insulin resistance and dysglycemia. This chapter is focused on the potential mediatory role of NO metabolism on hyperuricemia-induced dysglycemia and insulin resistance.

Keywords: Hyperuricemia, Insulin Receptor, Insulin Resistance, Type 2 Diabetes, Uric Acid.

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INTRODUCTION

Uric acid (UA), the end product of adenine- and guanine-based purines catabolism in humans, is synthesized by xanthine oxidoreductase (XOR) from xanthine [1]. Both endogenous (*i.e.*, de novo purine biosynthesis as well as cell and tissue turnover) and exogenous (*i.e.*, dietary purines occurring in the seafood, meats, and legumes) sources of purines are involved in UA production [2 - 4]. Increased plasma concentrations of UA above 7 mg/dL (1 mg/dL ≈59.48 μM), which accompanies by a doubled amount of exchangeable pool of UA (from 1200 to 2027 mg) and also hyperuricosuria (urinary excretion of UA> 800 mg/day in men and >750 mg/day in women) [4, 5], is the primary cause of gout [6].

Hyperuricemia is a risk factor for developing cardiometabolic disorders, including hypertension [7, 8], cardiovascular diseases [9, 10], chronic kidney disease [11], type 2 diabetes (T2D) [12 - 15], and mortality [16, 17]. In addition, hyperuricemia has been suggested as a component of metabolic syndrome, the cluster of metabolic and hemodynamic abnormalities, including abdominal obesity, glucose intolerance, insulin resistance, dyslipidemia, and hypertension [18]. Although not fully established, current documents imply that increased plasma UA concentration is a causative factor for developing insulin resistance and dysglycemia [12, 13].

Several mechanisms may explain the cause-and-effect relationship between hyperuricemia and the progression of T2D; high-UA concentrations can induce pancreatic β-cell death and impaired insulin secretion [19, 20]. High-UA can also disrupt insulin-induced glucose disposal and insulin signaling in various insulin-sensitive tissues, including cardiomyocytes [21], skeletal muscle cells [22], adipocytes [23], hepatocytes [24], and endothelial cells [25]; these events consequently lead to the development of systemic insulin resistance [26, 27]. Induction of oxidative stress, inflammatory processes, and impaired nitric oxide (NO) metabolism are crucial underlying mechanisms by which high-UA concentration imposes detrimental effects on glucose and insulin homeostasis.

In this chapter, considering both epidemiological and experimental evidence, we discuss the potential cause-and-effect relation between hyperuricemia and T2D and insulin resistance development. Furthermore, we focus on the possible mediating role of NO in hyperuricemia-induced dysglycemia and insulin resistance.

A BRIEF OVERVIEW OF URIC ACID METABOLISM AND FUNCTION

Uric Acid Synthesis In Human: Role of XOR

Uric acid is a weak hydrogenated organic acid (pKa_1 of 5.75 and pKa_2 of 10.3); under normal conditions, *i.e*., pH 7.4 and 37°C, the most predominant (~98-99%) form of UA within the circulation and synovial fluid is urate anion (*i.e*., mono-deprotonated ionic form) [28]. Uric acid synthesis mainly occurs in the liver, whereas other tissues, such as the intestine, myocardium, kidney, and vascular endothelium, also contribute to UA synthesis to a lesser extent [29]. UA is the metabolic end product of purine in humans (due to lack of uricase); it can be, however, be diverted into further catabolism by uricase to produce allantoin in other mammals [2, 30]; loss of uricase activity in humans has been an evolutionary process, which allowed them to readily accumulate fat *via* the metabolism of fructose from fruits [31].

Many enzymes, including nucleotidase, adenosine deaminase, and purine nucleoside phosphorylase, participate in the purine catabolism [*i.e*., converting adenosine monophosphate (AMP), and guanine monophosphate (GMP) to inosine and guanosine, respectively, and then into hypoxanthine (HPX)]. XOR, which converts HPX into xanthine and then to UA, is the key and rate-limiting enzyme in the pathway [1].

Positive regulators of XOR gene expression are hypoxia, lipopolysaccharide, inflammatory cytokines [interferon γ, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)], dexamethasone, cortisol, and prolactin [32]. In addition to oxygen pressure, NO can also modify XOR activity; exogenous NO and also NO produced by XOR (reduction of nitrite to NO *via* xanthine as reducing substrate) are accompanied by the enzyme inactivation (through NO-induced conversion of xanthine to its desulfo-form) and inactivates XOR [33, 34].

The maximum XOR activity in mammals is in the liver and intestinal epithelial cells; XOR is mainly located in the cytoplasm and cell membrane through cell surface binding mediated by glycosaminoglycans [35, 36]. As reviewed elsewhere, besides purines, XOR can also catabolize different endogenous metabolites (*i.e*., aldehydes, pyrimidines, pteridines, azopurines, and heterocyclic compounds) and different xenobiotics (*e.g.*, antiviral and anticancer agents) [37, 38].

Regulation of Circulating Uric Acid Levels

Normal serum UA concentration is 3.5-7.2 mg/dL in adult men and postmenopausal women and 2.6-6.0 mg/dL in premenopausal women; however, a

CHAPTER 10

Therapeutic Management of Type 2 Diabetes: The Nitric Oxide Axis

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Abstract: According to the World Health Organization (WHO), the prevalence of obesity across the globe has nearly tripled since 1975, with 39 million children under the age of 5 being overweight or obese in 2020. Obesity is the most common risk factor for developing type 2diabetes (T2D), which may lead to elevated serum triglycerides, hypertension, and insulin resistance. In the pathogenesis of T2D, there is a reduction in nitric oxide (NO) bioavailability. Restoration of NO levels has been associated with many favorable metabolic effects in T2D. Drugs that potentiate NO levels may have a role in improving T2D-associated adverse effects. Current medications approved for use in the management of T2D include biguanides, thiazolidinediones, sulfonylureas, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, alpha-glucosidase inhibitors, and sodium-glucose co-transporter 2 (SGLT2) inhibitors. These drugs mitigate the many adverse effects associated with T2D. This chapter discusses these classes of drugs, examines their mechanism of action, and presents evidence that these drugs directly or indirectly modulate NO levels.

