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HETEROCYCLIC AMINES AND ARYLAMINE *N*-ACETYLTRANSFERASE 2
POLYMORPHISM IN PATHOGENESIS OF INSULIN RESISTANCE

By

Kennedy M. Walls

B.S. Chemistry, track in biochemistry, University of Louisville, 2019

A Thesis submitted to the faculty of the School of Medicine of the University of
Louisville in partial fulfillment of the requirements for the degree of

Master of Science in Pharmacology and Toxicology

Department of Pharmacology and Toxicology

University of Louisville

Louisville, Kentucky

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A Thesis approved on

April 15, 2021

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DEDICATION

I dedicate this thesis to my mother and sister for supporting me in my academic career and making it possible for me to pursue and complete my degree.

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I'd like to thank my lab members, Mariam Habil, Dr. Raul Salazar-Gonzalez, Dr. James Wise, and Mark Doll for their help and guidance with my work. I'd also like to thank my mentors, Drs. Kyung Hong and David Hein, for their patience and support with my project. I offer special thanks to my committee members, Dr. Joshua Hood, Dr. Bradford Hill, and Dr. Daniel Conklin, for their insight and suggestions. Lastly, I'd like to express gratitude to my family and friends who have supported me throughout my academic career and aided in my success.

ABSTRACT

HETEROCYCLIC AMINES AND ARYLAMINE *N*-ACETYLTRANSFERASE 2 POLYMORPHISM IN PATHOGENESIS OF INSULIN RESISTANCE

Kennedy M. Walls

April 15, 2021

Heterocyclic amines (HCAs) are formed when cooking meats at high temperatures or for long periods of time, and their major metabolic pathway includes hepatic *N*-hydroxylation by CYP1A2 followed by *O*-acetylation by *N*-acetyltransferase 2 (NAT2). *NAT2* is a highly polymorphic gene involved in metabolism of drugs and xenobiotics, and its single nucleotide polymorphisms (SNPs) produce phenotypes characterized as slow, intermediate, or rapid acetylators. Recent studies have documented associations between HCAs and insulin resistance and between certain NAT2 phenotypes and insulin resistance. In this study, we demonstrated that NAT2 activity and exposure to certain HCAs may alter cellular glucose and lipid homeostasis. Additionally, these results demonstrate changes in sensitivity to insulin following exposure to some HCAs. These findings are significant because they indicate a genetic component (NAT2 polymorphism) and an environmental component (dietary HCA exposure via consumption of cooked meat) in the pathogenesis of insulin resistance in hepatocytes of human origin.

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INTRODUCTION

Arylamine *N*-acetyltransferases (*NATs*)

Arylamine *N*-acetyltransferases (*NATs*) are enzymes that metabolize xenobiotics and drugs, and they are known to play a role in detoxification of many compounds [1]. Specifically, they add an acetyl group from acetyl coenzyme A (CoA) to arylamines and arylhydrazines, which may detoxify or bioactivate the compound. Two *NAT* genes (*NAT1* and *NAT2*) have been identified and characterized in humans. *NAT1* and *NAT2* are similar in structure, but they differ in substrate selectivity and physiological function [2]. For example, *NAT1* demonstrates an affinity for p-aminobenzoic acid (PABA), and *NAT2* demonstrates affinity for sulfamethazine [4]. *NAT1* is expressed ubiquitously, while *NAT2* expression is restricted to the liver and gastrointestinal tract [8, 9]. The importance of *NAT2*'s substrate selectivity and organ-specific expression will be further discussed in later sections.

NAT1 and *NAT2* function as catalysts for both *N*-acetylation and *O*-acetylation of drugs, aromatic amines, and heterocyclic amines. *NAT2* catalyzes *N*-acetylation of drugs like sulfamethazine, hydralazine and dapson, and this is usually a deactivation [5]. Following oxidation by cytochrome P450 (CYP450), *NAT2* catalyzes *O*-acetylation of heterocyclic amines like 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline

(MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) found in cooked meats, and this is usually an activation process [6].

NATs and metabolism

NATs catalyze both *N*-acetylation and *O*-acetylation of drugs, aromatic amines, and heterocyclic amines. Figure 1 illustrates the reaction pathways undergone by aromatic and heterocyclic amines [34]. *N*-conjugation of NATs, UDP-glucuronosyltransferases (UGTs), or sulfotransferases (SULTs) produces non-toxic, stable *N*-acetates, *N*-glucuronides, and *N*-sulfates, respectively. In contrast, *N*-oxidation of arylamines or *N*-acetamides, mediated by cytochrome P450 1A2 (CYP1A2) or by prostaglandin H synthase (PHS) peroxidase, is a vital step in metabolic activation, generating more reactive hydroxylamines or hydroxamic acids [35, 36]. Further *N*-acetylation, *O*-acetylation and intramolecular *N,O*-acetyltransfer alters levels of chemically unstable acetoxy esters and *N*-acetyl-acetoxy esters, which are considered the ultimate carcinogens that will spontaneously decompose to electrophilic DNA- or protein-binding nitrenium ions [37].

In addition to NAT's ability to catalyze acetyl CoA-dependent conjugation on nitrogen atoms to produce arylamides and hydrazides, NATs can also mediate *O*-acetylation (*OAT*) and, to a lesser extent, *N,O*-acetyltransfer (*NOAT*) reactions, as demonstrated in Figure 1 below [38]. SULTs and UGTs can also biotransform hydroxylamines and hydroxamic acids formed by NAT/CYP1A2 into

sulfate or glucuronide esters that decompose to nitrenium ions [39, 40]. Lastly, microsomal deacetylases (DAC) can reverse acetylation reactions by influencing levels of substrates for activating pathways. Tumorigenicity of 2-acetylaminofluorene (AAF) may require its deacetylation to the arylamine [41], which is then presumably activated by pathways previously described.

Figure 1. Pathways of arylamine activation and detoxification. Adapted from [34].

To provide an example of NAT2 metabolism, NAT2's *N*-acetylation of aromatic amines like 4-aminobiphenyl (ABP) is a detoxification process. *N*-acetylation competes with P450-catalyzed *N*-hydroxylation followed by *O*-acetylation of *N*-hydroxy-amine (catalyzed by NAT2), which generates acetoxy-derivatives that are highly unstable. This leads to the formation of arylnitrenium ions that bind to DNA, resulting in mutagenesis and carcinogenesis [18]. Subsequently, *NAT* allelic variations producing various acetylator phenotypes impact the extent of these reactions' effects (see discussions below).

