INHIBITION OF PROTEIN GLYCATION BY POLYPHENOLIC EXTRACTS OF CULINARY HERBS AND SPICES

by

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(Under the Direction of James L. Hargrove, Ph.D.)

ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by a deficiency and/or absence of insulin that is associated with chronic pathology including retinopathy, nephropathy, neuropathy and atherosclerosis. These diabetic complications are caused in part by the build-up of advanced glycation endproducts (AGEs) that are formed non-enzymatically between reducing sugars and proteins. These AGEs elicit a pro-inflammatory response overwhelming the body's natural antioxidant defense and creating oxidative stress. There is growing interest in polyphenolic compounds due to their antioxidant and anti-inflammatory capacities. Potential health benefits of herbs and spices may derive from their high concentrations of phytochemicals including polyphenolics and other bioactive compounds. In this study, the total phenolic content and antioxidant activity of herb and spice extracts were determined and their abilities to inhibit albumin glycation in vitro was examined.

INDEX WORDS: Diabetes Mellitus, Advanced Glycation Endproducts (AGE), Polyphenolics, Antioxidant, Herbs and Spices

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August 2007

DEDICATION

This work is dedicated to those who are passionately interested in the mysterious link between food and human health.

ACKNOWLEDGEMENTS

Throughout the preparation of this work I have realized that we have yet to scratch the surface of understanding when we look at the consequence of diet on human health. This is not a new theory. However, when it comes to collecting data and drawing specific conclusions on this broad topic there are numerous roadblocks. Anyone who has performed research in this area is aware of these limitations related to in vitro and animal models, physiological relevance of the findings and an overall lack of understanding regarding the complexity of natural chemical constituents that may affect human health. I could not have risen above these obstacles without the assistance and guidance from my graduate committee members, Dr. James Hargrove, Dr. Diane Hartle and Dr. Ruthann Swanson. I would like to thank them for all they have contributed towards the completion of this project. I would also like to recognize Dr. Phillip Greenspan for his prior work on protein glycation and for his contribution to the present manuscript. Thank you to my family and friends for your support. This work was made possible by the UGA departments of Foods and Nutrition and the Nutraceutical Research Labs of the Department of Pharmaceutical and Biomedical Sciences and I would again like to thank all of those involved for allowing me this opportunity.

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CHAPTER ONE

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by a deficiency or resistance to insulin. Diabetes affects an estimated 20.8 million people in the United States, which is 7% of the US population (1). Many chronic pathologies such as retinopathy, nephropathy, neuropathy and atherosclerosis are associated with diabetes. As a result, diabetic patients generally have a life expectancy two-thirds that of a healthy adult (2). Hyperglycemia and oxidative stress are suspected of leading to the development of these diabetic complications in part due to glycated proteins and the build up of advanced glycation endproducts (AGE) (3).

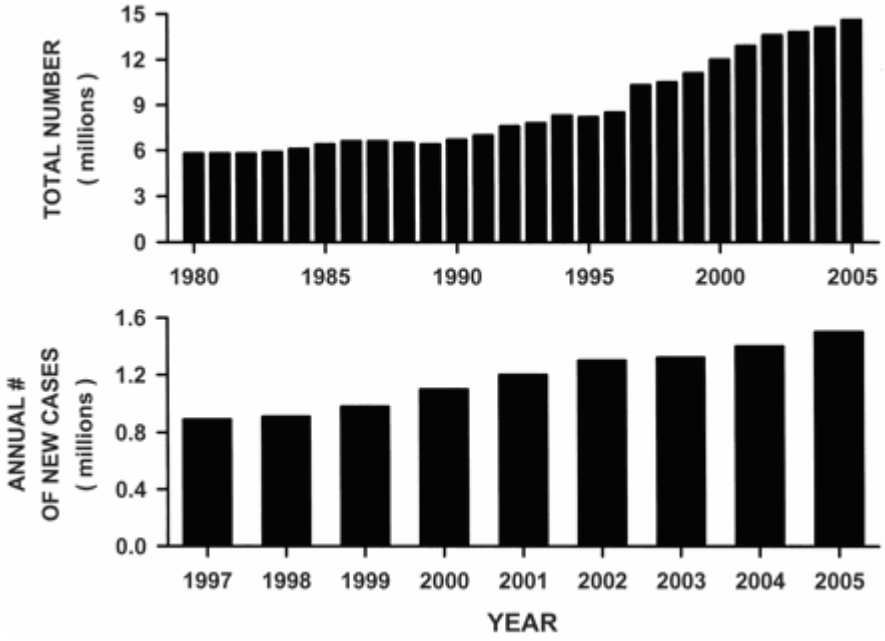

Figure 1. Diagnosed cases of diabetes in the United States. Copyright © 2006 American Diabetes Association From Diabetes Care®, Vol. 29, 2006; 2158-2164 Reprinted with permission from *The American Diabetes Association* (4).

Inhibition of AGEs is a tactical approach in the treatment of diabetic complications because glycation is involved in many pathological processes. Several potential pharmacological AGE inhibitors have been tested in human or animal models. Inhibitors of glycation include aminoguanidine, OPB-9195, ALT 711, N-phenacyl thiazolinium, pyridoxamine (provitamin B6) and a soluble receptor of advanced glycation end-products (sRAGE). Many of these inhibitors are inappropriate for pharmacologic use due to serious adverse effects (2, 5).

The body has a series of mechanisms for dealing with and preventing AGE formation. Protection is afforded by detoxifying liver enzymes, as well as plasma amines and antioxidants. Antioxidants, such as natural plant polyphenolics (or flavonoids), are a promising treatment option as many of these compounds may have reduced toxicity with either dietary or supplemental intake, when compared to standard pharmacological interventions (2). This author does not mean to imply that "natural" products cannot be toxic, merely that phytochemicals are a relatively new possibility of pharmacological treatment that may allow us to explore novel and potentially safer means of treatment for chronic disease.

There is growing interest in polyphenolic compounds due to their antioxidant capacity and potential importance to human health. Studies have addressed phenols in relation to a reduced risk of cancer, cardiovascular disease, neurodegenerative disease, retinopathy, osteoporosis and diabetes (2, 3, 6, 7). The relationship of these disease states with polyphenol consumption has yet to be defined, although numerous mechanisms have been proposed.

Herbs and spices may play a role in disease prevention through their antioxidant abilities and prevention of lipid peroxidation within the body. Herbal remedies have long been used in traditional medicine and numerous bioactive phytochemicals have been reported (8, 9). Common treatments include use of turmeric, green tea and rosemary for reducing inflammation; garlic, turmeric, flaxseed and saffron for cancer prevention and treatment; and garlic, rosemary, sage, thyme, turmeric and saffron for the lowering of cholesterol. Many of these herbs and spices are used in the development of pharmacological treatments of these diseases. The health benefits of herbs and spices are partially attributable to polyphenolic compounds (8, 10).

To date, few studies have reported the effects of plant polyphenolics on protein glycation. Kim and Kim (2003) tested 25 plant extracts for their antioxidant potentials, total polyphenolic content and glycation inhibitory activity. They found 22 of their samples inhibited glycation including Allium cepa (onion skin), Illicium religiosum bark (Star Anise) and Fagopyrum esculentum hull (buckwheat) (11). Lunceford and Gugliucci (2005) analyzed the mechanism by which AGE formation was inhibited by extracts of Ilex paraguariensis, a shrub grown in South America known as “mate”. Using tyrptophan fluorescence and sodium dodecyl sulfate polyarylamide gel (SDS-PAGE) electrophoresis they concluded that the inhibition of AGE formation by Ilex paraguariensis extract was due to the quenching of free radical production from the AGE related Amadori products (12). Another study showed that inhibition of AGE formation can be due to the antioxidant activity of the inhibitors or by a carbonyl blocking mechanism which prevents the formation of Amadori products (13). These studies become significant when addressing the proposed mechanism of inhibition within the present study.

In summary, studies have shown that many herbs and spices have high polyphenol content and antioxidant activities. Few studies have tested whether plant extracts can inhibit protein glycation and whether this inhibition may be related to the antioxidant abilities of those extracts. In the present study, the relationship between the high polyphenolic content in herb and spice extracts and the inhibition of glycation are examined. These herbs and spices might be considered for therapeutic use in the treatment or prevention of diabetic complications caused by AGE formation. The hypothesis of this study is that extracts of herbs and spices with high polyphenol content and antioxidant activity inhibit albumin glycation in vitro. A specific aim of this study was to analyze the differences between herb and spice extracts regarding albumin glycation inhibition due to total phenolic and antioxidant measures. A 50% ethanolic extraction was determined to maximize phenolic extraction in previous studies by our laboratory. 50% ethanolic extractions of culinary herbs and spices were utilized in this study.

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CHAPTER TWO

REVIEW OF LITERATURE

The following chapter will briefly review diabetic pathology and its significance to the present study. Related to this, the chemistry of glycation, the role of advanced glycation endproducts (AGEs) and means of glycation inhibition will also be discussed. Finally, the importance of polyphenolics in human health will be reviewed along with the not so novel idea of herbs and spices as medicinal agents. The information discussed here will serve as a background to demonstrate the significance of the present study of the inhibition of protein glycation with herb and spice polyphenolics.

DIABETIC PATHOLOGY AND CLASSIFICATION

Diabetes mellitus is a metabolic disorder characterized by a deficiency or resistance to insulin and is marked by chronic hyperglycemia. The two main classifications of diabetes are type 1 and type 2, but other less common classifications have been made.

Type 1 diabetes is an autoimmune disease that develops when the body attacks the insulin producing beta cells of the islets of Langerhans in the pancreas. Insulin is required for glucose uptake in muscle, hepatocytes and adipocytes in the human body. Other roles of insulin include stimulating the synthesis of fatty acids, stimulating the uptake of triglycerides into adipose tissue, inhibiting lipolysis and stimulating the production of proteins. These and other roles of insulin can be viewed in **Figure 2**

relating to the propagation of metabolic dysfunction. Type 1 diabetes accounts for 5-10% of all diagnosed cases (1). There are numerous factors that may spark this autoimmunity including genetic predisposition and environmental exposures. The rate of beta cell destruction varies, usually occurring rapidly in children and more slowly in adults (2). The lack of insulin in those with type 1 confers an inability to use glucose as an energy source. Lipolysis provides free fatty acids as an alternative energy source and results in some of the key characteristics used to distinguish type 1 from type 2 diabetes mellitus including a lean body type and a susceptibility to ketoacidosis. Those with type 1 diabetes are usually dependent on insulin injections for survival (1, 2).