Keywords: Alpha-glucosidase inhibitors, Biguanides, Dipeptidyl peptidase-4 inhibitors, Glucagon-like peptide-1, Glucagon-like peptide-1 receptor agonists, Meglitinides, Metformin, Nitric oxide, Sodium-glucose co-transporter, Sodium-glucose co-transporter inhibitors, Sulfonylureas, Thiazolidinediones, Type 2 diabetes.

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INTRODUCTION

In 1825, Jean Anthelme Brillat-Savarin wrote, in *Physiologie du Gout, ou Meditations de Gastronomie Transcendante*: "Dis-moi ce que tu manges, je te dirai ce que tu es." (Tell me what you eat and I will tell you what you are) [1]. The German philosopher and anthropologist Ludwig Andreas von Feuerbach, in an essay titled *Concerning Spiritualism and Materialism*, wrote: "Der Mensch ist, was er ißt" (man is what he eats) [2, 3]. The food you eat is a reflecting image of yourself. Thus, maintaining a well-balanced and healthy diet is essential for good health.

According to the World Health Organization (WHO), the prevalence of obesity across the globe has nearly tripled since 1975, with 39 million children under the age of 5 being overweight or obese in 2020 [4]. In the United States, approximately one-third of the adult population is obese, and an additional one-third is overweight [5]. Obesity is the fastest-growing lethal disease in Western and developing countries. People do not die from obesity itself but from its complications, which shorten their lifespan [6, 7]. Type 2 diabetes (T2D), referred to as adult-onset or non-insulin-dependent diabetes, accounts for over 90-95% of all diabetes. It is a complex chronic metabolic disorder characterized by alterations in lipid metabolism, insulin resistance, hyperglycemia, and pancreatic β-cell dysfunction [8]. Since in T2D, patients are unable to store and utilize their glucose within the muscles, adipose tissues, and liver [9], these patients are in a constant hyperglycemic state [10]. Risk factors associated with the development of T2D include obesity, especially in the intra-abdominal region, sedentary lifestyle, and family history [11]. For this reason, T2D patients are often overweight, but weight loss and exercise can improve their blood glucose levels.

Many complications are associated with T2D, including cardiovascular disease, kidney disease, peripheral neuropathy, and cataracts [12]. One mechanism by which high glucose levels can affect the heart is its non-enzymatic glycation and cross-link, which produces "advanced glycosylation end products" (AGEs) [13]. The AGEs trap circulating low-density lipoprotein cholesterol (LDL-C) within the medium and large-sized vessels leading to atherosclerosis [14]. Depending on the location of this atherosclerosis, it can lead to coronary artery disease, stroke, and peripheral vascular disease. When the AGEs are within the smaller sizes arteries and arterioles, this may cause diabetic kidney disease and renal failure [15]. For these reasons, type 2 diabetic patients need to maintain appropriate blood glucose levels to reduce the risks of these complications.

In the pathogenesis of T2D, there is a reduction in nitric oxide (NO) bioavailability [16]. Restoration of NO levels has been associated with many

favorable metabolic effects in T2D [16]. NO is produced in all tissues by NO synthase (NOS)-dependent and independent pathways [16]. There are three isoforms of NOS, neuronal (nNOS/NOS-1), inducible (iNOS/NOS-2), and endothelial (eNOS/NOS-3), with all three being expressed in the pancreatic β-cells [16]. Drugs that potentiate NO levels may have a role in improving T2D-associated adverse effects. Current drugs approved for use in the management of T2D include biguanides, thiazolidinediones, sulfonylureas, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, alpha-glucosidase inhibitors, and sodium-glucose co-transporter 2 (SGLT2) inhibitors [17, 18]. These drugs mitigate the many adverse effects associated with T2D. This chapter discusses these classes of drugs, examines their mechanism of action, and presents evidence that these drugs directly or indirectly modulate NO metabolism.

BIGUANIDES

General Mechanism of Action

Although the term biguanides refer to a whole class of oral T2D drugs, metformin (N, N-Dimemethylbiguanide) [widely known as glucophage] is the only approved biguanide derivative currently available for the treatment of hyperglycemia. It is on the World Health Organization's List of Essential Medicines [19]. Metformin is a first-line T2D drug used to reduce blood glucose levels [20] and body weight [21]. Generally, it is prescribed as monotherapy for treating T2D, but it can also be used in combination with other medications, such as pioglitazone and vildagliptin, which are thiazolidinediones and DPP-4 inhibitors, respectively [22]. The functional purpose of metformin is to lower both the basal and postprandial plasma glucose levels [23]. It decreases hepatic glucose production, reduces intestinal glucose absorption, and improves insulin sensitivity by increasing peripheral glucose uptake/utilization by the skeletal muscles and adipocytes [22, 23]. Metformin has also been shown to be an effective drug to reduce weight in insulin-sensitive and insulin-resistant overweight and obese patients [24].