NAT Single Nucleotide Polymorphisms

Both *NAT1* and *NAT2* exhibit single nucleotide polymorphisms (SNPs), and *NAT2* genotypic variants result in slow, intermediate, and rapid acetylator phenotypes [14, 15]. SNPs occur when a DNA sequence variation occurs due to a single nucleotide substitution, producing varying alleles within a locus. For any given SNP, the SNP can occur in a coding region but not result in a change in amino acid; it can occur in a coding region and result in an amino acid change; it can occur in a regulatory region resulting in a change in gene expression; or it can occur in a region between genes [3]. In the context of NAT2 SNPs discussed here, these SNPs occur in the coding region of the gene, resulting in amino acid changes and thus generating different alleles that produce slow, intermediate, or rapid phenotypes [3].

There are 28 *NAT1* alleles and 108 *NAT2* alleles, and allelic frequencies differ among different populations [10,11]. As mentioned, these SNPs are associated with varying enzymatic activity and stability, which will be further described in the following sections [12-14]. To mention some of the more common *NAT2* alleles, *NAT2**4 makes up 20–25% of alleles in the population study and is thus often considered the reference allele, and this is a rapid acetylator phenotype. The reference allele occurs at a somewhat higher frequency in African Americans (36–41%) and Hispanics (41%) [27, 28]. Reference allele frequency is highest in Asians (50% in Chinese and 70% in Japanese) [29-33]. The effects of the rapid acetylator phenotype of this reference allele will be discussed in the following section.

An additional frequent *NAT2* allele, *NAT2**5 and its subtypes, were most frequent among Caucasians (44%), Gabonese (41%), Hispanics, and those of African descent (25–27%) [26]. This variant is associated with the slow acetylation phenotype, in contrast to the reference allele, which generates a rapid acetylator phenotype. *NAT2**6 was distributed evenly across ethnic groups, while *NAT2**7 was more prevalent among Asians. Although only a select few of the *NAT2* allelic variation have been mentioned here, it should be noted that slow acetylator phenotype is likely not homogeneous, but rather that multiple slow acetylator phenotypes exist, and these result from different mechanisms inferred by various SNPs and haplotypes [14]. Thus, there are varying degrees of slow acetylation within *NAT2* polymorphisms.

NAT2 SNPs and drug toxicity or cancer risk

Increased risk for drug toxicity and cancer are associated with different NAT2 genotypes and phenotypes [15]. For example, people with the slow NAT2 phenotype have an increased risk of isoniazid-induced liver injury relative to those with the rapid NAT2 phenotype [16]. Isoniazid is a drug used for the treatment and prevention of tuberculosis, but treatment had frequently been compromised by isoniazid-induced hepatotoxicity and liver failure [95]. A previous study found that the incidence of isoniazid-induced hepatotoxicity differed among rapid (2.9%), intermediate (9.8%), and slow (22%) NAT2 acetylators [95]. Slow acetylators are significantly more likely to experience hepatotoxicity from isoniazid treatment for tuberculosis than rapid acetylators because slow acetylators retain a higher plasma concentration of the drug for an extended time [96].

Additionally, carriers of the slow acetylator genotype are correlated with greater adverse health responses after hydralazine treatment relative to rapid acetylators. Hydralazine is indicated in treatment of chronic hypertension, short-term therapy of pregnancy-induced hypertension and eclampsia, and in therapy of hypertensive crisis [97,98]. A rare side effect of hydralazine, a systemic lupus erythematosus (SLE)-like syndrome, has been observed and can have serious adverse health consequences. Hydralazine treatment for hypertension is modified by acetylator phenotype following oral administration [99], and there is substantial evidence supporting a correlation between NAT2 acetylator phenotype or genotype and urine and plasma hydralazine concentrations. Like

the previously discussed drug isoniazid, slow acetylators are more likely to experience adverse effects of hydralazine because they maintain higher plasma concentrations of the drug for a longer period, and thus *NAT2* genotype should be considered when dosing hydralazine.

In addition to alterations in drug toxicity, both rapid and slow *NAT2* acetylator phenotypes have been associated with increased risk of cancer in different target organs following arylamine exposure [21]. Rapid acetylators detoxify aromatic amines to a greater extent than slow acetylators via *N*-acetylation so they may be excreted from the body more quickly. However, following *N*-oxidation, the metabolites can undergo bioactivation via the *O*-acetylation pathway. The *O*-acetylation pathway often leads to formation of aryl nitrenium ions and DNA adducts, then carcinogenesis if DNA cannot be repaired. For this reason, slow *NAT2* acetylation has been associated with higher risk of developing urinary bladder cancer due to decreased ability to detoxify aromatic amines by *N*-acetylation [19, 20].

In contrast to urinary bladder cancer, the risk of colorectal cancer from well-done cooked meat was higher among rapid *NAT2* acetylators compared to slow acetylators resulting from increased rate of metabolism of heterocyclic amines (HCAs), carcinogens produced when cooking meat at high temperatures or until well-done [22-25]. Additionally, rapid acetylators are more prone to liver cancers following exposure to carcinogenic aromatic amines [35, 38, 39]. To elaborate on this paradoxical phenomenon, as mentioned previously, CYP450 mediates *N*-hydroxylation followed by *NAT2* *O*-acetylation of aromatic amines, resulting in a

carcinogenic metabolite. Since this metabolic process occurs in the liver, rapid acetylators are at greater risk of developing liver cancers [110]. However, in the case of urinary bladder cancer, *N*-hydroxylated aromatic amines exit the liver, unaltered by slow NAT2 acetylation, and reach the bladder, where DNA adducts are formed following an acid-catalyzed reaction with urothelial DNA [8].

Therefore, rapid NAT2 acetylation increases risk of liver cancer, while slow NAT2 acetylation increases risk of urinary bladder cancer. These data indicate the association of cancer risk with NAT2 phenotype may be exposure and organ specific.

Heterocyclic Amines (HCAs)

HCA Structures and Classifications

Heterocyclic amines (HCAs) are polycyclic aromatic molecules and known mutagens [42]. They are primarily formed in muscle foods including meats and fish, which provide precursors like creatinine, amino acids, and aldehydes [43-45]. Cooking meats at high temperatures and for long periods of time including frying, grilling, and roasting cooking methods substantially enhance HCA formation [46-48].

More than 25 HCAs have been isolated from food samples and their structures defined, some of which will be described further in the following section [49]. All HCAs contain at least one aromatic structure and one

heterocyclic structure, and most of them contain an exocyclic amino group. The five-membered heterocyclic aromatic ring of one class of HCAs, the amino-carbolines, is wedged between two six-membered aromatic rings. In contrast, another class of HCAs, aminoimidazo-azaarenes (AIAs), have an *N*-attached methyl group at the imidazole ring. Amino-carbolines, instead, either lack a methyl group entirely, or the methyl group is attached to a six-membered ring.