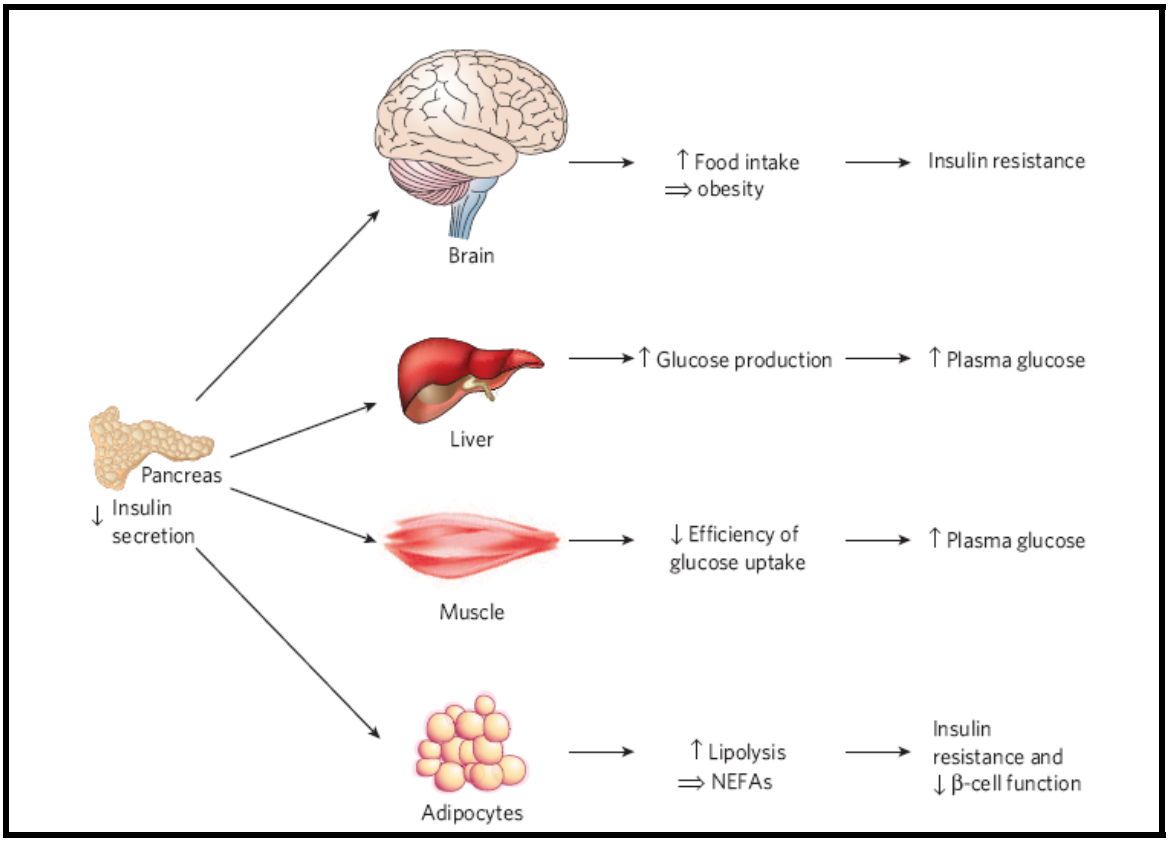


Figure 2. Impact of decreased insulin secretion on the body. Abbreviation: NEFA, non-esterified fatty acids. Copyright © 2006 Nature, Vol 444, 2006;840-846 Reprinted by permission from *Macmillan Publishers Ltd* (3).

Type 2 diabetes begins with an insulin deficiency or resistance to insulin action in peripheral tissues. Subsequently, hyperinsulinemia occurs due to the pancreas's attempt to compensate for the body's relative lack of insulin. Over time, the pancreas gradually experiences beta cell decline and a reduction in insulin output occurs. Fasting and postprandial glucose elevations can be reviewed in **Figure 3**. Type 2 diabetes accounts for 90-95% of all diabetes diagnoses (1). Type 2 diabetes has been associated with "metabolic syndrome", which is characterized by the presence of three out of five cardiac risk factors including abdominal obesity, dyslipidemia and hypertension (4). Additional risk factors for type 2 diabetes include age, obesity, family history, history of gestational diabetes, impaired glucose tolerance, physical inactivity and ethnicity. Glucocorticoid treatments also precipitate type 2 diabetes. The prevalence of type 2 diabetes in children and adolescents is rapidly increasing; however, data regarding the increasing prevalence is lacking (5). Other less common types of diabetes exist due to pregnancy, genetic mutations, surgery, drug/chemical exposure and infections. These cases make up 1-5% of all diagnoses (1, 2).

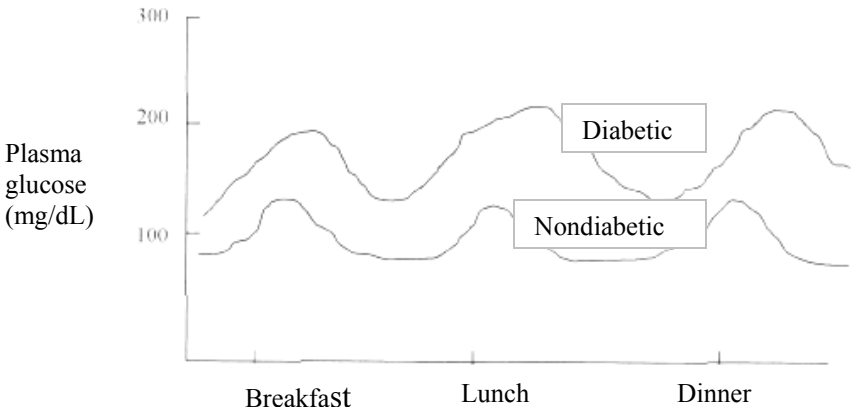


Figure 3. Fasting and postprandial glucose elevations. Adapted from Rizvi, 2004 (5).

Chronic hyperglycemia linked with diabetes leads to both micro- and macro vascular damage and can develop into long term complications such as retinopathy, neuropathy, nephropathy and atherosclerosis (6). It is now believed that the underlying cause of these complications is hyperglycemia and the over production of reactive oxygen species (ROS) by mitochondria. Oxidative stress is clearly associated with diabetes mellitus in a multi-factorial mechanism which drives the development and progression of the complications often experienced by diabetic patients (7). Brownlee et al. (8) has proposed a "unifying mechanism" involving oxidative stress and the development of diabetic complications. Pathways that are associated with hyperglycemic oxidative damage include the polyol pathway, activation of protein kinase C, the hexosamine pathway and buildup of advanced glycation endproducts (AGEs). Each of these pathways drives the others forward creating a cascade of metabolic dysfunction. While the focus of this study lies mainly with AGEs, there is an unavoidable overlap of AGEs and the above mentioned sources of oxidative stress (8). It is logical to assume there may be a benefit seen with antioxidant therapy in preventing/lessening diabetic complications related to oxidative stress.

CHEMISTRY OF GLYCATION

Protein glycation is an indicator of poor control of blood glucose over a period of weeks. Clinically, glucose control is monitored through measurement of glycated hemoglobin A1c in red blood cells and fructosamine in blood plasma. Both of these are advanced glycation endproducts (AGEs) that are formed when an amino group in a protein attaches to the carbonyl group of a non-cyclical reducing sugar forming a Schiff

base. The Schiff base rearranges into a more stable and irreversible Amadori product. These glycated proteins may undergo further reaction with dicarbonyls, such as 3-deoxyglucosone or methylglyoxal, to form AGEs. The autoxidation of glucose and Amadori products can also occur in the presence of transition metals and can participate in AGE formation. Glycation may involve amino groups of nucleic acids, such as adenine and guanine, and phospholipids, such as phosphatidylserine and phosphytidylethanolamine. The rate of production of AGE depends on the duration of hyperglycemia and the half-life of the protein, as the formation of stable reaction products takes several days to occur (6, 9, 10). Dietary AGEs can also significantly contribute to serum AGE levels (11). The formation as well as chemical structures of well known AGEs may be reviewed in **Figure 4**.

Common AGEs include carboxymethyl lysine (CML) protein adducts, carboxyethyl-lysine (CEL) protein adducts, pentosidine-adducts, pyrallines and crosslines (12). In addition, N-terminal amino acids in proteins may be glycated, as occurs in hemoglobin A1c. These individual AGEs may have specific roles in the development of diabetic complications. AGEs may directly impact extracellular proteins by impairing structural stability of the tissue, crosslinking, inducing conformational changes of proteins or by preventing cell communication. More recently, it has been discovered that AGEs may also form on intracellular proteins such as growth factors via intracellular sugars such as fructose, glyceraldehyde-3-phosphate or glucose-6-phosphate (9). The best known example is intracellular formation of glycated hemoglobin within the red blood cell, which requires uptake of glucose via a specific hexose transporter in erythrocyte membranes. There are also receptor mediated responses to glycated proteins.

AGEs bind to numerous receptors in vivo which can influence cells in many ways. These receptors include macrophage scavenger receptor (MSR) type II, galectin-3 and receptor of advanced glycation endproducts (RAGE). RAGE is a multi-ligand receptor that specifically activates an immune response causing inflammation and the activation of specific growth factors and nuclear factor (NF)-kB (12). NF-kB increases the production of cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF)-α and adhesion molecules (6) and has been linked with several inflammatory diseases, including cancer, atherosclerosis, osteoporosis, arthritis and diabetes (13). An increase in the presence of AGEs will increase gene expression of RAGEs leading to an exacerbated state of chronic inflammation within the body (9).

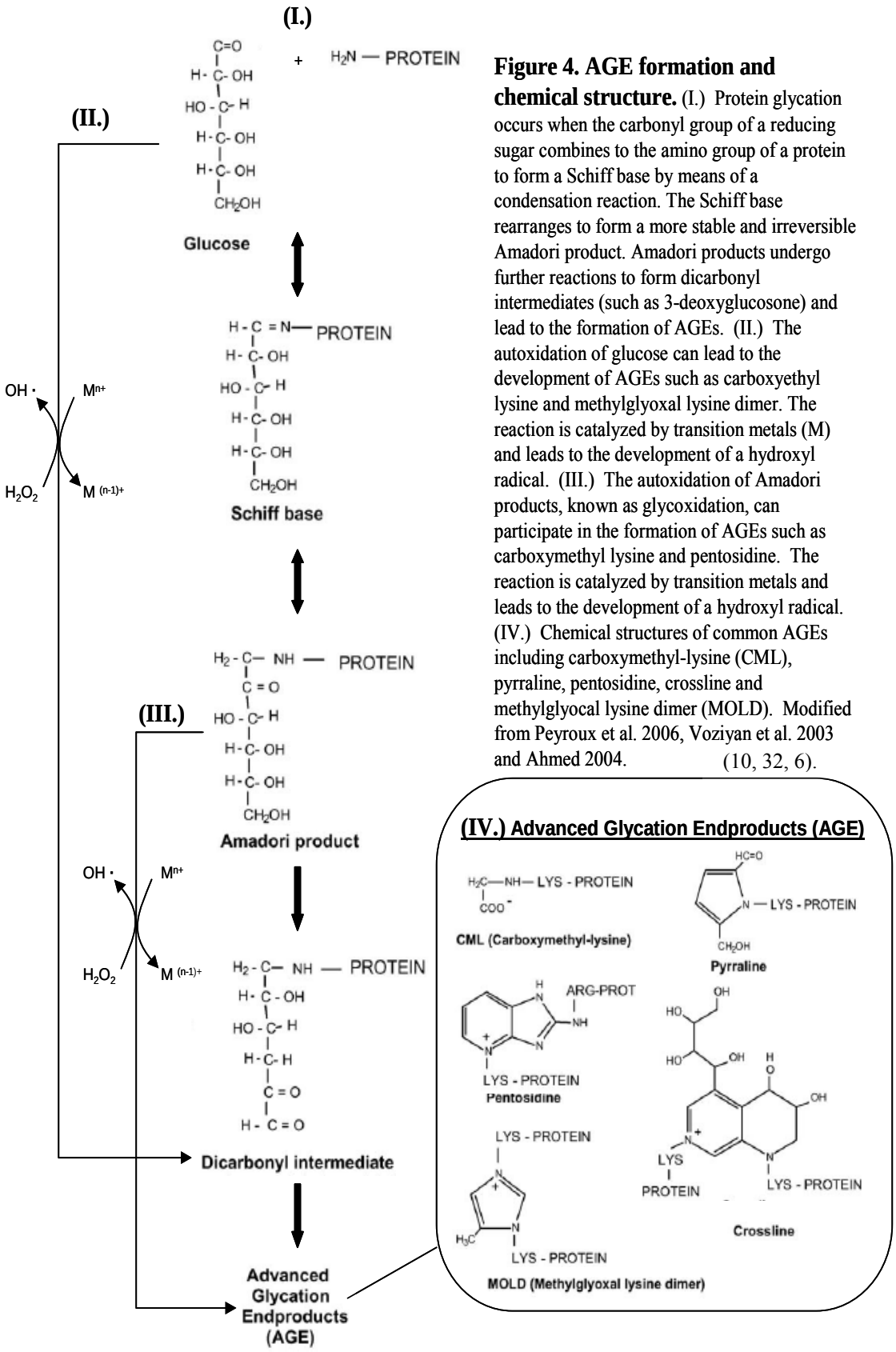


Figure 4. AGE formation and chemical structure. (I.) Protein glycation occurs when the carbonyl group of a reducing sugar combines to the amino group of a protein to form a Schiff base by means of a condensation reaction. The Schiff base rearranges to form a more stable and irreversible Amadori product. Amadori products undergo further reactions to form dicarbonyl intermediates (such as 3-deoxyglucosone) and lead to the formation of AGEs. (II.) The autoxidation of glucose can lead to the development of AGEs such as carboxyethyl lysine and methylglyoxal lysine dimer. The reaction is catalyzed by transition metals (M) and leads to the development of a hydroxyl radical. (III.) The autoxidation of Amadori products, known as glycoxidation, can participate in the formation of AGEs such as carboxymethyl lysine and pentosidine. The reaction is catalyzed by transition metals and leads to the development of a hydroxyl radical. (IV.) Chemical structures of common AGEs including carboxymethyl-lysine (CML), pyrraline, pentosidine, crossline and methylglyocal lysine dimer (MOLD). Modified from Peyroux et al. 2006, Voziyan et al. 2003 and Ahmed 2004. (10, 32, 6).