The hepatocytes take up metformin through the organic cation transporter-1 (OCT-1) [25]. Its mechanism of action involves reducing hepatic glucose production by inhibiting gluconeogenesis while maintaining insulin uptake by the periphery [26]. At the molecular level, metformin has been found to inhibit the hepatic mitochondrial respiratory chain at complex I [27], which ultimately leads to the activation of AMP-activated protein kinase (AMPK) [28]. AMPK is a heterotrimeric enzyme involved in metabolic regulation [29] and is composed of a catalytic subunit and two regulatory subunits [30]. The catalytic subunit of this

CHAPTER 11

Brain Insulin Resistance, Nitric Oxide and Alzheimer's Disease Pathology

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Abstract: Alzheimer's disease (AD) is a devastating age-related neurodegenerative disease characterized by progressive pathological changes and functional and cognitive impairments. Brain insulin resistance appears to contribute significantly to the pathology and cognitive deficits among several pathological mechanisms. Brain insulin resistance has been demonstrated in animal models of AD and postmortem human brain tissue from patients with AD dementia. Studies conducted in AD models and humans suggest attenuating brain insulin resistance by agents such as glucagon-like peptide1 (GLP-1) analogs and small molecule drug candidate PTI-125 reduces many AD pathologic features and symptoms. Insulin affects NO levels by activating endothelial and neuronal nitric oxide synthase (eNOS, nNOS), and systemic insulin resistance has been linked to reduced nitric oxide (NO) bioavailability. Increasing NO availability reduces systemic insulin resistance, and the insulin signaling pathway is associated with the activation of eNOS, implying a causal relationship. This chapter explores this relationship and the role of impaired NO availability in brain insulin resistance in AD dementia.

Keywords: α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) Receptor, CaMKII (Calcium-Calmodulin-Dependent Kinase II), Gamma-Aminobutyric acid (GABA) Receptor, Glutamate, Insulin Resistance, NADPH Oxidase 2 (Nox2), NADPH Oxidase Subunit NOX2, NG-Monomethyl-*L*-Arginine (*L*-NMMA), Nitric Oxide Synthase (NOS), N-methyl-*D*-Aspartate (NMDA) Receptor, Reactive Oxygen Species (ROS), Type-2 Diabetes (T2D).

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with many underlying pathophysiological changes that gradually lead to dementia [1 - 4].The lack of effective treatments for AD dementia and the enormous socioeconomic impact on society underscores the urgent need to develop effective treatments for this devastating disease [5, 6]. Many promising therapeutic agents in development for AD aim to reduce brain insulin resistance, a common early pathological feature of AD dementia with or without diabetes [7 - 10]. The pathological factors that contribute to brain insulin resistance are not fully understood. Nitric oxide (NO) is one of several biological molecules that interact with the insulin signaling pathway bi-directionally. In this chapter, we discuss the role of the NO system in the development of brain insulin resistance and explore the possibility that manipulating NO might be therapeutic for AD dementia.

INSULIN RECEPTOR SIGNALING AND ITS INTERACTION WITH NO SYSTEM

Insulin, a peptide secreted by the beta (β) cells in the pancreas, crosses the blood-brain barrier in a regulated and saturable manner to enter the central nervous system (CNS). Although de novo synthesis of insulin in the brain is still debated, support for local brain insulin synthesis includes the detection of C-peptide and insulin mRNA in various brain regions in humans, with the mRNA levels, being especially high in the hippocampus, striatum, and thalamus [11 - 15]. Insulin expression is decreased in AD compared to normal controls [14].

Insulin produces its cellular actions by binding its cognate insulin receptors (IRs) present on all cells, including neurons and glia in brain regions such as the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and amygdala [8, 16, 17]. IRs are more concentrated in neurons relative to glial cells and are particularly highly expressed in postsynaptic densities [8, 16 - 18]. Upon insulin binding to the extracellular α-subunit domains of IRs, the intracellular IR β-subunit domains dimerize, leading to activation of their intrinsic tyrosine kinase to cause autophosphorylation. Insulin-like growth factor-1 (IGF-1) also binds and activates IRs with lower affinity, leading to the same trophic and metabolic actions as insulin, including neuronal plasticity [19, 20].

In addition to regulation of glucose utilization and homeostasis, insulin activates PI3K-Akt (Phosphoinositide 3-kinase - Protein kinase B/Akt) and mTOR (Mechanistic target of rapamycin) signaling *via* recruitment of insulin receptor substrate family (IRS) proteins, such as IRS-1 and IRS-2. This insulin-stimulated PI3K/Akt/mTOR pathway has many other functions in cells throughout the body, including the neuronal and vascular systems. Insulin activates Akt *via* IRS1-PI3K

to directly phosphorylate serine1177 residues and activate vascular endothelial NO synthase (eNOS), leading to NO production and consequent vasodilation and increased capillary blood flow [21, 22]. Insulin signaling promoting NO-mediated vasodilation in the brain is supported by increased blood flow in the insular cortex following intranasal insulin in men, independent of cortisol manipulation [23]. Expression of eNOS has been shown not only in the endothelium of the cerebrovasculature but, more importantly, in dendritic spines [24].

Innate eNOS activity confers protection against secondary neuronal injury; thus, impaired eNOS due to insufficient insulin signaling in the brain can conceivably contribute to pathologies in AD, leading to cognitive impairments [25].

Insulin has been shown to modulate a wide range of neuronal functions. Insulin regulates 1) trafficking of ligand-gated ion channels, 2) expression and localization of GABA (γ-Aminobutyric acid), NMDA (N-Methyl-*D*-aspartic acid or N-Methyl-*D*-aspartate), and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, 3) catecholamine release and uptake, and 4) synaptic plasticity shown by long-term potentiation (LTP) and depression (LTD) in an NMDA receptor and PI3K dependent manner [26 - 29]. Insulin also promotes dendritic spine formation and excitatory synaptic development, and insulin regulates the development and health of excitatory synapses by activating PI3K/Akt/mTOR and Rac1/Cdc42 signaling [30].