Formation of different classes of mutagens from proteinaceous foods is determined by the critical heating temperature of 300 degrees Celsius [50]. Protein pyrolysates form above 300 °C, and these are characterized as 2-amino-pyridine-mutagens, also known as amino-carbolines [51, 52]. Those formed below 300 °C are considered 2-amino-imidazole-type mutagens, also known as aminoimidazo-azaarenes (AIAs) [53]. Pyrolytic mutagens can be further divided into five groups, including pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrene, and benzimidazole. AIAs are divided further into three groups, including IQ, 2-amino-3-methyl-imidazo[4,5-*f*]quinoxaline (IQx), and imidazopyridine.

An additional method of classifying HCAs includes polarity. Polar HCAs include the IQ, IQx-type, and imidazopyridine type (AIAs), while nonpolar HCAs have a common pyridoindole or dipyrdoimidazole moiety [54]. Polar HCAs are formed from amino acids, carbohydrates, and creatinine when a temperature between 150 and 250 °C is reached. Non-polar HCAs form at higher temperatures and are usually assigned as pyrolysis products of the amino acids

[53]. Figure 2 shows structures of common HCAs that are found most frequently in meat and known to be carcinogenic.

Figure 2. Structures of common HCAs. Adapted from [42].

Formation of HCAs

Formation of HCAs is a series of complex chemical reactions. The browning reaction was proposed by Maillard to account for the brown pigments and polymers produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar [16]. The Maillard reaction encompasses a network of reactions, including the formation of pyrazines, quinoxalines, and pyrido[3,4-d]imidazoles, which are involved in the formation of HCAs [46-48].

Formation of amino-carbolines

Amino acids or proteins are the precursors of amino-carbolines, and creatinine is not required for their formation. Therefore, they can be formed in foods originating from both animals and plants [43]. However, creatinine may function as an additional source of structural fragments. Free radical reactions occurring within drastic thermal environments is the suggested pathway for producing reactive fragments in amino-carbolines [56]. However, this hypothesis has yet to be fully investigated due to difficult manipulation of high temperatures required for experimental setups.

Formation of aminoimidazole-azaarenes

AIAAs can be responsible for most of the mutagenic activity in cooked foods, especially in Western diets [57]. IQ- and IQx-type HCAs (structures found in Figure 2 above) and their methylated forms are likely formed from creatinine, sugars, free amino acids, and dipeptides through Maillard reaction and Strecker degradation upon heating [44, 58].

Creatinine was determined to be important for HCA formation because HCA yield was increased with the addition of creatinine to the surface of meat prior to frying it [59]. It was demonstrated that cyclization and water elimination of creatinine formed the amino-imidazo structure of IQ and IQx, and the Maillard reaction between amino acids and hexose formed the Strecker degradation products like pyridines or pyrazines, which formed the remainder of the molecule,

likely by aldol condensation [43, 61]. While the reaction is favored at temperature above 100 °C, pyrazine and pyridine yield from Maillard reaction and Strecker degradation is still low [46]. This phenomenon was considered a factor for low HCA yield in foods and common model systems, making it difficult to investigate AIAs [60].

Metabolism of HCAs and cancer

Meat consumption has been associated with increased risk of many common cancers, including prostate, lung, breast, and colon cancer [100-103]. Genetic variation in *NAT2* has been suggested to modify the association of meat intake with some cancers through its influence on metabolism of HCAs [103]. Thus, epidemiological studies have reported inconsistent associations between dietary HCA intake via meat consumption and increased cancer risk, as *NAT2* allelic variations occur naturally in the sampled human population.

Despite inconsistent epidemiological reports, HCAs are highly mutagenic *in vitro* and *in vivo* [104]. Additionally, when HCAs were fed in the diet, rodents developed cancers in many organs, including the colon, breast, and prostate, and one HCA produced hepatomas in monkeys [104]. Many HCAs require metabolic activation to result in carcinogenesis. The initial step of HCA metabolism and activation is the generation of *N*-hydroxy-HCA derivatives, and this is catalyzed by cytochrome P450, mainly isoenzyme *CYP1A2*. Further metabolism and activation is carried out by *NAT2* and *SULTs*, which catalyze

esterification of *N*-hydroxy-HCAs, as previously described in the *NATs* metabolism section. The resulting products of these reactions are highly genotoxic and capable of direct interaction with DNA by adduct formation. HCA-DNA adducts can cause errors in DNA replication and result in mutations, which, when not repaired, may contribute to carcinogenesis. In contrast, UDP-glucuronosyltransferases and glutathione S-transferases are enzymes involved in detoxification of HCAs [103 – 105]. Balance between activation and detoxication processes of HCAs, together with genetically determined differences in HCA metabolism (from *NAT2* SNPs), are crucial for the understanding of HCA-dependent cancer risk among individuals [105].

Insulin

Insulin is an endocrine peptide hormone that binds plasma membrane-bound receptors in target cells to orchestrate an anabolic response to nutrient availability [64]. Insulin is made in the pancreas and secreted from cells called the Islets of Langerhans. These cells release a small amount of insulin into the body continuously and release surges of insulin in response to a rise in the blood glucose level.

Insulin's role in glucose homeostasis is characterized by insulin's direct effects on skeletal muscle, liver, and white adipocytes. In skeletal muscle, insulin promotes glucose uptake by increasing glucose transport and glycogen

synthesis. In the liver, insulin stimulates glycogen synthesis, increases lipogenic gene expression, and decreases gluconeogenic gene expression. In white adipocyte tissue, insulin suppresses lipolysis and increases glucose transport and lipogenesis [64].

Signaling Pathways

There are 2 known insulin-signaling pathways, and they are presented in Figure 3 below. These pathways are activated by insulin binding to the insulin receptor (labeled as IR) at the plasma membrane. Insulin/receptor interaction activates an intrinsic tyrosine protein kinase, which auto-phosphorylates the receptor and downstream substrates. In the main pathway, proteins known as IR substrates (IRS) are the immediate downstream substrates, which activate a cascade of serine-protein kinases [67, 68]. Akt (protein kinase B) is a major division point with numerous downstream substrates that lead to a variety of physiological functions like regulation of energy homeostasis [69], and this pathway will be discussed further in the following sections. The alternate pathway (“ras/mitogen-activated protein kinase”) is a serine-protein kinase cascade as

well, and this pathway regulates transcription, cell growth and differentiation, and protein synthesis [69, 70].

Figure 3. Two insulin signaling pathways mediate actions of insulin.

Adapted from [64].

Metabolic Syndrome and Insulin Resistance

Metabolic syndrome is clinically defined by having at least 3 of the following conditions: hypertension, elevated fasting blood sugar, obesity, low high-density lipoprotein cholesterol, and elevated triglycerides [71]. An individual with metabolic syndrome is at an increased risk of complications including atherosclerosis, type II diabetes mellitus, heart attack, kidney disease, fatty liver,

vascular disease, and stroke [72, 73]. Many have argued that the underlying etiology of metabolic syndrome could be insulin resistance [73, 74].