ROLE OF AGEs IN DIBETIC PATHOLOGY

The pro-inflammatory effects of AGEs are thought to be directly involved in the development of micro and macro vascular diabetic complications including retinopathy, nephropathy, neuropathy and atherosclerosis.

Retinopathy

Diabetic retinopathy is one of the most common diabetic complications (14) and is the most common cause of blindness (6). Many specialized cells of the eye are unable to regenerate, making them susceptible to AGE-induced impairment or necrosis (14). A study by Pachydaki et al. (15) found that the vitreous levels of AGEs and soluble receptor of advanced glycation endproducts (sRAGE) were significantly increased in proliferative diabetic retinopathic human eyes when compared to a control. AGEs and RAGE were also present in epiretinal membranes. Their presence may be linked to the increased proliferation of blood vessels, vascular occlusion, thickening of capillary basement membranes and increased permeability of retinal endothelial cells that characterize diabetic retinopathy (6). AGE stimulated NF-kB affects the expression of specific genes that increase free radicals and lead to apoptosis of pericytes and endothelial cells. AGEs were found to increase intracellular adhesion molecule-1 (ICAM-1) on retinal endothelial cells, which may lead to capillary occlusion and vascular dysfunction (14). AGEs enhance the gene expression of vascular endothelial cell growth factor (VEGF). VEGF is involved in increased angiogenesis and microvascular permeability. Yatoh et al. (16) found that the accumulation of AGEs on retinal microvascular cells leads to increased apoptosis of these cells in Goto-Kakizaki (GK) diabetic rats. They also observed that

antioxidants and an inhibitor of AGEs, known as OPB-919, inhibited retinal cell death. Diabetic cataract is another complication caused by chronic hyperglycemia. Lens crystalline glycation and polyol accumulation are thought to be the cause (17).

Neuropathy

Diabetic neuropathy is characterized by impaired nerve conduction and damaged neural microvasculature. The symptoms of diabetic nerve damage are often numbness of the limbs, pain and/or sexual dysfunction. Although the direct role of AGEs is unclear, it is thought that glycated myelin on nerve fibers are taken up by macrophages leading to demyelination (6). Other consequences of glycation may include axonal atrophy and abnormal Schwann cells (18). Studies have demonstrated a significant accumulation of AGEs on the peripheral nerve of diabetic humans compared to controls, and these results are correlated with a reduction in myelinated fibers (19) and severity of structural changes of nerve components (20). Cellek et al. (21) found that AGEs induced nitric oxide (NO) dependent apoptosis in human neuroblastoma cells, likely due to an increase in oxidative stress. Aminoguanidine, a known inhibitor of AGEs, has been found to improve nerve transmissions, prevent demyelination and axonal atrophy, and improve neural blood flow. Other AGE inhibitors such as pyridoxamine have shown similar results (18).

Nephropathy

AGEs may play a role in diabetic nephropathy by accumulating on collagen within the basement membrane of the renal tubules. This impairs glomerular filtration

and may cause compression of the blood vessels. Circulating AGEs are not readily filtered from the blood with the development of diabetic nephropathy, and may promote the rapid development of atherosclerotic plaques in patients with diabetes via AGE buildup on vessel walls (6). Urinary excretion of AGE was found to increase with the advancement of proteinuria experienced in later stages by diabetic renal patients (22). Mao et al. (23) found carboxymethyl lysine (CML) in the mesangial layer of diabetic nephropathy patients. Pentosidine was found in the glomerular basement membranes and this was significantly correlated with glomerulosclerosis. Pyrraline was present in tubular basement membranes and was correlated with immune cell perfusion. These results indicate the possible role of specific AGEs in tissues of the kidney including proliferation of mesangial cells and changes in interstitial cells. In diabetic apoE knockout mice, AGE deposits were found in the glomeruli and tubulointerstitium of the kidneys. There was also increased expression of RAGE, cytokines and growth factors such as transforming growth factor-ß (TGF-ß1), connective tissue growth factor (CTGF) and platelet derived growth factor (PDGF). Inhibitors of AGE, aminoguanidine and ALT-711 improved renal functions (24).

Atherosclerosis

Diabetic atherosclerosis is arguably the most common diabetic complication as it plays an etiological role for all of the complications described above. Atherosclerosis is characterized by the accumulation of plaque in arterial walls. This is propagated by hyperglycemia and the glycation and oxidation of low-density lipoprotein (LDL). The LDL receptor does not recognize glycated LDL and this complex is unable to be taken

into cells, resulting in hyperlipidemia. These LDL complexes are instead taken up by macrophages, which then accumulate as foam cells, forming atherosclerotic plaques. The glycation of high-density lipoprotein (HDL) reduces cholesterol clearance from peripheral tissues. AGEs also promote the production of cytokines, such as insulin-like growth factor-I (IGF-I) and PDGF, which stimulates macrophages and the proliferation of smooth muscle cells within the vessels (6). AGEs also inhibit NO by deactivating or reducing the activity of endothelial NO-synthase (NOS). This results in impaired vasodilation, increased platelet aggregation and increased smooth muscle growth in the vasculature (9). Wang et al. (25) found AGE-RAGE interaction and AGE stimulated growth factors increased vascular smooth muscle cells (VSMC) in diabetic rat sera. AGE and RAGE antibodies inhibited VSMC proliferation. AGE inhibitors aminoguanidine and ALT-711 were found to reduce total plaque area of the thoracic and abdominal aorta and to reduce collagen accumulation in diabetic apoE knockout mice (24).

GLYCATION INHIBITION

Inhibition of protein glycation is a complex process that could occur at any step in the formation of AGEs. The body possesses a series of mechanisms for dealing with and preventing AGE formation. Protection is afforded by detoxifying liver enzymes, as well as plasma amines and antioxidants. The efficacy of these physiological systems is unknown (6). A means of inhibition or protection from AGEs could occur by blocking the free amino group of a protein, blocking the free carbonyl group of a sugar, creating antibodies for Amadori products, chelation of transition metals, antioxidant protection from free radical damage, deglycation enzymes, dissolution of crosslinking or blocking

of RAGEs. There are several known inhibitors of AGEs (6). These inhibitors and their proposed mechanisms are reviewed here; however many of these inhibitors have multiple targets.

Amadori Inhibition

Amidorase enzymes have been explored as a possible deglycating approach. Fructosylamine oxidase was able to deglycate small substrates, yielding glucosone and an amine; however it was ineffective against glycated proteins. Fructosamine 3-kinase is an ATP dependant enzyme that phophorylates Amadori products and inhibits the formation of intracellular AGEs (26). Brown et al. tested the role of amidorase enzymes on tissue specific glycated proteins. They found that extracellular tissues such as skin collagen, which lack the deglycating enzymes and its substrate ATP, had a significant increase in the Amadori product fructolysine in diabetic Sprague-Dawley rats. Red blood cells (RBC) are not afforded any protection via deglycating enzymes, but it is interesting to note that these cells are freely permeable to glucose (27). Other groups have found that fuctosamine 3-kinase inhibited fructolysine in RBC in vitro. Muscle, liver and brain tissues all seemed to have some protection against hyperglycemia and the development of Amadori products. Tissue specific responses to blood glucose levels in diabetic and non-diabetic models can be reviewed in **Figure** 5, as observed by Brown et al. (27). While there are other protective mechanisms in these tissues discussed by Brown et al., it is possible that the decreased fructolysine and glycated protein could be due to the intracellular deglycating enzyme fructosamine 3-kinase (27).

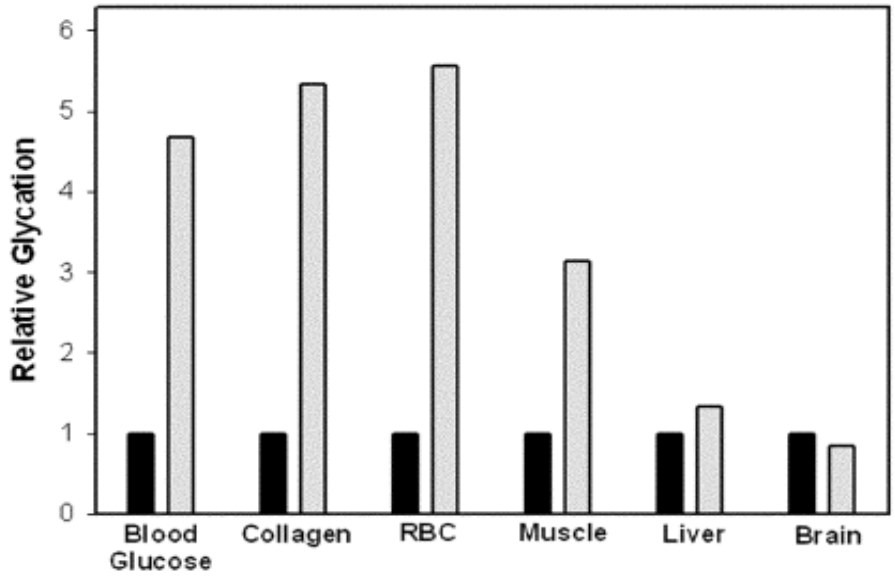


Figure 5. Brown et al.'s ratio of changes in blood glucose and glycation of protein in specific tissues. Copyright © 2005 Ann. N. Y. Sci., Vol 1043, 2005;817-823 Reprinted by permission from *Blackwell Publishing* Ltd. Non-diabetic animals (black bars) are compared to diabetic animals (gray bars) and values are expressed as an increase in glycation in the form of a ratio to the non-diabetic value (27).

Dicarbonyl Scavenging

Dicarbonyls can form via fragmentation of Amadori products or through metabolic pathways. Methylglyoxal can be derived from glycolysis. 3-deoxyglucosone can be produced from fructose shuttled through the sorbitol pathway. These carbonyl compounds tend to react with lysine or arginine residues to form complex AGEs (26).

Aminoguanidine, known as Pimagedine, is one of the first and most studied AGE inhibitors. The primary mechanism of action is thought to involve the scavenging of α,ß-dicarbonyls preventing the formation of AGEs and AGE crosslinking. The product of this reaction is an inhibitor of NOS (28). At high concentrations, aminoguanidine also scavenged other carbonyl compounds including pyruvate. In states of hyperglycemia, excessive pyruvate oxidation in the Krebs cycle may reduce oxidative stress associated

with mitochondrial dysfunction (28). A Phase III randomized, double blind clinical trial has been completed using pimagedine. ACTION I trial evaluated the efficacy of aminoguanidine on improving diabetic nephropathy in 690 type 1 diabetics. ACTION I trial found some improvements in diabetic factors but failed to reach its primary endpoint, the doubling of serum creatinine with deteriorating renal function (29). ACTION II was conducted under the same conditions using 599 type 2 diabetics (30), but was discontinued due to safety concerns (10, 28).