Activation of the NMDA receptor recruits and activates neuronal NO synthase (nNOS) *via* Akt- and CaMKII(Ca^{2+}/calmodulin-dependent protein kinase II)-mediated phosphorylation of nNOS to promote the production of NO in the postsynaptic field [31, 32]. The activation of nNOS was also found to elevate AMPA receptor levels [32]. Thus, insulin can increase NO production in postsynaptic neurons by stimulating nNOS *via* activation of NMDA receptors. Increased NO promotes NADPH oxidase 2 (NOX2)-dependent ROS production postsynaptically, which may damage the dendritic field. Dendritic field destruction is one of the pathological changes in AD [33].

The importance of NO in modulating insulin receptor activity has also been illustrated by the blockade of the phosphatases SHP-1(Src homology region 2 domain-containing phosphatase-1), SHP-2(SH2 domain-containing protein tyrosine phosphatase-2), and PTP1B (Protein Tyrosine Phosphatase 1B) by S-nitrosylation of the cysteine residue at the enzyme's active sites concomitantly with a burst of NO production in response to insulin [34, 35]. Inhibition of the PTP1B, SHP-1, and SHP-2 by S-nitrosylation release inhibition of tyrosine phosphatases on insulin signaling. Hence, increased NO levels can promote NO-dependent tyrosine-phosphorylated insulin receptors and its downstream effector

CHAPTER 12

Arginine, Nitric Oxide, and Type 2 Diabetes

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Abstract: *L* Arginine (Arg), a semi-essential essential amino acid, has received significant research interest over the last two decades as nitric oxide (NO) precursor. Arg is widely used as a complementary treatment in various NO-disrupted conditions, *e.g.*, hypertension, preeclampsia, and endothelial dysfunction. Here, we provide an overview of the potential efficacy of Arg as a NO precursor and its effects on glucose and insulin homeostasis and diabetes-induced cardiovascular complications.

Keywords: Arginine, Argininosuccinate lyase, Argininosuccinate synthase, Citrulline, Nitric oxide, Nitric oxide synthase, Ornithine aminotransferase, Ornithine transcarbamylase and ornithine decarboxylase, Type 2 diabetes.

INTRODUCTION

L-Arginine (Arg), a semi-essential or conditionally essential amino acid, is involved in synthesizing proteins, creatine, polyamines, agmatine, urea, and the metabolism of proline and glutamate in the body [1, 2]. Arg is provided by either the exogenous (dietary intake) or endogenous [*de novo* synthesis from *L*-citrulline (Cit) and turnover of proteins] sources; the sufficient magnitude of *de novo* synthesis makes Arg a non-essential amino acid in healthy adult humans. How-

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ever, during early development and pathologic conditions, such as infection, inflammation, and impaired renal or intestinal metabolic functions, *de novo* synthesis is not sufficient to meet metabolic needs [3].

Arg has received significant research interest over the last two decades as nitric oxide (NO) precursor [1, 2]. This property has led to the widespread use of Arg as a complementary treatment in various NO-disrupted conditions, including hypertension [4 - 6], preeclampsia [7], and endothelial dysfunction [8]. Because of its ability to increase NO production, Arg has been considered a potential complementary treatment in type 2 diabetes (T2D) to improve glucose and insulin homeostasis and diabetes-induced cardiovascular complications [6, 8 - 10].

In this review, the potential efficacy of Arg as a NO precursor is discussed in T2D. First, we provide an overview of the amino acid metabolism (biosynthesis pathways, catabolic pathways, and pharmacokinetics of oral ingested doses) and its contributions to cellular and whole-body NO synthesis. Then, we provide some evidence to investigate the undesirable change of metabolism of Arg in T2D. Finally, the effects of supplementation with Arg on glucose and insulin homeostasis, and diabetes-related cardiometabolic complications, investigated in animal experiments and human clinical trials, are discussed.

PLASMA ARG FLUX

Plasma levels of Arg range from 80-120 μM in healthy adults [1]. Plasma flux of an amino acid represents the difference between arterial and venous concentrations of the amino acid multiplied by plasma flow; a positive value indicates net influx (uptake), and a negative value indicates net efflux [11]. Fluxes of plasma Arg in healthy humans on a normal diet were estimated to be 63-70 and 70-95 μmol/kg/h in fasted and fed states, respectively [12, 13]; fed flux is higher because of increased entry of dietary Arg into plasma [14]. In healthy adults, about 10-15% (~5.5-9.2 μmol/kg/h) of the plasma Arg flux originates from *de novo* synthesis from Cit [12, 15]. Arg derived from protein turnover is ~ 36 μmol/kg/h [16]. As indicated in Fig. (**1**), the endogenous flux of Arg in the fed state is lower than in the fasted state (52 *v.* 63 μmol/kg/h), probably because of the availability of Arg from exogenous sources, which decreases protein turnover and *de novo* synthesis of Arg.

Endogenous Arg flux, estimated after exclusion of dietary Arg intake from the plasma flux, remained unchanged following either free- and Arg-rich diets (~53 μmol/kg/h); contributions of protein breakdown and *de novo* synthesis of Arg were also similar in endogenous Arg flux in both conditions [16].