When higher insulin levels are required to achieve the same glucose-lowering response previously described, a subject is considered insulin resistant. Plasma lipids, hormones and adipokines, and their respective signaling pathways regulate insulin sensitivity [79,80], and pathological resistance to insulin (or lowered insulin sensitivity) develops through multifaceted interactions of genotype and lifestyle (including exercise habits and diet) [75-78].

Our understanding of the physiology of insulin resistance largely relates to defective insulin action at target cells. However, when insulin resistance affiliated with human type II diabetes was shown to involve both a right-shifted dose-response curve to insulin and a decreased overall maximum insulin response, it was inferred that both receptor and “post-receptor” (insulin signal transduction) defects contribute to insulin resistance [75].

The pathophysiology of insulin resistance in skeletal muscle is well described and can be found in reference texts [64]. For the purposes of this text, we will instead focus on the pathophysiology of hepatic insulin resistance.

Selective Hepatic Insulin Resistance

The liver plays a crucial role in systemic regulation of glucose and lipid metabolism and aberrant hepatic insulin action is thought to be a primary factor in insulin resistance [89]. In individuals with a normal physiologic fasting state,

high glucagon-to-insulin ratio will decrease glucose consumption and shift the liver into glucose production by consuming stored glycogen (glycogenolysis) and from glucogenic precursors (gluconeogenesis) [90]. Following a meal, decreased glucagon and increased insulin levels signal the liver to increase glucose consumption, stop glucose production, and store excess nutrients in the form of glycogen and lipids [91]. In obese individuals or those with pathological insulin resistance, insulin fails to regulate hepatic metabolism, leading to excess production of glucose despite accelerated rates of lipid synthesis. This condition is known as selective hepatic insulin resistance [92].

Hepatic Insulin Signaling and Lipid Metabolism

As previously mentioned, evidence has indicated that the phosphoinositide 3-phosphate kinase (PI3K)/Akt pathway is the primary signaling pathway that mediates insulin's effects on anabolic metabolism [93]. Figure 4 models the primary insulin signaling pathway in hepatocytes.

Figure 4. PI3K/Akt signaling in hepatocytes. Adapted from [89].

Insulin binds to the insulin receptor on the surface of the liver following a meal. The receptor recruits and activates IRS, which then activates PI3K [94]. PI3K phosphorylates PIP2 into PIP3 in a process that is opposed by PTEN. PIP3 activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates Akt at Thr308. mTORC2 also phosphorylates Akt at Ser473 to fully activate Akt. Various pathways for controlling glucose and lipid homeostasis branch out from Akt. Glycogen synthesis is induced through Akt inhibition of

GSK3. Akt can also promote glycogen independent of GSK3 via activation of GYS2 by glucose-6-phosphate (G6P). Akt inhibition of TSC activates mTORC1, which then activates SREBP1c and GCK, resulting in phosphorylation of glucose to G6P. G6P feeds into glycolysis and glycogen synthesis. Additionally, G6P activates ChREBP, which activates lipogenesis along with SREBP1c. Akt inhibits FOXO1, causing inhibition of gluconeogenesis by suppressing expression of the proteins, such as glucose-6-phosphatase (*G6PC*) and phosphoenolpyruvate carboxykinase 1 (*PCK1*). Free fatty acids (FFAs) can also promote gluconeogenesis and be taken up by the liver and converted to acetyl-CoA, which activates pyruvate carboxylase and contributes to insulin resistance [89].

Mechanisms of Hepatic Insulin Resistance

Insulin orchestrates a switch from glucose production to glucose uptake in the liver. This involves suppression of gluconeogenesis and glycogenolysis and the activation of glycogen synthesis. Suppression of gluconeogenesis involves acute and chronic processes. Rates of hepatic gluconeogenesis are increased in type II diabetes and are the cause of fasting hyperglycemia that defines the disease [119]. Chronic suppression of gluconeogenesis by insulin, better observed in fasting-refeeding studies than acute insulin stimulation studies, is a direct hepatocellular effect mediated by inhibition of the FOXO transcription factors, especially FOXO1.

Removal of the hepatic insulin receptor is associated with unrestricted FOXO1 activity, which is required to drive pathological gluconeogenesis [119]. This phenotype is also seen in mice with severe hepatic insulin resistance resulting from removal of hepatic *Irs1* and *Irs2*, in which *Foxo1* deletion normalizes glucose tolerance [120]. Mouse models of hepatic insulin resistance, like *ob/ob* and lipodystrophic mice, display increased *G6pc* expression, indicating increased FOXO1 activity [121]. However, hepatic insulin resistance of fat-fed rats is not accompanied by alterations in gluconeogenic enzyme content, arguing against a role for FOXO1-driven gluconeogenic gene transcription in genetically normal rodents [122]. Reducing hepatic *Pck1* expression by 90% in mice caused only a ~40% reduction in gluconeogenic flux, suggesting that fluctuations in *Pck1* expression exert limited control over gluconeogenesis [123]. Lastly, a study of humans with type II diabetes found no increase in the hepatic expression of *FOXO1* targets *G6PC* and *PCK1*, despite fasting hyperglycemia [124]. Thus, depression of *FOXO1* can drive pathological gluconeogenesis in rodents, but there is no strong evidence that this is a functional mechanism for fasting hyperglycemia in typical human hepatic insulin resistance. The complex control of hepatic gluconeogenesis indicates transcriptionally mediated increases in gluconeogenic capacity may not be sufficient to drive gluconeogenesis in genetically normal subjects. Therefore, increased hepatic gluconeogenesis of humans with type II diabetes is a complex and poorly understood phenomenon [64].

Consumption of Cooked Meat Associated with Insulin Resistance

Heterocyclic amines (HCAs), such as 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), represent a group of compounds that are mutagenic and are known human carcinogens [42 – 45]. HCAs are formed via condensation of creatinine with amino acids when meats are cooked at high temperatures or for long periods of time [42]. When ingested, HCAs are absorbed and rapidly distributed. The major metabolic pathway for HCAs includes hepatic *N*-hydroxylation by CYP1A2 followed by *O*-acetylation by arylamine *N*-acetyltransferase 2 (NAT2) [21]. This leads to formation of arylnitrenium ion, which results in DNA damage. Previously, studies on HCAs and exposure via cooked meat have focused on their carcinogenic potential and association to common cancers [109]. Recently, an epidemiological study found a novel link between HCA exposure via cooked meat consumption and insulin resistance [111]. High HCA intake was associated with increased prevalence of insulin resistance, even after the multivariate analysis was adjusted for saturated fatty acid and cholesterol intake. However, this epidemiological association has not yet been validated in cell or animal models.