OPB-9195 has a similar chemical structure as aminoguanidine and likewise it has a similar, but more effective mode of action. Clinical trials were halted due adverse side effects (10). Pyridoxamine is a vitamin B-6 derivative investigated in AGE inhibition due to its interaction with α-carbonyls in carbohydrate metabolism (31). Pyridoxamine has been found to be an effective scavenger of reactive dicarbonyls. It may also significantly prevent the oxidation of Amadori products and work as a metal ion chelator. Toxicity studies are promising and clinical trials are underway (32). A relative vitamin B6 deficiency has been observed in experimental diabetic animals (33), which would be consistent with the idea that provitamins react with carbonyls generated in this condition.

Transition Metal Chelation

Many carbonyl trapping inhibitors also possess a powerful chelating ability. As shown in **Figure 4**, the metal-catalyzed autoxidation of reducing sugars and glycoxidation of Amadori products contributes to the formation of AGEs and hydroxyl radicals. Price et al. determined the chelating abilities of several AGE inhibitors including aminoguanidine, pyridoxamine, carnosine, phenazinediamine, OPB-9195,

tenilsetam, N-phenacylthiazolium bromide (PTB) and phenacyldimethylthiazolium bromide (PMTB) via inhibition of copper catalyzed autoxidation of ascorbic acid. While all inhibitors tested were found to have chelating abilities, it was concluded that carnosine, tenilsetam and OPB-9195 inhibit primarily as chelators as opposed to carbonyl trapping mechanisms. AGE crosslink breakers PTB and PMTB were observed to have potent chelating abilities (34). Chelators are not generally used in clinical practice due to the sequestering of vital metal ions used to carry out metabolic processes (6).

Protein Crosslink Cleavage

It is possible to break up AGE crosslinks in vitro. N-phenacyl thiazolinium bromide (PTB) is rapidly hydrolysed in water and the products of this reaction can reduce disulfide crosslinks (35). PTB has been reported to prevent collagen and albumin crosslinks and was found to prevent AGE accumulation in STZ-diabetic rats. PTB's unstable nature led to the development of several analogs including N-phenacyl-4,-dimethylthiazolium chloride (ALT-711) (6, 10), also known as alagebrium (35). Thallas-Bonke et al. found that ALT-711 had several renoprotective effects including a reduction in protein kinase C, reduced expression of vascular endothelial growth factors (VEGF) and a reduced accumulation in AGEs (36). ALT-711 is not projected to cleave common AGE crosslinks such as pentosidine, glucosepane, methylglyoxal lysine dimmer (MOLD) and glyoxal lysine dimmer (GOLD) (35). It has been proposed that ALT-711 may act in ways other than AGE-breakers, however clinical trials are underway and appear to be well tolerated (10, 27).

RAGE Blocking

A RAGE blocking agent could act by blocking of AGE with its receptor or impairing signal transduction between RAGE and its intracellular components. Soluble RAGE (sRAGE) is an extracellular AGE-binding form of RAGE. The binding of sRAGE with AGE interferes with the activation of cellular RAGE and has been associated with reduced cellular dysfunction and vascular injury (37). Park et al. demonstrated the attenuation of atherosclerotic factors in accelerated atherosclerosis apoE deficient mice. Such inhibition of RAGE signaling may reduce oxidative stress that has been associated with diabetes (38). Yonekura et al. found that human vascular cells expressed their own form of naturally occurring soluble RAGE and they named this endogenous secretory RAGE (esRAGE). The presence of esRAGE expressed cytoprotective properties including inhibition of VEGF. It was concluded that individual variations in esRAGE levels may account for resistance or rate of the development of diabetic complications in vivo (39). The inhibition of ligand binding to RAGE has therapeutic potential.

POLYPHENOLS

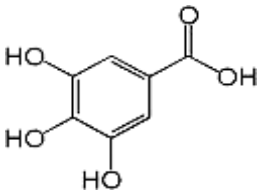
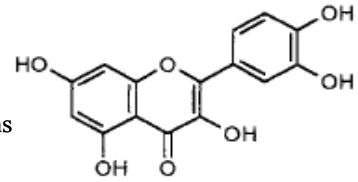
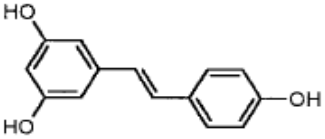
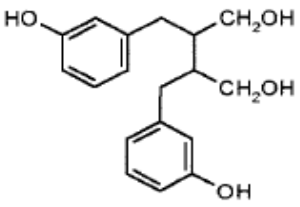
Polyphenols are phytochemicals and are the main sources of antioxidants in the human diet. Consumption in the western diet equates to about 1g/day of dietary polyphenols (40). These include numerous compounds each of which is present in different concentrations based on the plant source as well as geographic region and other growth conditions. They are reducing compounds that work by accepting electrons from free radicals to form stable phenoxyl radicals (41). Phenolic compounds are formed from phenylalanine or shikimic acid and usually occur as glycones (42). These are sugar

residues attached to hydroxyl groups or aromatic carbons on phenols. Polyphenolic compounds also function as metal chelators and enzyme modulators (43). Polyphenols maintain the health of the plant by providing UV protection, limiting photosynthetic stress, protecting against microbes, preventing fungal growth and inhibiting reactive oxygen species (42). The array of biological activities attributed to polyphenolic compounds may be due to the vast differences in chemical structures.

Classification and Metabolism

The main classes of polyphenols are based on chemical structure and include phenolic acids, flavonoids, stilbenes and lignans. Common phenolic acids include caffeic acid found in coffee and ferulic acid found in wheat bran. Phenolic acid derivatives include gallic acid and tannins. Flavonoids contain a set of subclasses which include flavones, flavonols, isoflavones, anthocyanins, flavanols, proanthocyanidins and flavanones. Common phenols found within these groups include catechin, hesperetin, cyanidin, daidzein, and proanthocyanidins. These compounds are found in various food sources such as tea, fruit, onions and chocolate. The most well known stilbene is resveratrol found in wine. Lignans are found in flaxseed though little in known about this class of phenols (44). The structural classification, chemical structure and food sources of common phenols can be reviewed in **Table 1**.

Table 1. Classification of polyphenolic compounds and various food sources.

Class	Polyphenols	Structure	Food Source (44, 41)
Phenolic acids	Caffeic acid Ferulic acid Gallic acid Eallic acid Tannins Coumaric acid Vanillic acid	 gallic acid	Coffee, Blueberries, Raspberries, Strawberries, Wheat bran
Flavonoids	Catechin Hesperetin Cyanidin Daidzein Proanthocyanidins Quercetin Epicatechin Genistein Myricetin Kaempferol	 quercetin	Onions, Tea, Soy, Wine, Citrus fruit, Chocolate, Berries, Apples
Stilbenes	Resveratrol	 resveratrol	Wine, Peanuts
Lignans	Enterodiol Enterolactone Secoisolariciresinol	 enterodiol	Flaxseed, Broccoli, Sesame seeds, Rye, Nuts, Whole grains

The chemical structures of these polyphenolic compounds affect uptake and metabolism of the active metabolites. Polyphenols are found in plants as glycoside conjugates. Glycosylation may occur at numerous positions on polyphenolic compounds,

generally occurring at functional hydroxyl groups (45). Once ingested, polyphenols are deglycosylated by ß-glucosidases in order to passively diffuse across the brush border of the small intestines. Aglycone forms are readily conjugated by sulfation, glucuronidation and/or methylation. Once absorbed into portal circulation, the liver further modifies polyphenol conjugates. After delivery to the peripheral tissues, these compounds are excreted in the urine. Alternatively, conjugated polyphenolic compounds may re-enter the intestinal lumen by reuptake from enterocytes or through bile secretions, reducing absorption. Colonic microflora may hydrolyze these polyphenolic compounds and may assist in greater absorption (44).

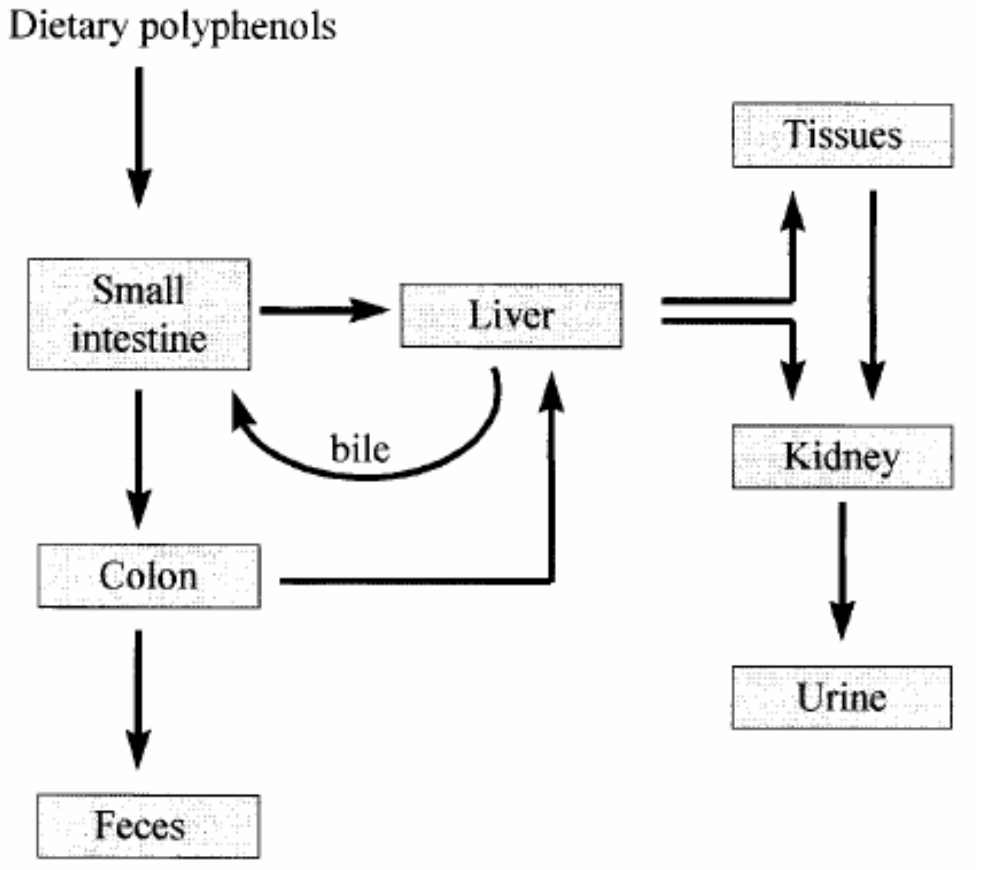


Figure 6. Absorption and excretion of polyphenols in humans. Adapted from Scalbert, 2000 (44).

Bioavailability and Plasma Concentration

Plasma concentrations of unconjugated aglycone forms of phenolic compounds are minimal, with maximum aglycone plasma levels reaching about 1-2 μmol/L (44, 46, 47). Erulund et al. explored the results of three intervention studies involving the bioavailability of the flavonoid quercetin from berries. All three studies showed a significant increase in plasma quercetin after the consumption of a variety of berries for varying lengths of time from 4 hours to 8 weeks, compared to baseline or a control. Plasma concentrations did not exceed 0.2 μmol/L (48). Silberberg et al. looked at the bioavailability of conjugated metabolites of ferulic acid, genistein and hesperetin in a rat *in situ* intestinal perfusion model at doses similar to human intake (15-120 μM). See **figure 7**. Intestinal conjugation of genistein and hesperetin amounted to greater than 95% of mesenteric metabolites of these compounds. Ferulic acid was 40% conjugated in mesenteric circulation, but it is believed further hepatic conjugation increased these metabolites to 75%. Ferulic acid appears to be more bioavailable than genistein or hesperetin. Perfusion of individual compounds simultaneously did not affect results. Data suggested that high polyphenolic intakes may saturate conjugation mechanisms and improve bioavailability (40).