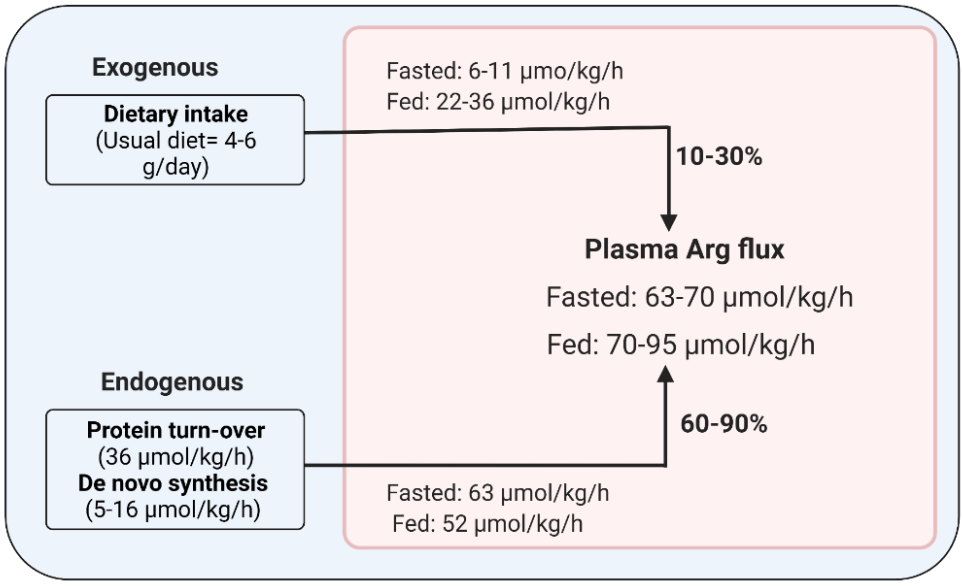

Fig. (1). Determinants of plasma flux of *L*-arginine (Arg). Both exogenous (10-30%) and endogenous sources (60-90%) contribute to Arg plasma flux. Created with BioRender.com.

The relative amount of Arg in various dietary proteins has been determined at 3-15% [17]. The usual daily intake of Arg in healthy adults has been estimated to be 4-6 g/day, which is ~20% of its plasma flux [18]. The contribution of diet to plasma Arg flux may be as low as ~8-20% in the fasted state (6-11 µmol/kg/h) to as large as ~35–40% in the postprandial state (~22-36 µmol/kg/h) [14, 16, 19]. Following a 6-day Arg-free diet, these values reduced to 52±7 and 63±14 µmol/kg/h, whereas, during the same period, an Arg-rich diet increased plasma Arg fluxes to 69±8 and 87±12 µmol/kg/h in the fasted and fed states, respectively [16]. Fig. (**1**) displays determinants of plasma Arg flux.

Arginine Biosynthesis Pathways

A significant fraction of Arg is synthesized from the amino acid Cit through an intestinal-renal axis. About 50-80% of Cit released by the intestine is taken up by the kidneys [20, 21] and then converted into Arg within the proximal tubule *via* argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) enzymes (Fig. **2**); about 75% [22] to 100% [20] of plasma Cit taken up by the kidney backs into the circulation as Arg. The extra-renal tissues are also involved (up to 40%) in Arg *de novo* synthesis for both meeting their needs for Arg and its release into circulation; in the nephrectomized mouse model (~80% nephrectomy), the amount of Cit accounted for plasma Arg decreased from 88 to 42% [23].

CHAPTER 13

Citrulline, Nitric Oxide, and Type 2 Diabetes

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Abstract: L-citrulline (Cit), a neutral, non-essential, and non-protein amino acid, is a precursor of *L*-arginine (Arg) and is involved in nitric oxide (NO) synthesis. Since oral ingestion of Cit can effectively elevate total Arg flux in the entire body and promote NO production, its supplementation has recently received much attention in the realm of cardio-metabolic diseases where NO metabolism is disrupted. Although preliminary data obtained from *in vitro* and *in vivo* animal experiments indicates that Cit improves glucose and insulin homeostasis and can effectively prevent hyperglycemia-induced complications such as inflammation, oxidative stress, renal dysfunction, and endothelial dysfunction, these findings are yet to be realized in well-designed long-term clinical studies in patients with type 2 diabetes (T2D). If Cit is shown to be an effective anti-diabetic agent with a good safety profile, its supplementation will be superior to that of Arg because it effectively increases systemic Arg availability more than Arg itself, and hence NO production.

Keywords: Arginine, Arginase, Argininosuccinate Synthase, Argininosuccinate Lyase, Carbamoyl Phosphate Synthetase I, Citrulline, Glutamine, Nitric Oxide, Ornithine Decarboxylase, Ornithine Transcarbamylase, Type 2 Diabetes.

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INTRODUCTION

L-Citrulline (Cit) is a neutral, non-essential, non-protein amino acid, which is best known as an intermediate in the urea cycle but has also garnered interest as a precursor of *L*-arginine (Arg) synthesis and nitric oxide (NO) production [1]. The name of this amino acid originated from the *Citrullus vulgaris* (watermelon); it was first isolated in 1914 from watermelon juice, but it was not until 1930 when Mitsunori Wada elucidated its structure and named it 'citrulline' [2]. Due to its relatively rare food sources (fresh watermelon containing about 1.6-3.5 g/kg), the amount of Cit provided through usual dietary intakes is somewhat limited. For example, one has to consume about 1-1.5 kg of fresh watermelon to achieve the minimum effective dose of Cit, which is about 3 g [3]. Watermelon rind contains more Cit than flesh watermelon, approximately 24.7 *versus* 16.7 mg/g dry weight [4]. There is no recommended dietary allowance for Cit [1].

The mean plasma concentration of Cit has been reported to be about 20-47 μM [5 - 10], and whole body Cit flux is estimated to be ~ 8.9 μmol/kg/h (~ 6-10 μmol/kg/h), in healthy human adults in the fasted state [5]. More quantitatively, whole body Cit flux has been estimated to be about 4g/day [11]. The mean concentration of Cit in red blood cells (RBC) was reported as approximately 31±13 μM [10]. Cit is the only precursor of Arg de novo synthesis in the body; the conversion of Cit into Arg occurs in the kidneys, endothelial, and immune cells, as exemplified by activated macrophages [12]. Several studies indicate that Cit raises plasma Arg concentrations and effectively promotes whole-body NO production [3, 12, 13]. Cit augments NO-dependent signaling pathways and exerts beneficial effects in pathologic conditions relating to reductions in NO availability, such as cardiovascular disease and hypertension [3, 12, 13].