NAT2 and Insulin Resistance

Arylamine *N*-acetyltransferase 2 (NAT2) is a highly polymorphic gene that catalyzes metabolism of arylamines, including HCAs. NAT2 plays key roles in

detoxification or activation of carcinogenic arylamines and xenobiotics by catalyzing acetyl coA-dependent biotransformation of arylamines to *N*-arylametamides [18]. Individuals can be categorized as rapid, intermediate or slow acetylators according to their combinations of NAT2 alleles, and NAT2 phenotype has been shown to influence metabolism, and thus the efficacy and toxicity, of arylamine drugs such as sulfamethazine [2]. NAT2 acetylator phenotype is associated with increased risk for colorectal cancer following exposure to HCAs [22 – 24].

Recently, certain genetic variants/polymorphisms of NAT2 have been linked to insulin resistance, high serum triglyceride, and coronary artery disease as well as high fasting plasma glucose level [112 – 116]. Analysis of Nat1-knockout (homolog of human NAT2) mice revealed that Nat1 deficiency leads to development of insulin resistance and metabolic defects at the systemic level, supporting the previously unrecognized role of NAT2 in regulating metabolism and insulin sensitivity [117 – 118]. However, it is currently unknown how NAT2 influences cellular metabolism and insulin sensitivity.

With newly documented associations between dietary HCA intake and insulin resistance and between NAT2 polymorphisms and insulin resistance, it is important we explore the novel link between HCA exposure to pathogenesis of insulin resistance in cell culture and animal models, and that we investigate the role of NAT2-mediated metabolism in this process. In the current study, we will analyze effects of HCAs and NAT2 phenotypes on insulin sensitivity in hepatocytes of human origin. Our results indicate that HCAs may directly induce

insulin resistance in human hepatocytes, and that NAT2 phenotype may impact sensitivity to insulin in human hepatocytes.

MATERIALS AND METHODS

Source and Processing of Cryopreserved human hepatocytes

Cryopreserved human hepatocytes were obtained from BioIVT and were stored in liquid nitrogen until use. Hepatocyte samples were collected from consenting donors under IRB approved protocols at the FDA licensed donor center at BioIVT (<http://www.bioivt.com/>). Hepatocytes were prepared from fresh human tissue and were isolated and frozen within 24 hours of organ removal. The hepatocytes are human transplant rejected and tested negative for hepatitis B and C and HIV 1 and 2. Hepatocytes were thawed according to the manufacturer's instructions once removed from liquid nitrogen by warming a vial of the hepatocytes at 37 °C for 90 seconds and suspending them in InVitroGRO HT medium (BioIVT, Baltimore, MD, USA) containing 1 mL TORPEDO™ Antibiotic Mix (BioIVT, Baltimore, MD, USA) per 45 mL media. Hepatocytes were then plated on Biocoat® collagen coated plates and remained cultured in this medium while incubated in humidified air (95%) and carbon dioxide (CO₂, 5%) conditions at 37 °C.

HepG2 cell culture

HepG2 cells were purchased from American Type Culture Collection (ATCC, ATCC® HB-8065™, Rockville, MD, USA). HepG2 was originally derived from a liver hepatocellular carcinoma in a male adolescent. The HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco), 5.5 mM D-glucose, 1 mM pyruvate, and 4 mM L-glutamine, and were kept in an incubator with a humidified air (95%) and carbon dioxide (CO₂, 5%) condition at 37 °C.

Cytotoxicity assay

For the cytotoxicity assay, HepG2 cells were treated in indicated compounds (MeIQ and PhIP) at the indicated concentrations (0, 0.1, 10, 100 µM) for 3 days in complete media. alamarBlue™ assay was used to measure cell viability.

Generating NAT2 small-hairpin-loop RNA (shRNA) lentivirus

Initially, we screened 8 different shRNA candidates for *NAT2* transcript, and the top two candidates (shRNA4 and shRNA7) were selected for present studies.

The target sequences for the shRNAs are the following: 5'-

GCTTGAACCTCGAACAATT-3' for shRNA4; and 5'-

GCAGACGTCTCCAACATCT-3' for shRNA7. The target sequence for control shRNA was 5'-ACTACCGTTGTTATAGGTG-3,' which does not match any

known gene transcripts. The templates for the corresponding shRNAs were synthesized at Integrated DNA Technologies (IDT), annealed, subcloned into a lentiviral vector (pLenti6-U6-shRNA-GFP-2A-Puro) (unpublished, K. U. Hong), and sequence-verified. In pLenti6-U6-shRNA-GFP-2A-Puro vector, the human U6 small nuclear RNA gene promoter drives the shRNA expression while *EGFP* and puromycin resistance gene (*pac*) are co-expressed under SV40 promoter. The shRNA lentiviral vectors were then transfected into HEK 293T cells with the packaging mix to produce lentivirus according to manufacturer's instructions (ViraPower™ Lentiviral Expression System; Invitrogen). The lentivirus-containing supernatant was harvested and stored in aliquots at -80 °C.

Generating NAT2 overexpression lentivirus

The coding sequences (870 bp) for human NAT2 reference gene (NAT2*4) were PCR amplified using the following primers: hNAT2 fwd EcoRI, 5'-TTTGAATTCATGGACATTGAAGCATATTTTGAAAG-3' and hNAT2 rev XhoI, 5'-TTCTCGAGTAATAGTAAGGGATCCATCACCAGG-3'. The PCR product was digested with EcoRI and XhoI and subcloned into the pLVX-2xmyc-E2A-Puro-T2A-EGFP vector (Unpublished, K. U. Hong), a modified pLVX lentiviral vector (Clontech), and sequence-verified. The pLVX-2xmyc-E2A-Puro-T2A-EGFP utilizes viral 2A peptide sequences and allows co-expression of the gene of interest, plus puromycin resistance gene (*pac*) and *EGFP* from the same CMV promoter. The pLVX NAT2 viral construct was transfected into HEK 293T cells with the packaging mix to produce lentivirus according to manufacturer's

instructions (ViraPower™ Lentiviral Expression System; Invitrogen). The lentivirus-containing supernatant was harvested and stored in aliquots at -80 °C.

Lentivirus transduction and selection

HepG2 cells on 6-well plates were transduced with the indicated lentivirus in a complete media containing 6 µg/ml polybrene. After 2 days, the cells were placed in complete media containing 2 µg/ml puromycin to select for virus-transduced cells. The puromycin selection and subsequent passaging of the cells continued for approximately 2 weeks. Following selection, the cells were examined under a fluorescent microscope, and as expected, virtually every cell in the culture was positive for GFP. The cells were then cryo-preserved in 1 ml aliquots in cell freezing media (90% FBS + 10% DMSO) and stored in liquid nitrogen until use.