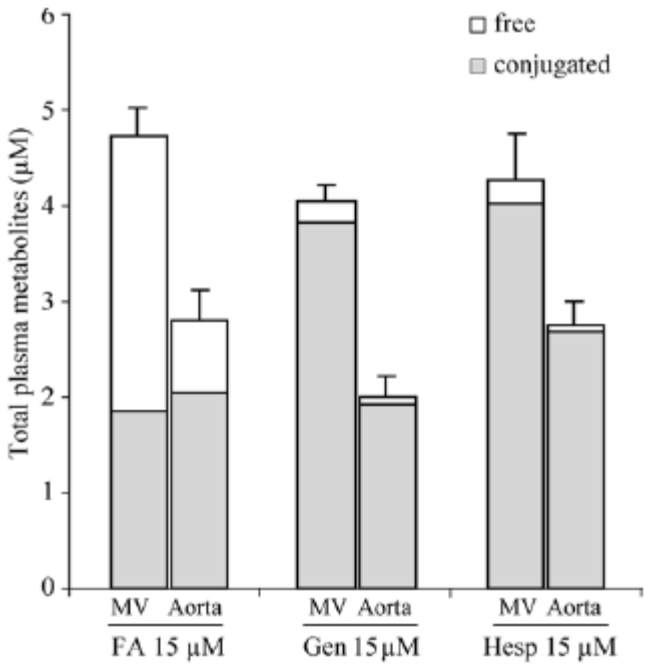


Figure 7. Silberberg et al.'s total plasma metabolites of selected polyphenols. Total plasma metabolites were determined in abdominal aorta and mesenteric vein after the perfusion of 15 µM of ferulic acid, genistein and hesperetin in the small intestine of a rat model. Data are means ± SEM, n = 8 (40).

The potent biological effects of polyphenolic parent compounds have been well studied, however they do not explain the total increase in the antioxidant capacity of plasma when ingested (44). This means the conjugated forms of these parent compounds retain some ability to exert biological influence. Cano et al. studied the superoxide anion scavenging abilities of flavonol conjugates quercetin-3-rhamnoglucoside, quercetin-3-sophoroside, quercetin-3-sulphate, quercetin-3-glucuronide, kaempferol-3-sophoroside and kaempferol-3-glucuronide at concentrations 0.5-10 µM. Several conjugates remained effective scavengers of superoxide anions including sophoroside, glucuronide and rhamnoglucoside conjugates (49). Day et al. performed a similar study of quercetin conjugates and determined that these compounds retained some biological activity including antioxidant potential and the ability to inhibit lipoxygenase and xanthine

oxidase at 0.25 μM. Quercetin aglycone was the most effective inhibitor (50). Both studies emphasize that activity depends on the position of conjugation.

The hypothesis of polyphenol synergism has also been addressed. Pignatelli et al. explored the use of red and white wine polyphenols in inhibiting oxidative stress. The IC50 for individual polyphenols to inhibit LDL oxidation is greater than 1μM, more than typical plasma concentrations. However, Pignatelli et al. showed a mixture of three polyphenols at concentrations less than 1μM significantly inhibited LDL oxidation. Consequently, it is likely that an additive or synergistic effect of polyphenols accounts for biological effects seen at physiologically relevant concentrations (47). Mertens-Talcott et al. came to similar conclusions when they examined the synergistic effects of quercetin and ellagic acid on cellular growth variables and apoptosis of MOLT-4 human leukemia cell lines at concentrations of 5-10μM (51). Seeram et al. examined antiproliferative, apoptotic and antioxidant activities on human oral, colon and prostate tumor cells being treated with punicalagin, ellagic acid and a total pomegranate tannin extract, found in pomegranate juice. Purified polyphenols and whole pomegranate juice were tested at concentrations of 12.5-100 μg/ml. Pomegranate juice, containing all of the tested purified polyphenols, displayed synergistic abilities concerning the experimental parameters (52). These studies suggest consumption of whole plant foods high in polyphenols may have superior bioactivities than individual polyphenols.

Relevance to Human Health

There is growing interest in polyphenolic compounds due to their antioxidant capacity and potential relevance to human health. Numerous studies have addressed

phenols in relation to a reduced risk of cancer (41, 42, 46, 53), cardiovascular disease (41, 42, 54), diabetes (41, 55), nephropathy (43), neurodegenerative disease (41, 56, 57), retinopathy (14), and osteoporosis (41, 58). The relationship of these disease states with polyphenol consumption has yet to be defined, however numerous mechanisms have been proposed. The above mentioned disease states are often associated with an increase in oxidative stress, in which case polyphenolic compounds may serve to scavenge ROS or regenerate endogenous defenses (41). It may also be possible for polyphenolic componds to trap reactive carbonyl species as a mechanism to improve human health (59).

The antioxidant abilities of polyphenols have been well studied using several in vitro assays that involve radical scavenging or the reduction of an oxidant compared to a control. It has been well established that the consumption of polyphenolic compounds increases the antioxidant capacity of plasma (40, 47, 48, 50). Possible consequences of increased antioxidant capacity of plasma may include a reduction in LDL oxidation, protection of endothelial cells, metal ion chelation and inhibition of DNA oxidation. Other non-antioxidant mechanism may exist which involve enzyme modulation, signal transduction modifications, inhibition of cell proliferation and suppression of inflammation factors (41). Various biological functions of polyphenolic compounds include antibiotics, antidiarrheal, antiulcer, hypoglycemic and anti-inflammatory abilities (42). More studies are needed to illustrate the relationship between polyphenolic consumption and protective effects against disease. There may also be a pro-oxidant risk from consumption of high concentrations of polyphenolic compounds (42), which requires further investigation.

HERBS AND SPICES

Generally, herbs are considered to be edible products that are derived from the leaves of plants, whereas spices include the bark, stem, bud, root or seed. Many herbs and spices have been examined for their use as functional foods.

Food Preservation

Herbs and spices serve the purpose of adding flavor to food but also in preventing lipid oxidation, thereby preserving food and extending shelf life. This ability to preserve food quality results from high concentrations of natural antioxidants such as vitamins and polyphenols present in many herbs and spices. Herbs and spices are replacing synthetic antioxidants/food preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which have been reported to have carcinogenic effects in several studies (60). Herbs and spices are also known to have antimicrobial activities due to aromatic and generally hydrophobic nature, which can affect membrane stability (61). Moreno et al. found rosemary extracts to have antimicrobial affects associated with its phenolic composition, namely carnosic acid, carnosol and rosmarinic acid, at minimal inhibitory concentrations of 2-15 μg/ml for gram positive bacteria, 2-60 μg/ml for gram negative bacteria and 4 μg/ml for yeast, using either methanolic or water extractions. These results indicate a potential for the use of rosemary in food preservation (62). Intense sensory characteristics are the limiting factor for the use of herbs and spices as food preservatives; however it has been proposed that their use in combination with low concentrations of synthetic agents may limit their carcinogenic effects (61).

Medicinal Use

Herbal remedies have long been used in traditional medicine and public interest in "natural" remedies is growing. Common treatments include turmeric, green tea and rosemary for reducing inflammation; curry, garlic, turmeric, flaxseed and saffron for cancer prevention and treatment; and garlic, rosemary, sage, thyme, turmeric and saffron for the lowering of cholesterol. Many of these herbs are used in the development of pharmacological treatments of these diseases. It is thought that the health benefits of herbs and spices are the result of the bioactive phytochemicals (63, 64). In the United States, the Food and Drug Administration categorizes most herbs and spices as GRAS, generally recognized as safe. However, the use of some medicinal plants in high concentrations can be toxic or can produce allergic reactions (63, 65).

Herbs and spices may play a role in disease prevention through their antioxidant abilities and prevention of lipid peroxidation within the body. A highly and positively correlated relationship between herb and spice total phenolic content and antioxidant potential has been reported (66), though it is likely that herbs and spices are involved in numerous physiological mechanisms. Several herbs and spices and their phytochemical components have been found to inhibit the activation of NF-kB through various pathways. Among those shown to have an effect are turmeric, cloves, ginger, cumin, rosemary and garlic. Their major polyphenolic compounds are curcumin, eugenol, gingerol, anethol, ursolic acid and diallyl sulifide, respectively (13). Zahid Ashraf et al. found garlic and turmeric to be vasodilators and possess the ability to improve cholesterol profiles in albino Wistar rats. Significant improvements were seen in arterial blood pressure in animals on herbal supplemented diets (67). In a review by Srinivasan, the

anti-diabetic properties of several spices were examined. Fenugreek, garlic, onion, turmeric and cumin are well studied spice extracts, which are thought to have anti-diabetic modes of action via stimulation of beta cells, decreasing glucose absorption and insulin protecting mechanisms (68).

Individual phenolic compounds within herbs and spices may be related to presumed physiological benefits. Sage contains phenols such as rosmarinic acid, carnosol, carnosic acid, caffeic acid and cirsimaritin. Oregano possesses rosmarinic acid and hydroxycinnamic acid. Thyme contains significant amounts of thymol, carvacrol, 3,4,3',4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl, eriodicytol, rosmarinic acid and luteolin. Rosemary contains such phenols as rosmanol, rosmarinic acid, naringin, cirsimaritin and carnosic acid. Many of these compounds possess greater antioxidant abilities than α-tocopherol, BHA or BHT (69). It is important to recognize the ratios of such phenolic compounds within herbs and spices will vary by source and location. As previously discussed, it appears consumption of a wide profile of polyphenolics seems to have a synergistic affect on physiological benefits. Hence, it is the whole herb or spice extract that will be examined in this study as opposed to an isolated polyphenolic compound.

GLYCATION INHIBITION WITH POLYPHENOLICS

It is clear that phenolic compounds have a role in health and the prevention of disease, including diabetes. Specifically, there is evidence to suggest the role of polyphenolics in inhibiting the degree of protein glycation that is thought to lead to many of the health complications experienced by diabetics.

Antioxidants have been shown to reduce diabetic complications and inhibit AGE formation (16, 41). Diabetes is a pro-inflammatory disease state, meaning antioxidants may limit the resulting oxidative stress brought about by hyperglycemia and the progression of the disease. It is not known how antioxidants limit diabetic complications. It may be that antioxidants quench the AGE-derived free radicals, preventing oxidative damage, or they may act by blocking the carbonyl group of reducing sugars or dicarbonyl compounds, thereby preventing AGE formation and buildup (6). It is also possible that polyphenols could act by chelating transition metal ions, which catalyze intermediate reactions in AGE formation (70). The formation of AGE products is complex; likewise a proposed mechanism of inhibition by polyphenols is equally complex as an inhibitor could act at any or all steps of formation.

Plant extracts and their polyphenolic constituents have been shown to inhibit protein glycation (71-73). Plant extracts that have been found to inhibit the formation of AGEs include garlic (74), ginseng (75), green tea (76) and Ilex paraguariensis (77). This inhibition may be due to the antioxidant activities of the polyphenolic compounds found within these extracts. Herbs and spices contain polyphenols implicating an antioxidant potential (63). Kim and Kim (71) analyzed the inhibitory effects of twenty-five plant extracts and found inhibition to be significantly correlated with phenolic content and antioxidant activities. Bousova et al. (72) analyzed the inhibition of aspartate aminotransferase (AST) glycoxidation by several phenolic compounds at varying concentrations. While many phenolic compounds inhibited the glycation of AST, it was also found that at higher concentrations (50 mM), some phenolic compounds had a negative effect on AST activity. It is likely that polyphenols react with functional groups

on proteins and that high concentrations may be inhibitory or alter protein conformation. Wu and Yen (73) investigated the inhibitory effect of ten naturally occurring flavonoids on the inhibition of protein glycation and specifically analyzed the inhibition at different stages of protein glycation including the presence of Amadori products, reactive carbonyl species and AGE/AGE crosslinking. Inhibition was observed at each stage and a significant correlation between inhibition and antioxidant potential was shown. These results suggest that polyphenolic inhibition of AGE formation may occur at multiple steps and that antioxidant abilities of these compounds play an important role in inhibition (73). Many studies have indicated that inhibition of AGE formation with natural products is possible (71, 72, 73); however appropriate physiological concentrations of inhibitors and possible mechanisms of inhibition need further investigation.