Cit appears much more effective than Arg for NO production [14]. Furthermore, oral doses of Arg impose some negative effects, including induction of arginase activity [15, 16] and urea production [17], inhibition of cellular uptake of Cit [18] and recycling of Arg form Cit [16], suppression of endothelial NO synthase (eNOS) expression and activity [19], and induction of cellar oxidative stress [19]. On the other hand, there is evidence indicating the existence of cellular "Arg tolerance" with long-term Arg exposure, a phenomenon that reduces the expected beneficial effects of long-term Arg supplementation [19]. For such reasons, Cit has recently received more attention than Arg as a NO-boosting supplement.

This chapter provides an overview of Cit metabolism in the body and its role in whole-body NO production. Then we discuss the efficacy and safety of oral doses of Cit in type 2 diabetes (T2D), a pathological condition of NO-disrupted metabolism.

CIT BIOSYNTHESIS PATHWAYS

Cit biosynthesis is a complex process and involves multiple precursors, enzymes and intracellular, and interorgan substrates transfer. Cit biosynthesis occurs in both the intestine and intra-intestine organs; intestinal biosynthesis is estimated to be responsible for 60-90% of whole plasma Cit flux, whereas extra-intestinal biosynthesis provides a fraction of Cit flux estimated to be as low as 10-30% [20].

Intestinal Biosynthesis of Cit

The intestine is the principal site of Cit biosynthesis, and it is the organ that supplies the entire body with Cit [20]. Enteral or plasma amino acids such as Arg, glutamine, glutamate, and proline are responsible for supplying the ornithine used for Cit biosynthesis in the enterocytes [21, 22]. The enzymes involved in the synthesis of ornithine are glutaminase, pyrroline-5-carboxylate synthase, proline oxidase, arginase, and ornithine aminotransferase (OAT), the latter being the key enzyme [21]. The ornithine is finally converted into the Cit by the action of ornithine transcarbamylase (OTC), one of the key enzymes in the urea cycle, which in humans is mainly present in the liver and intestinal mucosa [9]. The presence of the urea cycle enzymes, which are carbamoyl phosphate synthetase I (CPS-I), ornithine decarboxylase (ODC), arginase, argininosuccinate synthase (ASS), and argininosuccinate lyase (ASL) in the enterocytes, provides a biochemical basis for synthesizing Cit and Arg from glutamine [23, 24].

The kinetic properties of the intestinal urea cycle enzymes, optimizing the net synthesis of Cit from glutamine, can explain the large amounts of Cit that are released by the intestine. First, compared to the liver, the intestinal urea cycle enzymes are likely to synthesize Cit rather than urea; second, co-localization of CPS-I and OCT, along with a high activity of OCT in the mitochondria, enables the enterocytes to synthesize Cit from ammonia, HCO_3^- and ornithine; and third, low activities of the cytosolic ASS and ASL minimize the Cit-Arg conversion and the recycling of Cit into ornithine *via* arginase in the enterocytes [25].

The extent of contribution of each of the amino acids glutamine, Arg, and proline to the intestinal biosynthesis of Cit is under debate. Initial investigations suggested glutamine as an important precursor (60-80%) for plasma Cit in humans [26, 27]. In healthy adults in the fed state, more than 70% of the ^{15}N label glutamine was found on the α-nitrogen of Cit [5]. About 13-26% of glutamine taken up by the intestines is converted to Cit [27], and the conversion rate has been estimated to be about 5.1±0.7 μmol/kg/h [27]. A significant association is observed between Cit flux and plasma glutamine concentration (r = 0.78) [5].

CHAPTER 14

Nitrate, Nitrite and Type 2 Diabetes

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Abstract: Recent research punctuates that the nitrate (NO_3)-nitrite (NO_2)-nitric oxide (NO) pathway may be a potential therapeutic target in type 2 diabetes (T2D), a NO-disrupted metabolic disorder. Nutritional aspects of the NO_3-NO_2-NO pathway has been highlighted by focusing on the protective effects of some traditional high-NO_3 diet, such as Mediterranean and DASH (Dietary Approaches to Stop Hypertension) diets and their NO_3-rich components, *i.e.*, fruits, vegetables, legumes, and green leafy vegetables, against the development of T2D. Both acute and long-term administration of inorganic NO_3 and NO_2 in animal experiments display anti-diabetic properties; inorganic NO_3 decreases fasting blood glucose, glycosylated hemoglobin, and proinsulin to insulin ratio and improves glucose tolerance. In contrast to animal experiments, NO_3/NO_2 therapy has failed to show anti-diabetic properties and beneficial effects on glucose and insulin homeostasis in humans. This lost-i--translation remains an open question, and long-term clinical trials are needed to confirm the salutary effects of inorganic NO_3 and NO_2 as the natural NO boosters in patients with T2D.

Keywords: Acceptable Daily Intake, Insulin Resistance, Hypertension, Metabolic Syndrome, Nitric Oxide, Nitric Oxide Synthase, Nitrate, Nitrite, Type 2 Diabetes.

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INTRODUCTION

Inorganic nitrate (NO_3) and nitrite (NO_2) can act as a substrate or backup system for non-enzymatic endogenous generation of nitric oxide (NO), and current data indicate that both NO_3 and NO_2 display NO-like bioactivity [1, 2]. The ability of inorganic NO_3-NO_2 to convert to NO highlights the physiological importance and novel aspects of the pathway in health and disease [3]. Boosting the NO_3-NO_2-NO pathway, where the classic L-arginine-NO pathway is compromised, has therefore become an attractive approach for potential prevention and treatment of cardiometabolic diseases, including hypertension (HTN), metabolic syndrome (MetS), and T2D [4, 5].