Protein quantification and Western blot analysis

HepG2 cells and primary human hepatocytes were lysed with Laemmli buffer (50 mM Tris-Cl [pH 6.8], 2% sodium dodecyl sulfate [SDS; wt/vol], 0.1% bromophenol blue, 10% [vol/vol] glycerol) and boiled for 10 minutes. Protein concentrations were determined using Pierce BCA Assay kit (Thermo Scientific) per manufacturer's instructions. One to two µL of 2-mercaptoethanol was added to each sample and boiled again for 5 minutes. Twenty-five to fifty µg of protein per sample was loaded and separated on 4–12% gradient Bis-Tris Plus polyacrylamide gels (Invitrogen). The gel was transferred to a PVDF membrane

and blocked in 5% (w/v) skim milk in tris buffered saline containing 0.1% Tween 20 (TBST) for 30 minutes. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed 3 times for 5 minutes each with TBST. Membranes were incubated for 1 hour at room temperature with secondary antibodies. Membranes were washed with TBST 3 times and then protein was detected with chemiluminescent substrate (Thermo Scientific Pierce).

Antibodies

The following antibodies were purchased from Cell Signaling: phosphorylated-protein kinase B (p-AKT S473) #4060 and protein kinase B (AKT) #4691. A-tubulin (clone B-5-1-2) (Sigma-Aldrich) was an additional antibody used as the control.

Gene Expression

RNA was isolated using E.Z.N.A. Total RNA Kit 1 (OMEGA) per manufacturer's protocol. cDNA was synthesized using High Capacity cDNA Reverse Transcriptase PCR (Thermo Fisher Scientific) per manufacturer's instructions. Gene-specific cDNA was quantified with qRT-PCR using self-designed SYBR Green-based assays. The following primers were designed:

- glucose-6-phosphatase catalytic subunit (G6PC; forward: 5'-ACGAATCTACCTTGCTGCTCA-3'; reverse: 5'-AAAATCCGATGGCGAAGCTG-3')
- phosphoenolpyruvate carboxykinase 1 (PCK1 or PEPCK; forward: 5'-TGATGAGCCGCTAGCTTCAG-3'; reverse: 5'-GCCTTTATGTTCTGCAGCCG-3')
- fatty acid synthase (FASN; forward: 5'-CACACACGATGGACCCTCAG-3'; reverse: 5'-GAAGAAGGAGAGCCGGTTGG-3')
- sterol regulatory element binding transcription factor 1 (SREBF1 or SREBP1; forward: 5'-CACCACCACCGGCGTAG-3'; reverse: 5'-GTACCCACTGGCCTTCTCAC-3').

iTaq Universal SYBR Green Supermix (172–5124, Bio-Rad) was used to perform qRT-PCR. Reaction conditions were determined per manufacturer's instructions.

Extracellular Glucose Measurements

Hepatocytes were seeded into Biocoat® collagen coated plates in normal media conditions for 24 hours to allow attachment. After 24 hours, media was replaced with complete media containing varying concentrations of HCAs for 2 days. Then, media was replaced with serum-free, glucose-free media containing varying concentrations of HCAs for 6 hours. After this completion, media was replaced in appropriate wells with serum-free, glucose-free media containing gluconeogenic substrates (2 mM pyruvate, 20 mM lactate, 0.5 mM L-lysine, 5

mM glycerol) for 24 hours. Finally, the media was collected for extracellular measurement of glucose and cells were harvested with Laemmli buffer for protein lysates for normalization to total protein content. Glucose levels were measured using a glucose (GO) assay kit (Sigma-Aldrich, GAGO-20). Total protein in each well was measured using the Pierce BCA Assay kit (Thermo Scientific), and the values were used to normalize the glucose levels.

Statistical analysis

Differences in gene expression among different NAT2 phenotypes and HCA treatments were tested for significance by One-Way or Two-Way ANOVA followed by Dunnett's Multiple Comparison Test or Bonferroni's Multiple Comparison Test, respectively. Statistical significance was determined per the following p-values:

* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

RESULTS

High-glucose model of insulin resistance in HepG2 cells. We optimized models that induced insulin resistance in HepG2 cells. Others have reported that high-glucose and high free fatty acid conditions can induce insulin resistance in hepatocytes [107, 108]. The high-glucose model here and the high free fatty acid model discussed later are used in later experiments in combination with heterocyclic amine (HCA) treatment and varying levels of NAT2 activity to determine if treatment with HCAs or changes in NAT2 activity can exacerbate effects of excess nutrient conditions on insulin resistance. To measure changes in insulin signaling in response to excess nutrients, we cultured HepG2 cells in low (5.5 mM) or high (25 mM) glucose-containing, serum-free media and then harvested for protein after 10 minutes of insulin treatment. Western blot was used to measure induction of p-AKT at the Ser473 site of phosphorylation. As mentioned previously, phosphorylation of AKT (at Thr308 and Ser473) plays a key role in the insulin signaling cascade [89]. Thus, we compared p-AKT (indicating induction of insulin signaling) to total-AKT in a p-AKT/total-AKT ratio. We found that cells cultured in high glucose conditions had a lower p-AKT induction (i.e., lower response to insulin) when compared to cells cultured in low glucose conditions. Consistent with previous reports [107, 108], these results

(Fig. 5A) indicate that high-glucose condition reduces insulin sensitivity in HepG2 cells.

Additionally, we confirmed our high glucose insulin resistance model by measuring transcripts of genes involved in gluconeogenesis (glucose-6-phosphatase, *G6PC*; and phosphoenolpyruvate carboxykinase 1, *PCK1*). HepG2 cells were cultured in low glucose (5.5 mM) or high glucose (25 mM) DMEM for 2 days and then treated with 10 nM insulin for 24 hours. The samples were harvested for RNA and analyzed by qRT-PCR. Although insulin downregulated *G6PC* and *PCK1* gene expression in high glucose, relative mRNA levels of both genes remain upregulated compared to low glucose with insulin (Fig. 5B). Thus, despite access to excess nutrients, the gluconeogenic gene transcripts are upregulated in these cells. These results further confirm induction of insulin resistance in HepG2 cells cultured with high glucose. However, insulin treatment should have significantly downregulated *G6PC* and *PCK1* in low glucose conditions. This was not observed and indicates that our insulin treatment was insufficient. We are optimizing this condition by testing different insulin concentrations at varying durations of treatment time to improve observed effects of insulin.

Figure 5. High glucose model of insulin resistance in HepG2 cells. (A) Cells were cultured in low glucose (5.5 mM) or high glucose (25 mM) DMEM for 2 days, then serum starved in their respective glucose conditions for 24 hours. We treated cells with 0, 10, or 100 nM insulin for 10 minutes prior to harvesting cells for protein. Western blot was used to measure p-AKT (Ser473) and total AKT levels. (B) Cells were cultured in low glucose (5.5 mM) or high glucose (25 mM) DMEM for 2 days and then treated with 10 nM insulin for 24 hours. Cells were then harvested for RNA and analyzed via qRT-PCR. Bars represent mean $\pm$ standard error of mean (SEM); n=3.