In the present study, we analyzed the anti-glycating ability of twenty herbs and spices using a modified procedure proposed by McPherson et al. (78). Antioxidant and total polyphenolic content were measured using Benzie and Strain's ferric reducing antioxidant potential (FRAP) method (79) and Singleton and Rossi's Folin-Ciocalteu total phenolic estimation method (80). We hypothesize that polyphenolics found in herb and spice extracts inhibit albumin glycation and that the antioxidant potential of the samples may be partially responsible for these effects.

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CHAPTER THREE

INHIBITION OF PROTEIN GLYCATION BY POLYPHENOLIC EXTRACTS OF CULINARY HERBS AND SPICES[1]

[1]Rebecca P. Dearlove, Phillip Greenspan, Diane K. Hartle, Ruthann B. Swanson, James L. Hargrove. To be submitted for publication in The Journal of Medicinal Food.

ABSTRACT

We hypothesized that substances in herbs and spices may inhibit protein glycation. Extracts of 20 herbs and spices were tested for their ability to inhibit glycation of albumin. Dry samples were ground and extracted with 50% ethanol (10% w/v), and total phenolic content and ferric reducing antioxidant potential (FRAP) were measured. Aliquots were incubated in triplicate at 37ºC in 0.2 M potassium phosphate buffer, pH.7.4, containing 0.25 M fructose and 10 mg/mL fatty acid-free bovine albumin with final concentrations of total phenolics from 33 to 267 μg/mL. After 3 days, fluorescence was measured at the wavelength pair of 370nm and 440 nm as an index of the formation of glycated albumin. In general, the spice extracts inhibited glycation more than the herb extracts. The most potent inhibitors included cloves (83.5%), ground Jamaican allspice (67.8%), and cinnamon (65.0%). Potent herbs tested included sage (49.1%), tarragon (48.1%), and rosemary (47.7%). Inhibition of glycation by spice extracts was concentration dependent and correlated with total phenolic and FRAP values (R^2=0.52 and R^2=0.51, respectively) whereas the inhibition by herb extracts was not highly correlated (R^2=0.14 and R^2=0.004, respectively) with these parameters. Total phenolics were highly correlated with FRAP values (R^2=0.93). The evidence shows that compounds found in herb and spice extracts inhibit albumin glycation in vitro. The low correlation between total phenolics and inhibition of glycation by herb extracts suggests that not all active substances are polyphenolics.

KEYWORDS: • advanced glycation endproducts (AGEs) • antioxidants • diabetes mellitus • polyphenols

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by a deficiency and/or absence of insulin and is associated with much chronic pathology including retinopathy, nephropathy, neuropathy and atherosclerosis. These diabetic complications are caused in part by the build up of advanced glycation endproducts (AGE), which are formed non-enzymatically between reducing sugars and proteins. Protein glycation occurs when the carbonyl group of a sugar combines to the amino group of a protein to form a Schiff base. The Schiff base rearranges to form a more stable and irreversible Amadori product. Post-Amadori reactions are catalyzed by transition metals and create reactive oxygen species (ROS). Amadori products react further with dicarbonyls to form AGEs (1). Protein glycation is an indicator of poor blood glucose control over a period of weeks. Clinically, glucose control is monitored through measurement of glycated hemoglobin A1c and fructosamine in blood plasma. These AGEs elicit a proinflammatory response which overwhelms the body's natural antioxidant defense to create oxidative stress (1, 2).

Polyphenolic compounds from plants are the main sources of antioxidants in the human diet (3). There is growing interest in polyphenolics due to their antioxidant and anti-inflammatory capacities. For example, numerous studies have implicated dietary phenolics in relation to a reduced risk of cancer, cardiovascular disease, neurodegenerative disease, retinopathy, osteoporosis and diabetes (1-4). It is thought that potential health benefits of herbs and spices may derive from their high concentrations of phytochemicals including polyphenolics and other bioactive compounds (5, 6).

Plant extracts have been shown to inhibit protein glycation (7-10). This may be due to the antioxidant activities of the polyphenolic compounds found within these samples.

Because many herbs and spices contain high concentrations of polyphenolics (11, 12), it is plausible that extracts of these substances may block the formation of AGE compounds. Though several means of glycation inhibition have been proposed, a specific mechanism has not been elucidated. In the present study, the total phenolic and antioxidant activity of herb and spice extracts were tested in vitro and the relationship with inhibition of albumin glycation examined.

MATERIALS AND METHODS

Chemicals and Plant Materials

Bovine serum albumin (BSA) (Fraction V, Essentially Fatty Acid Free), D- (-) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent and TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) were purchased from Sigma Chemical Company, (St. Louis, MO). Dried McCormick's brand (Hunt Valley, MD) herbs and spices were purchased at a chain supermarket in Athens, Georgia.

Preparation of Extracts

Samples were ground into a fine powder and were extracted with a 1:10 (w/v) with 50% ethanol at room temperature. The extracts were centrifuged at 1100 rpm for 10 minutes at 10°C to remove the percipitate. In this study, 40 commercially available herb and spice samples were tested, as well as various liquid extracts, for antioxidant activity and total phenolic content. From those tested, 20 samples were chosen that demonstrated high values in these measures and/or particular samples of interest based on the literature. These samples included marjoram, sage, cinnamon, clove, ground oregano, ground

Jamaican allspice, gourmet Italian seasoning, apple pie spice, rosemary, pumpkin pie spice, thyme, Italian seasoning, chili, turmeric, tarragon, black pepper, table salt, ginger, mint and poultry seasoning.

Total Phenolic Content

Total phenolic content was estimated using the Folin-Ciocalteu reagent as described by Singleton and Rossi (13). The assay was read using a Beckman DU 600 series spectrophotometer at a wavelength of 760nm. A standard using gallic acid was established and results expressed as mg gallic acid equivalents (GAE) per gram of dry sample. All experiments were performed in triplicate.

Ferric Reducing Antioxidant Potential (FRAP)

FRAP values were determined using a modified Benzie and Strain method (14). Freshly prepared FRAP reagent (25ml acetate buffer; 2.5ml TPTZ solution; 2.5ml ferric chloride solution) was used to estimate the antioxidant potentials of the extracts. Ferrous sulfate was used as a reference standard. The assay was read using a Beckman DU series spectrophotometer at a wavelength of 593nm with results expressed as millimoles ferrous sulfate per 100 gram of dry sample. All experiments were performed in triplicate.

Protein Glycation

Albumin glycation was estimated using the McPherson et al. (15) method and was modified as follows: bovine serum albumin (10 mg/ml) was incubated with 250 mM D-fructose and a 200 mM postassium phosphate buffer (pH 7.4; 0.02% sodium azide) for 72

hours at 37°C and 5% CO_2. Results were read using a Perkin-Elmer LS 55 Luminescence Spectrometer with an excitation/emission wavelength pair of 370/440 nm. The effects of herb and spice extracts on albumin glycation were analyzed at five concentrations including 33, 67, 133, 200 and 267μg/mL. All experiments were performed in triplicate.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Glycation inhibition results are expressed as a percentage of the control ± SD. Simple linear regression equations were used to determine a dose response of standard solutions and correlation coefficients (R^2) for total phenolic and FRAP measures as well as to quantify a relationship between these measures and the inhibition of albumin glycation. Data were analyzed using one-way analysis of variance (ANOVA). A p-value < 0.05 was considered statistically significant. Statistical tests were performed using Microsoft Excel software.

RESULTS

Effect of Herb and Spice Extracts on Albumin Glycation

Total phenolic and FRAP measures are presented in **Table 2** as a mean ± SD**.** The percentage of albumin glycation inhibition at the maximum concentration tested (267μg/mL) is presented in **Table 2** as a mean ± SD. These results are arranged in descending order of percent inhibition. Clove, ground Jamaican allspice and cinnamon

were among the most potent inhibitors at 83.5 ± 0.7, 67.8 ± 0.3 and 65.0 ± 3.6% respectively. Ginger and table salt did not display a dose-response relationship at the concentrations tested and for this reason were eliminated from further analyses.

Figure 8 displays simple linear regression and correlation coefficients of total phenol and FRAP measures of all samples tested (a), albumin glycation inhibition at maximum concentration tested and total phenol content of samples (b) and albumin glycation inhibition at maximum concentration tested and FRAP values for samples tested (c). Correlation coefficients were 0.93, 0.51 and 0.48 respectively and were considered significantly and positively related with p-values <<0.001, <<0.001 and 0.001 respectively.

Effect of Spice Extracts on Albumin Glycation

Because the spices tested generally had higher antioxidant potential and total phenolic contents when compared to the herbs, spices and herbs were also analyzed separately as groups regarding their antioxidant measures and abilities to inhibit albumin glycation. Spices included cloves, ground Jamaican allspice, cinnamon, turmeric, chili, black pepper, as well as spice blends such as apple pie spice and pumpkin pie spice. **Figure 9** presents simple linear regression and correlation coefficients of albumin glycation inhibition at maximum concentration tested and total phenolic content (a), as well as albumin glycation inhibition at maximum concentration tested and FRAP values (b). Correlation coefficients were 0.66 and 0.51 respectively with corresponding p-values of 0.04 and 0.04. Concentration dependence of glycation inhibition is shown in **Figure 10** at 33, 67, 133, 200 and 267 μg/mL for the most potent spice inhibitors.

Effect of Herb Extracts on Albumin Glycation

Herbs analyzed included sage, tarragon, rosemary, thyme, ground oregano, marjoram, mint, as well as herb mixes such as Italian seasoning, gourmet Italian seasoning and poultry seasoning. **Figure 11** presents simple linear regression and correlation coefficients of albumin glycation inhibition at maximum concentration tested and total phenolic content (a), as well as albumin glycation inhibition at maximum concentration tested and FRAP values (b). Correlation coefficients were 0.14 and 0.004 respectively and were not considered statistically significant (p-value > 0.05).

DISCUSSION

The present data show that extracts of herbs and spices inhibit albumin glycation in vitro. Moreover, the total phenolic content of herbs and spices is correlated with their antioxidant potentials and both measures are predictive of the samples' ability to inhibit protein glycation. Spices generally possess a higher total phenolic content and these measures are strongly correlated with albumin glycation inhibition. Inhibition is also correlated with antioxidant potential, suggesting inhibition may be the result of the antioxidant capabilities of polyphenolic compounds. Herbs, however, did not demonstrate a correlation between total phenolic content and protein glycation, suggesting that not all of the active substances of herbal extracts resulting in protein glycation inhibition are polyphenolics.

Among the top-ranking samples regarding albumin glycation inhibition included cloves, ground Jamaican allspice and cinnamon. Cloves and allspice are in the plant

Family Myrtaceaeae. Cinnamon is in the plant Family Lauraceae (6). Major phenolic constituents of cloves include eugenol, caryophyllin, vanillin and eugenin. Allspice contains eugenol, cineole, phellandrene and caryophyllene. Cinnamon contains cinnamaldehyde, furfural, cuminaldehyde, eugenol and caryophyllene (6, 16). Due to the complexity of the phenolic composition of each extract, it would be a large undertaking to assess and quantify each chemical compound and determine its biological role. This study examined only the total phenolic content with the understanding that this value represents a diverse chemical makeup and a potential synergistic effect of its individual components. The concept of a synergistic effect is supported by the fact that many of the herb and spice blends were effective inhibitors. These samples included apple pie spice, pumpkin pie spice, gourmet Italian seasoning, poultry seasoning and Italian seasoning.