Both *in vitro* and *in vivo* animal experiments greatly support the hypothesis that the NO_3-NO_2-NO pathway plays a critical role in regulating glucose and insulin metabolism and insulin signaling [6 - 8]. Supplementation with inorganic NO_3-NO_2 in animal models of T2D resulted in improved hyperglycemia, insulin sensitivity, and glucose tolerance; their supplementation was also accompanied by elevated pancreatic islet insulin content and insulin secretion, improved inflammation, dyslipidemia, liver steatosis, and oxidative stress [9 - 11]. Favorable effects of NO_3 therapy on glucose and insulin homeostasis have been reported to be head-to-head with metformin, the first-line anti-diabetic agent [12]. In animal experiments, NO_3 had equipotent anti-diabetic effects compared to metformin, while it was superior to protecting against diabetes-induced cardiovascular dysfunction and liver steatosis [12].

Although the beneficial effects of inorganic NO_3-NO_2 supplementation have received increasing appreciation in animal experiments, their efficacy on glucose and insulin homeostasis has remained unproven in humans. Supplementation with inorganic NO_3 and NO_2 (as KNO_3, $NaNO_3$, $NaNO_2$, or NO_3-rich food products) in acute [13], mid-term [14, 15], and long-term [16 - 18] experiments, have failed to show beneficial effects on glucose and insulin parameters including fasting and postprandial plasma glucose and insulin, insulin resistance indices, and HbA1c levels in patients with T2D.

This chapter reviews available data, animal experiments and human clinical trials, addressing the potential effects of inorganic NO_3/NO_2 on glucose and insulin homeostasis in T2D. We also try to provide some plausible reasons to address the challenge of lost-in-translation of beneficial effects of inorganic NO_3 and NO_2 from bench-to-bedside.

INORGANIC NITRATE AND NITRITE: A NUTRITIONAL PERSPECTIVE

Due to the historical background about potential hazardous effects of inorganic NO_3 and NO_2 [19, 20], strict regulations and limitations have been legislated for exogenous exposure of NO_3-NO_2 from both dietary intake and drinking water [21]. In 1975, US Environmental Protection Agency (EPA) established the maximum contaminant level for NO_3 at 10 mg/L, and in 1993, the world health organization (WHO) established the maximum concentration limit as 11.3 mg/L nitrate-nitrogen in drinking water; the permissible NO_3 level in drinking water is now 50 mg/L in the European Union and 44 mg/L in the US [22]. The acceptable daily intake (ADI) values are 3.7 and 0.06 mg/kg body weight for NO_3 and NO_2, respectively [22]. The acute oral LD_{50} values of sodium nitrate ($NaNO_3$) in rats, mice, and rabbits were reported in a range of 2480-9000 mg/kg; for sodium nitrite ($NaNO_2$), these values range 180-214 mg/kg in rats, rabbits, and mice [23].

Food and Dietary Sources of Inorganic NO_3 and NO_2

The main dietary sources of inorganic NO_3 are vegetables, especially green leafy vegetables, beetroot, and celery [24]. The NO_3 levels of vegetables are commonly categorized as low (<100 mg/kg), medium (100-1000 mg/kg), and high-NO_3 (>1000 mg/kg) [25, 26]. Celery, mint, lettuce, tarragon, beetroot, and radish are considered very-high NO_3-containing vegetables, while scallion, cauliflower, zucchini, eggplant, parsley, leek, dill, spinach, cabbage, turnip, cress, and basil are classified as high NO_3-containing vegetables [27]. Other vegetables, including carrots, onions, mushrooms, fenugreek, cucumber, and green pea, contain a medium level of NO_3, whereas corn, garlic, potato, green beans, and tomato are categorized as low- and very low-NO_3 vegetables [27]. A recent systematic search reviewed 255 published documents (1980-2016) on NO_3 content of vegetables database, reported that NO_3 content of vegetables ranged from Chinese flat cabbage (median=4240, range= 3004-6310 mg/kg of fresh weight) to corn (median=12; 5-1091 mg/kg of fresh weight) [24].

The NO_2 content of vegetables has been reported in a range of less than 1 mg/kg, however in some Asian countries (*e.g.*, Iran, Japan, Turkey, and South Korea), NO_2 contents of vegetables were reported to be in a wide range of 1.7-7.4 mg/kg [27 - 30]. European countries usually report a lower range of NO_2 content in vegetables to be 0.1-2.5 mg/kg [30]; however, some countries, *e.g.*, Denmark and Germany, reported a maximum level of 11.0 and 19.6 mg/kg, respectively [30]. In some reports, NO_2 concentrations ranged between 1.1-54 mg/kg [31]; a wide range of NO_2 levels were reported in vegetables especially in lettuce (2.9-8.8 mg/kg), spinach (3.8-12.7 mg/kg), and radish (2.4-14.2 mg/kg) [32]. Several

CHAPTER 15

Potential Applications of Nitric Oxide Donors in Type 2 Diabetes

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Abstract: Nitric oxide (NO) donors are chemical agents that produce NO-related activity in biological systems, mimic endogenous NO-related responses, or compensate for NO deficiency. NO donors have been increasingly studied as promising therapeutic agents for insulin resistance and type 2 diabetes (T2D). Here, we provide evidence, which investigated the effects of the most frequently studied and implemented NO-releasing compounds, including sodium nitroprusside (SNP), *S*-nitrosothiols [RSNOs, *i.e*., S-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetyl-penicillamine, (SNAP)], and N-Diazeniumdiolates (NONOates, *i.e*., spermine NONOate, diethylamine NONOate) on glucose and insulin homeostasis. Available evidence could not draw a clear conclusion regarding therapeutic applications of NO donors in T2D due to different methodological approaches (*i.e*., *in vitro vs*. *in vivo*) and different doses and formulations used to assess the potential effects of NO donors on carbohydrate metabolism. Considering key properties and different kinetic behaviors between various classes of NO donors, targeted compound selection, defining optimum doses, and appropriate use of NO-releasing platforms (topical *vs*. systemic delivery mode) seem to be critical issues that can accelerate the bench-to-beside translation of NO donors in T2D.