High free fatty acid (FFA) model of insulin resistance in HepG2 cells. We optimized an additional model that induces insulin resistance in HepG2 cells. Like the high glucose model, this model is used in later experiments in combination with HCA treatment and varying levels of NAT2 activity to determine if treatment with HCAs or changes in NAT2 activity can exacerbate effects of excess nutrient conditions on insulin resistance. To measure changes in insulin signaling in response to excess FFA, we cultured HepG2 cells in DMEM containing 1% BSA or varying concentrations of palmitate-BSA (0.125, 0.25, 0.5 mM) for 24 hours, then serum starved them in their respective FFA conditions for 24 hours. We treated cells with 10 nM or 100 nM insulin for 20 minutes prior to harvesting them for protein. Western blot was used to measure induction of p-AKT compared to total-AKT, as previously described. We found that cells cultured in high FFA conditions had a lower p-AKT induction (i.e. lower response to insulin) when compared to cells cultured in BSA-only conditions. These results (Fig. 6A) indicate that high FFA reduces insulin sensitivity in HepG2 cells in a dose-dependent manner, similarly to results reported by others [108].

A.

Figure 6. High free fatty acid (FFA) model of insulin resistance in HepG2 cells. (A) We cultured HepG2 cells in DMEM containing 1% BSA or varying concentrations of palmitate-BSA (0.125, 0.25, 0.5 mM) for 24 hours, then serum starved them in their respective FFA conditions for 24 hours. We treated cells with 10 nM or 100 nM insulin for 20 minutes prior to harvesting cells for protein. Western blot was used to measure p-AKT and total AKT levels.

Generating shRNA cell lines to knockdown NAT2 in HepG2 cells. NAT2 is a highly polymorphic gene, and its SNPs produce a variety of phenotypes, including slow, intermediate, and rapid acetylators. To mimic the NAT2 slow acetylator phenotype, we generated shRNA NAT2 knockdown in HepG2 cells using lentiviral vectors. For our studies, we chose the top two candidates (shRNA4 and shRNA7) of 8 candidates screened. The templates for the corresponding shRNAs were synthesized, annealed, subcloned into a lentiviral vector, and sequence-verified. In our vector, the human U6 small nuclear RNA gene promoter drives the shRNA expression while *EGFP* and puromycin resistance gene (*pac*) are co-expressed under SV40 promoter. Details regarding transfection are discussed in the Materials and Methods section of the text. HepG2 cells on 6-well plates were transduced with the lentivirus in a complete media containing 6 µg/ml polybrene. After 2 days, the cells were placed in complete media containing 2 µg/ml puromycin to select for virus-transduced cells. The puromycin selection and subsequent passaging of the cells continued for approximately 2 weeks. Following selection, the cells were examined under a fluorescent microscope (Fig. 7A), and as expected, virtually every cell in the culture was positive for GFP. NAT2 activity was measured in the generated cell lines as described in the Materials and Methods section, using sulfamethazine (SMZ) as a substrate to measure *in situ* activity (Fig. 7B). These results confirm that the NAT2 activity was successfully knocked down in our HepG2 NAT2 shRNA cell lines.

Figure 7. Generation and characterization of HepG2 cell lines stably expressing control or NAT2 shRNAs. (A) Control and NAT2 shRNA HepG2 cells were examined under a fluorescent microscope following lentiviral transduction and antibiotics selection. (B) NAT2 activity was measured *in situ* in HepG2 shRNA cell lines using SMZ as substrate. Bars represent mean $\pm$ standard error of mean (SEM); n=3.

Generating HepG2 cell lines that overexpress *NAT2*. *NAT2* is a highly polymorphic gene, and its SNPs produce a variety of phenotypes, including slow, intermediate, and rapid acetylators. To mimic the *NAT2* rapid acetylator phenotype, we generated HepG2 cell lines that overexpress *NAT2* using lentiviral vectors. Information regarding generation of lentiviral vectors and transfection can be found in the Methods and Materials section of this text. Cells here were transduced with lentivirus and selected the same as shRNA cell lines, as described above. Fig. 8 provides evidence of successful lentivirus-mediated transduction and upregulation of *NAT2* activity measured by acetylation of sulfamethazine *in situ*, compared to vector control, although these initial results show only moderate upregulation of *NAT2* activity. Additional cell lines overexpressing *NAT2* were generated by two separate *NAT2* clones (*NAT2*-1 and *NAT2*-3) that had greater increase in activity (Fig. 9).

Figure 8. Generation and characterization of HepG2 cell lines stably expressing vector or NAT2. (A) We examined HepG2 cells under a fluorescent microscope following lentiviral transduction. (B) NAT2 activity was measured *in situ* in HepG2 cell lines using SMZ as substrate. Bars represent mean $\pm$ standard error of mean (SEM); n=3.

Figure 9. Generation and characterization of additional HepG2 cell lines stably expressing vector or NAT2. (A) We examined HepG2 cells under a fluorescent microscope following lentiviral transduction. (B) NAT2 activity was measured *in situ* in HepG2 cell lines using SMZ as substrate. ***, $p < 0.001$. Bars represent mean $\pm$ standard error of mean (SEM); $n=3$.

Significant changes in gluconeogenic and lipogenic gene expression are observed in HepG2 shRNA knockdown cell lines in low and high glucose conditions. To investigate the effects of low NAT2 activity on transcripts of gluconeogenic and lipogenic genes (insulin target genes), we cultured HepG2 NAT2 shRNA cells in low (5.5 mM) and high (25 mM) glucose serum-free media for one day and then harvested for RNA. As previously described, gluconeogenesis and lipogenesis are altered with changes in metabolic function (glucose and lipid production). We measured gluconeogenic (*G6PC*, *PCK1*) and lipogenic (*FASN*, *SREBF1*) gene transcripts via qRT-PCR. Even in the absence of high glucose, gluconeogenic and lipogenic gene expression was downregulated in NAT2 shRNA cells, compared to control shRNA cells. These results (Figs. 10 and 11) indicate that NAT2 activity may affect regulation of glucose and lipid production. Additional experiments incorporating insulin into the experimental design are required to determine the effect of NAT2 activity on insulin sensitivity.