The two samples that did not demonstrate a dose response in relation to the inhibition of albumin glycation, powdered ginger and table salt, also ranked among the lowest in regards to total phenolic content and FRAP values. It should be noted that table salt is not a plant tissue and hence does not contain phytochemicals. These two samples were purposefully chosen as samples of interest due to their common use and previously reported benefits regarding ginger, including reducing nausea and inflammation (5, 17). Preliminary data in our lab showed powdered garlic similarly possessed only minimal values for total phenolic and antioxidant measures. Garlic is known for its therapeutic uses such as the lowering of cholesterol, inhibiting platelet aggregation and improving immune function (5). It may be concluded that the processing of some of these well-known medicinal plants may remove some if not all of the therapeutic benefits that might

be derived from antioxidant mechanisms. There may be some remaining effects regarding secondary metabolism.

Antioxidants have been shown to reduce diabetic complications and inhibit AGE formation (3, 18). Diabetes is a pro-inflammatory disease state, meaning antioxidants can limit the resulting oxidative stress brought about by the progression of the disease. It is not known how antioxidants limit diabetic complications. It may be that antioxidants quench the AGE derived free radicals, preventing oxidative damage, or they may act by blocking the carbonyl group of reducing sugars or dicarbonyl compounds, thereby preventing AGE formation and buildup (1). It is also possible that polyphenols could act by chelating transition metal ions which catalyze intermediate reactions in AGE formation (19).

Plant extracts have previously been shown to inhibit protein glycation (7-10). This may be due to the antioxidant activities of the polyphenolic compounds found within these samples. In our study, the ethanolic extracts of 20 commonly used and commercially available herbs and spices were assessed, 18 of which were found to significantly inhibit albumin glycation in a dose response manner. Overall, the ability to inhibit albumin glycation was found to be significantly correlated with their total phenolic content and antioxidant potentials. The strong correlation between total phenolic and antioxidant measures suggest that the phenolic compounds found in the 20 herb and spice samples tested were responsible for their antioxidant capacity. In this study, it appears spice extracts may inhibit protein glycation via an antioxidant mechanism, while herb extracts do not. It may be hypothesized that herb extracts inhibit protein glycation via the binding of transition metals and not from its antioxidant

properties. Herb and spice extracts inhibit the non-enzymatic process of AGE formation and may be useful in reducing inflammatory disease complications seen in diabetes.

Limitations to this study are the application of in vitro experiments are not always indicative of in vivo results and factors such as metabolic breakdown and poor bioavailability may prevent this effect within the human body. While there is a growing literature on the bioavailability of polyphenols, this has yet to be fully explored in herb and spice extracts. Maximum plasma concentrations of polyphenolics lie between 1-2 μmol/L of aglycone forms (4, 20, 21). Crude extract concentrations tested in this study ranged from 33-267 μg/mL; however it is unknown whether the active compounds are present in physiologically relevant concentrations. The incorporation of herbs and spices into the human diet may be a strategic means of increasing plasma concentrations of polyphenols to attain their possible benefits, but many questions are left unanswered. More research into the bioavailability of polyphenols from herb and spice dietary intake is needed to assess the feasibility of our results in vivo. The pharmacological effect of these active compounds deserves further investigation. This experimental survey of common culinary herbs and spices suggests important chemoprotection can be added to the diet through liberal use of these generally recognized as safe (GRAS) products. Herbs and spices may augment health benefits of the foods they flavor. Protein glycation contributes to the aging process in general and is accelerated in metabolic syndrome and diabetes. This study provides a health rationale for seasoning food with ample herbs and spices.

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Table 2. ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF HERB AND SPICE EXTRACTS RELATIVE TO INHIBITION OF ALBUMIN GLYCATION IN VITRO.

Extract	Phenolic Content (mg/g)	FRAP Value (mmol/100g)	Inhibition at Max Conc. Tested 267µg/mL (%) [a]
	Mean ± SD		
Cloves	330.8 ± 3.7	404.1 ± 6.9	83.5 ± 0.7
Cinnamon	200.6 ± 11.5	136.7 ± 3.1	65.0 ± 3.6
Ground Jamaican Allspice	122.2 ± 7.0	134.1 ± 0.6	67.8 ± 0.3
Apple Pie Spice	163.0 ± 2.8	178.5 ±2.1	64.2 ± 0.02
Black Pepper	5.1 ± 0.1	4.7 ± 0.002	57.1 ± 0.3
Sage	59.8 ± 3.0	62.8 ± 3.1	49.1 ± 6.1
Turmeric	25.9 ± 1.6	10.2 ± 0.06	51.9 ± 1.2
Rosemary	48.2 ± 1.0	47.9 ± 2.7	47.7 ± 4.0
Tarragon	51.0 ± 2.7	46.1 ± 0.9	48.2 ± 2.1
Thyme	52.0 ± 2.0	59.1 ± 0.6	45.3 ± 2.8
Ground Oregano	82.3 ± 0.9	84.0 ± 0.7	43.4 ± 4.3
Mint	50.9 ± 2.3	60.9± 0.6	42.1 ± 2.0
Pumpkin Pie Spice	86.4 ± 7.0	81.0 ± 0.3	37.2 ± 3.2
Gourmet Italian	51.9 ± 1.2	47.9 ± 0.3	37.5 ± 1.1
Marjoram	69.0 ± 2.1	66.0 ± 0.9	33.1 ± 4.3
Poultry Seasoning	37.6 ± 6.5	39.0 ± 0.3	25.7 ± 5.6
Italian	41.7 ± 1.4	51.9 ± 1.6	20.1 ± 3.5
Chili	19.5 ± 1.2	11.7 ± 0.2	13.3 ± 1.2
Ginger [b]	17.7 ± 1.7	19.5 ± 0.3	Did not display a dose response.
Salt [b]	0.0 ± 0.0	0.0 ± 0.0	Did not display a dose response.

[a] Percent inhibition = (fluorescence intensity (sample) / fluorescence intensity of (control)) x 100. Result subtracted by 100 to obtain a negative % inhibition. [b]Ginger and salt did not display a dose response relative to the inhibition of albumin glycation.

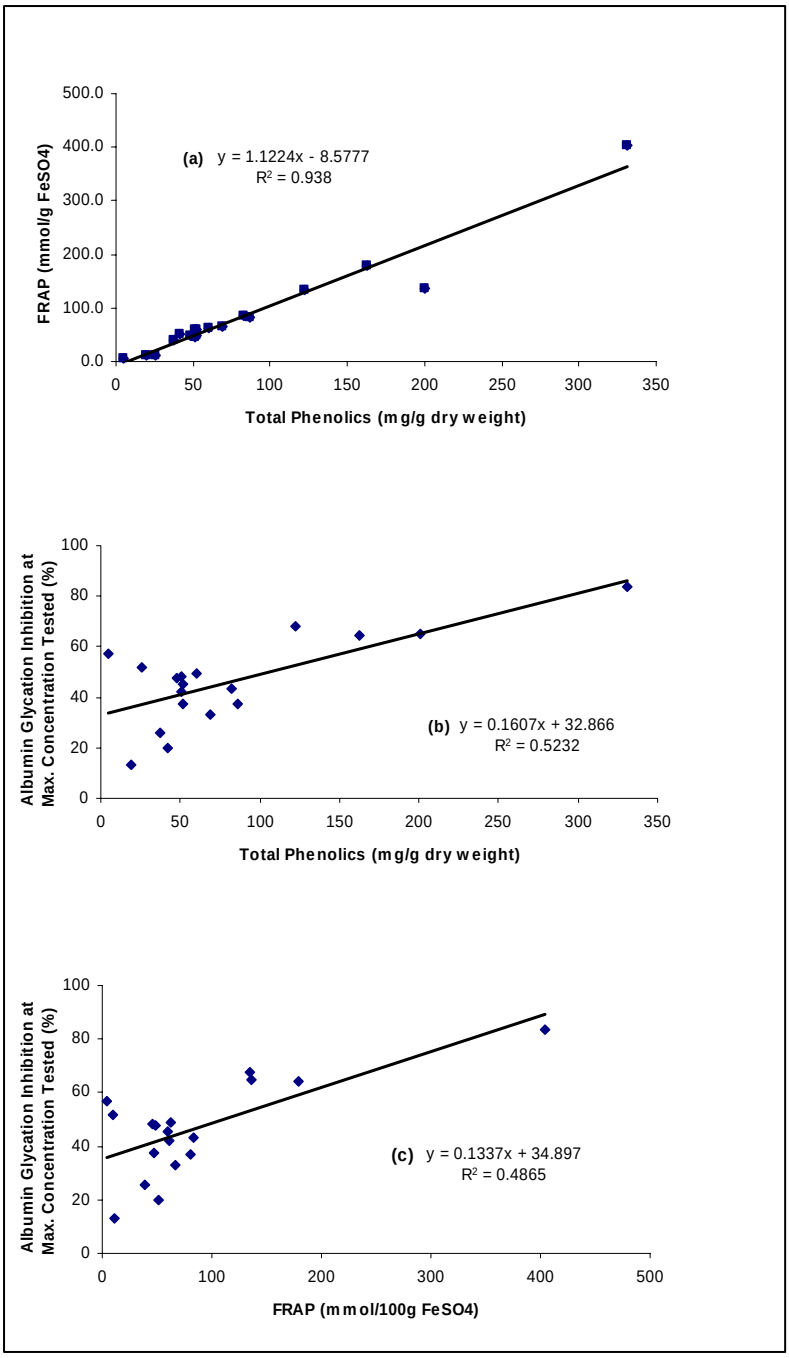

Figure 8. A relationship of (a) total phenolic and FRAP values of all samples tested and a summary of the effect of (b) total phenolic and (c) FRAP measures on albumin glycation inhibition. Samples included marjoram, sage, cinnamon, cloves, ground oregano, ground Jamaican allspice, gourmet Italian, apple pie spice, rosemary, pumpkin pie spice, thyme, Italian seasoning, chili, turmeric, tarragon, black pepper, mint and poultry seasoning.