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Keywords: Diethylamine NONOate, Insulin resistance, *N*-Diazeniumdiolates, Nitric oxide, Nitric oxide donors, Nitroprusside, Sodium nitroprusside, *S*-nitrosoglutathione, *S*-nitrosothiols, *S*-nitroso-*N*-acetyl-penicillamine, Spermine NONOate, Type 2 diabetes.

INTRODUCTION

Impaired nitric oxide (NO) homeostasis and function are associated with the development of insulin resistance and type 2 diabetes (T2D) [1]. As discussed in Chapter **3**, reduced NO production [due to impaired *L*-arginine-NO synthase (NOS) pathway [2] and the nitrate (NO_3)-nitrite (NO_2)-NO pathway [3, 4], inducible NOS (iNOS)-induced overproduction of NO [5], impaired NO transport within the circulation and its delivery to target cells, reduced NO availability [*e.g.*, *via* oxidative-induced NO quenching [6, 7], and impaired NO signaling pathway [*i.e.*, NO-soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway [8, 9] contribute to impaired NO bioactivity in hyperglycemia and insulin resistance.

To overcome the impaired NO homeostasis and increase NO bioactivity, several therapeutic approaches, including supporting NO precursors for both NOS and NO_3-NO_2-NO pathways (see Chapters **12, 13, 14**), providing co-factors of cNOS (*e.g.*, tetrahydrobiopterin, BH_4) [10], targeted-inhibition of iNOS [11, 12], using sGC activators [13, 14] (ongoing clinical trials: NCT03091920 and NCT02906579), restoring NO bioavailability and its function by antioxidants [15, 16] have been evaluated in NO-disrupted situations like T2D [17 - 19]. Using NO donors, *i.e.*, chemical substances that generate NO *via* NOS-independent mechanisms [20], has been increasingly studied as a promising NO-based therapeutic approach in T2D.

As a brief history, the first NO donor, glyceryl trinitrate (GTN), has been clinically used for 150 years for acute angina pectoris [21]. However, it was first recognized as a NO donor in the 1980s [21]. Glyceryl trinitrate was synthesized by an Italian chemist, Ascanio Sobrero, in 1847; however, its *in vivo* mechanism of action remained unknown until the discovery of NO as a naturally occurring messenger molecule in the 1980s [22]. The second most-known NO donor, sodium nitroprusside (SNP), was initially discovered in 1849 by Playfair [23] and was initially used as an anti-hypertensive agent by Johnson in 1922 [24]. In 1979, it was revealed that SNP acts *via* NO in the bovine coronary artery, where NO activates guanylate cyclase, resulting in vascular relaxation through cyclic guanosine monophosphate (cGMP) production [25].

During the past two decades, the development of influential NO donors and advanced NO-releasing materials for clinical therapies has been a significant

research interest [26 - 31]. Several synthetic compounds have been developed to chemically stabilize and release NO, in a controlled manner [27, 29, 32]. As exposure time with NO and its kinetic behavior critically affect NO biological applications, researchers have tried to develop various NO donors, mimicking continuous NO production in a wide range of time intervals from seconds to days [27, 33]. On the other hand, despite the simplicity of the chemical nature, exogenous applications of NO are challenging due to the complex reactions in which NO is involved, tissue specificity, and concentration-dependence behaviors of the molecule [34].

This chapter gives a brief overview of NO donors and provides evidence that investigated the effects of the most frequently studied and implemented NO-releasing compounds (*i.e*., SNP and S-nitrosothiols) on carbohydrate metabolism.

A BRIEF OVERVIEW OF NITRIC OXIDE DONORS

Nitric oxide donors are a heterogeneous group of compounds able to release NO-related species, including NO radical, the nitrosonium ion (NO^+), or the nitroxyl anion (NO^-), *in vitro* or *in vivo*, independently of its endogenous sources [35]. However, there is a substantial difference between the compounds as to which NO-related species (*i.e*., NO^+, NO radical, or NO^-) they release in biological systems (See Box. **1**); the reactivity towards other biomolecules, the produced by-products, and the response are affected by different NO-related species released by donor compounds [36]. No clear-cut definition has been provided for NO donors; some definitions have restricted NO donors to those generating NO through mechanisms that are independent of the enzymatic action of NOSs [20] and endogenous sources [35], while others discussed that the term NO donor implies on the compound releases the active NO [36], thus included *L*-arginine and inorganic NO_3 and NO_2 as NO donors [34, 37].

Nitric oxide donors have been classified according to different points of view, including their chemical structures of NO-releasing moieties, the form of NO released by the compound (*e.g*., NO donors *vs.* HNO donors), the mode of actions (*i.e*., spontaneously released or those required metabolic activations), and their typical working mechanisms [35, 38 - 40]; furthermore, others classified NO donors as natural *vs.* synthetic donors [34], exogenous *vs.* endogenous stimuli sensitive donors [28], and short- *vs.* long-acting NO donors [41]. The American Chemical Society comprehensively reviewed nomenclatures and major classes of NO donors in 2002 [20]. Table **1** provides a brief overview of the most common NO donors [20, 30, 31, 42 - 49]. The most frequently employed NO donors in basic and clinical studies are organic nitrates (*e.g*., glyceryl trinitrate, isosorbide dinitrate), SNP [$Na_2(FeCN)_5NO$)], sydnonimines [*e.g*., molsidomine, morpholino-

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