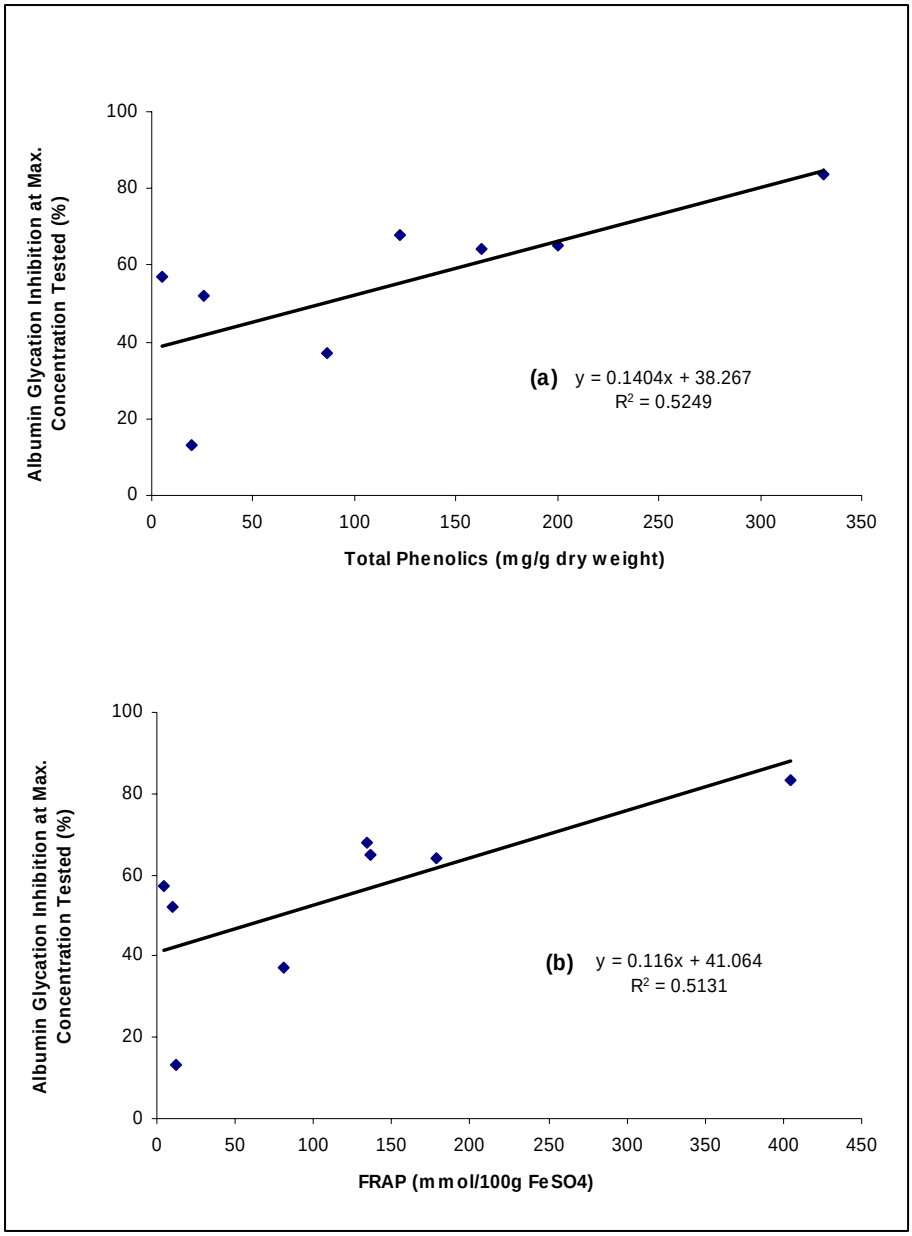

Figure 9. A relationship of (a) total phenolic and (b) FRAP values of all spices tested and a summary of the effect of these measures on albumin glycation inhibition. Spices included cloves, ground Jamaican allspice, cinnamon, turmeric, chili, black pepper, as well as spice mixes such as apple pie spice and pumpkin pie spice.

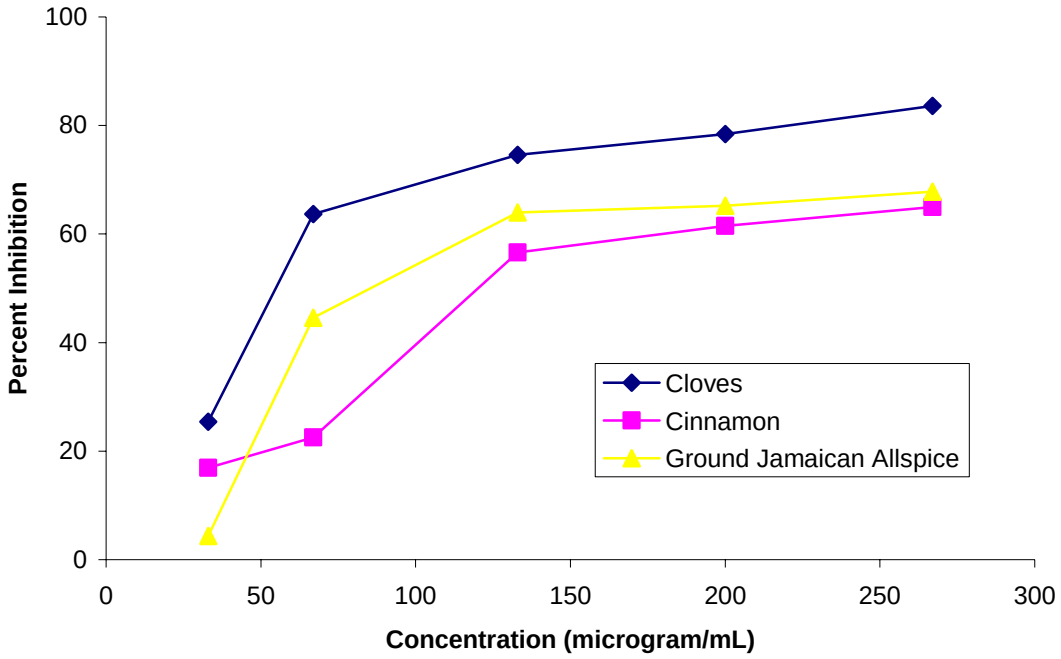

Figure 10. Inhibition of protein glycation dependence on extract concentration at 33, 67, 133, 200 and 267 µg/mL.

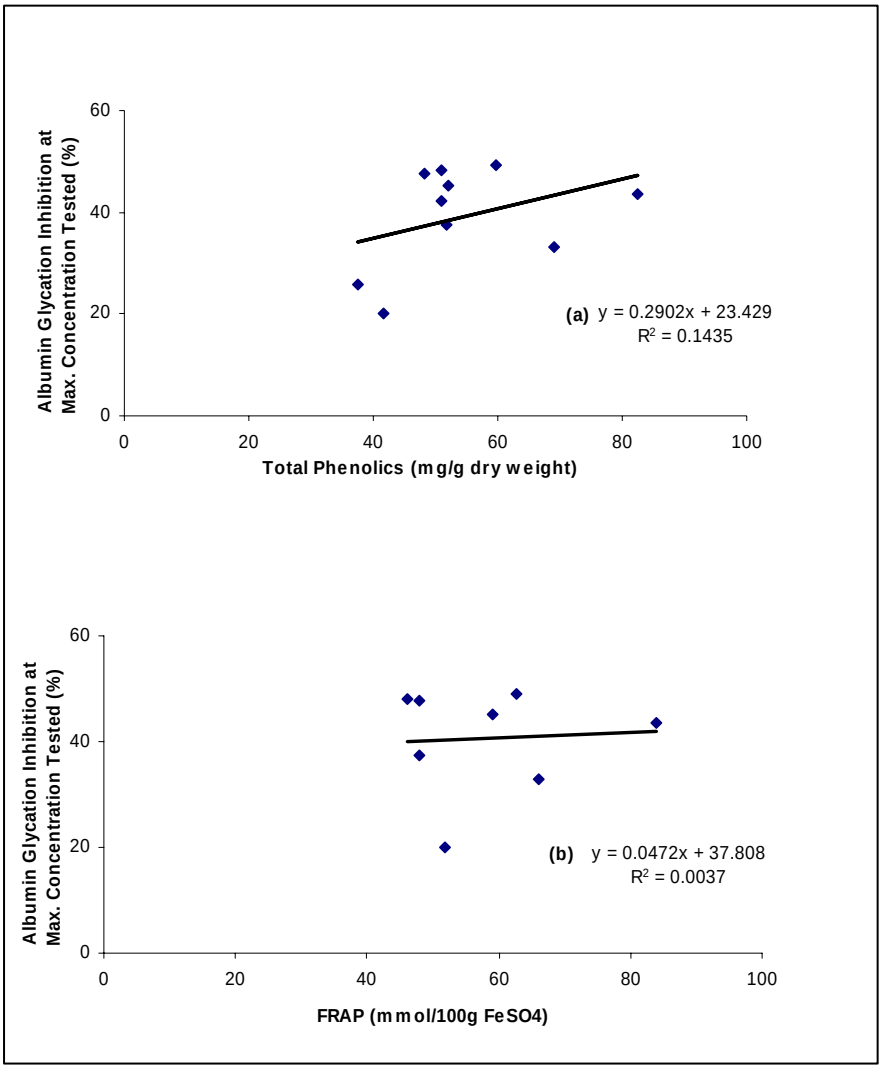

Figure 11. A relationship of (a) total phenolic and (b) FRAP values of all herbs tested and a summary of the effect of these measures on albumin glycation inhibition. Herbs included sage, tarragon, rosemary, thyme, ground oregano, marjoram, mint, as well as herb mixes such as Italian seasoning, gourmet Italian seasoning, and poultry seasoning.

CHAPTER FOUR

SUMMARY

The goals of this study were to test the ability of herb and spice extracts to inhibit albumin glycation and to relate this potential to the polyphenolic content of these samples. The results show that inhibition was positively correlated with polyphenolic content (R^2 =0.51). Polyphenolic content was positively correlated with antioxidant potential (R^2 =0.48), indicating a possible means of protein glycation inhibition. When analyzed independently, it was found that inhibition of protein glycation via spice extracts was related to polyphenolic content, whereas inhibition by herb extracts was not. This may mean there are additional bioactive compounds found in herb extracts responsible for the observed inhibition. There are non-phenolic compounds in plant extracts that may have biological activity.

The implications of this study lie in the potential of herb and spice intake to augment consumption of antioxidants in a bland diet and to influence the formation and buildup of AGEs, which have been shown to be involved in the development of diabetic complications (1-3). Various herb and spice extracts inhibited albumin glycation in vitro in a concentration dependent manner. Whereas not all extracts tested inhibited glycation, several were particularly effective. For example, it may be appropriate to use herbs and spices as nutritional therapy to increase plasma concentrations of the bioactive compounds that are demonstrating these physiological benefits. This study suggests that polyphenolic compounds may be largely responsible for the inhibition of protein

glycation. Polyphenolic content and inhibition of protein glycation were strongly correlated with antioxidant potential. It may be possible that other antioxidative compounds may be therapeutically used to inhibit protein glycation. This study supports previous studies that implicated an antioxidative mechanism in the inhibition of AGE formation via plant extracts (4-7).

The major limitations of this study involve the validity of extrapolating these in vitro results to efficacy within the human body. Certain factors such as bioavailability and metabolic breakdown or conjugation may influence the ability of bioactive components of herbs and spices to influence protein glycation. It has been reported that maximum plasma concentrations of polyphenolics lie between 1-2 μmol/L of aglycone forms (8-10). Crude extracts were used in this study in concentrations ranging from 33-267 μg/mL, however it is unknown what bioactive compounds are responsible for the inhibition of protein glycation and at what concentrations they are present in crude extracts. It is unlikely these are physiologically attainable concentrations; however the consumption of herbs and spices may still be beneficial in strategically increasing plasma concentrations of polyphenolics in humans. The potential benefits involving inhibition of albumin glycation and antioxidant abilities of herb and spice extracts have been demonstrated in this study. In addition, there are other mechanisms whereby flavonoids and stilbenes can modulate physiological function, such as activation of transcription factors that influence protective genetic pathways. These mechanisms were beyond the scope of the present investigation.

Future studies may investigate the physiological benefits of conjugated forms of polyphenols. It is well known that the metabolism of polyphenolics leaves minimal

traces of the aglycone form (8, 11), which also happens to be the form primarily used in scientific studies. Studies such as Cano et al. which studied the superoxide anion scavenging abilities of flavonol conjugates quercetin-3-rhamnoglucoside, quercetin-3-sophoroside, quercetin-3-sulphate, quercetin-3-glucuronide, kaempferol-3-sophoroside and kaempferol-3-glucuronide at concentrations 0.5-10 μM are needed to demonstrate potential physiological benefits of these compounds (12). The present study used crude plant extracts with the anticipation of a synergistic effect of active compounds that has been reported in the literature (10, 13). Investigation into the polyphenolic profiles of specific bioactive herb and spice compounds may be useful in the development of new pharmacological treatments of specific disease states, including diabetes. Exploration into in vivo inhibition of protein glycation and other possible physiological benefits of herbs and spices are needed. Furthermore, studies into strategically increasing human plasma polyphenolic concentrations are needed as the benefits of these compounds are becoming better understood.

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