Figure 10. Gluconeogenic gene expression in control and NAT2 shRNA

HepG2 cells (low vs. high glucose). HepG2 shRNA cell lines were cultured in low glucose (5.5 mM) or high glucose (25 mM) serum-free DMEM conditions for 1 day and harvested for RNA. G6PC and PCK1 levels were measured via qRT-PCR. (A) depicts data grouped by glucose treatment and compares control to NAT2 shRNA4 and shRNA7. (B) depicts data grouped by shRNA cell line and compares low to high glucose treatment. Statistical significance was determined by two-way ANOVA followed by Bonferroni's Posttest; ***, $p < 0.001$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Figure 11. Lipogenic gene expression in control and NAT2 shRNA HepG2 cells (low vs. high glucose). HepG2 shRNA cell lines were subjected to low glucose (5.5 mM) or high glucose (5.5 mM) serum-free DMEM conditions for 1 day and harvested for RNA. FASN and SREBF1 levels were measured via qRT-PCR. (A) depicts data grouped by glucose treatment and compares control to NAT2 shRNA4 and shRNA7. (B) depicts data grouped by shRNA cell line and compares low to high glucose treatment. Statistical significance was determined by two-way ANOVA followed by Bonferroni's Posttest; *, < 0.05, **, $p < 0.01$, ***, $p < 0.001$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Significant changes in gluconeogenic and lipogenic gene expression are observed in HepG2 shRNA knockdown cell lines in the high FFA model. In addition to the high glucose model, the previously described high FFA model was used as a system of insulin resistance with the HepG2 shRNA cells. We cultured HepG2 shRNA NAT2 knockdown cells in 1% BSA or 0.5 mM palmitate-BSA serum-free media for one day and then harvested for RNA. We measured gluconeogenic (G6PC, PCK1) and lipogenic (FASN, SREBF1) gene expression via qRT-PCR. In response to high FFA, NAT2 shRNA cells showed significant alterations in the pattern and magnitude of gene expression. The high FFA treatment downregulated *FASN* and *SREBF1* transcripts in control cells. NAT2 shRNA cells, however, showed increases in expression of the lipogenic genes under the high FFA condition. In addition, *PCK1* mRNA was significantly upregulated in high FFA-treated NAT2 shRNA cells, while the treatment did not affect *PCK1* expression in control cells. These results (Fig. 12, Fig. 13) suggest that depletion of NAT2 leads to reductions in glucose and lipid production in HepG2 cells at both normal and excessive levels of nutrients and that hepatocytes expressing low NAT2 activity may be less able to effectively respond to the switch between fed and fasted states. Further investigation of the dysregulation of gluconeogenic and lipogenic genes in NAT2 knockdown cells will aid in understanding how NAT2 influences hepatic glucose and lipid homeostasis. Incorporating insulin into the experimental design will aid in understanding how changes in NAT2 activity affect insulin sensitivity.

Figure 12. Gluconeogenic gene expression in control and NAT2 shRNA

HepG2 cells (high FFA model). HepG2 shRNA cell lines were subjected to 1% BSA or 0.5 mM Palmitate-BSA for 1 day and harvested for RNA. G6PC and PCK1 levels were measured via qRT-PCR. (A) depicts data grouped by free fatty acid treatment and compares control to NAT2 shRNA4 and shRNA7. (B) depicts data grouped by shRNA cell line and compares control (BSA) to high free fatty acid (PA) treatment. Statistical significance was determined by two-way ANOVA followed by Bonferroni's Posttest; **, < 0.01, ***, p < 0.001. Bars represent mean +/- standard error of mean (SEM); n=4.

Figure 13. Lipogenic gene expression in control and NAT2 shRNA HepG2 cells (high FFA model). HepG2 shRNA cell lines were subjected to 1% BSA or 0.5 mM Palmitate-BSA for 1 day and harvested for RNA. FASN and SREBF1 levels were measured via qRT-PCR. (A) depicts data grouped by free fatty acid treatment and compares control to NAT2 shRNA4 and shRNA7. (B) depicts data grouped by shRNA cell line and compares control (BSA) to high free fatty acid (PA) treatment. Statistical significance was determined by two-way ANOVA followed by Bonferroni's Posttest; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Cytotoxicity assay was performed in HepG2 cells to determine optimal concentrations of HCA treatments. We wanted to ensure HCAs would not result in cell death during experiments, so we tested MeIQ and PhIP, two common HCAs found in cooked meats [42], at different concentrations in HepG2 cells to detect cell viability. Cells were treated with the indicated compounds at varying concentrations for 3 days in complete media with no media change during this time. alamarBlue™ assay was used to measure cell viability. There was relatively little to no cell death in each of the compounds tested until the concentration of 100 µM, where nearly half of the cells died in MeIQ (Fig.14). Moving forward, most experiments used a range of HCA concentrations to investigate dose-response, and these concentrations ranged from 0 to 100 µM, although we later switched to ranges from 0 to 50 µM to prevent cell death.

Figure 14. Cell viability in HepG2 cells after treatment with MelQ and PhIP.

Cells were treated with either MelQ or PhIP at concentrations of 0, 0.1, 1.0, 10, or 100 μ M in complete media for 3 days. alamarBlue™ assay was used to measure cell viability. Bars represent mean $\pm$ standard error of mean (SEM); n=4.

No statistically significant change in gluconeogenic or lipogenic gene expression in HepG2 cells following exposure to PhIP. HCAs are known human carcinogens and have recently been associated with insulin resistance and high serum triglycerides [100 – 103]. HCAs are produced when cooking meats at high temperatures or for long periods of time. Cooking method, temperature, type of meat, and length of cooking time impact the type of HCA and level present in the cooked meat [46 – 48], and this is further described in previous sections of this text. PhIP is the most common HCA found in cooked meats, though it is less genotoxic compared to other HCAs [44, 109]. To investigate if exposure to PhIP impacts gluconeogenic or lipogenic gene expression, we cultured HepG2 cells in low-glucose or high-glucose media containing PhIP at varying concentrations (0 – 100 μ M) for 3 days and then harvested for RNA. The samples were analyzed by qRT-PCR to measure transcripts of genes involved in gluconeogenesis and lipogenesis. There were no statistically significant changes observed in gluconeogenic (G6PC, PCK1) or lipogenic (FASN, SREBF1) gene expression following exposure to PhIP at any concentration in either low glucose or high glucose conditions. These results (Fig. 15) suggest that PhIP has little impact on regulation of glucose and lipid production in hepatocytes.

Figure 15. Gluconeogenic and lipogenic gene expression in HepG2 cells

following PhIP treatment. HepG2 cells were cultured in low glucose (5.5 mM)

or high glucose (25 mM) DMEM and DMSO or varying concentrations (0, 1, 10 or

100 μ M) of PhIP for 3 days and then harvested for RNA. Samples were analyzed

with qRT-PCR. Statistical significance was determined by one-way ANOVA. Bars

represent mean $\pm$ standard error of mean (SEM); n=4.

Gluconeogenic and lipogenic gene expression in HepG2 cells is

dysregulated by exposure to particular HCAs. To investigate the effects of HCAs on gluconeogenic and lipogenic gene transcription, we screened a variety of HCAs, including MeIQ, MeIQx, IQ, and IQx, as these are some additional common HCAs found in cooked meats. We cultured HepG2 cells with each HCA for 3 days and then harvested for RNA. The samples were analyzed by qRT-PCR to measure transcripts of genes involved in gluconeogenesis and lipogenesis. We observed a variety of results among the different HCAs. G6PC was upregulated in MeIQx, while G6PC and SREBF1 were downregulated in IQ and IQx. There were no statistically significant changes in FASN among any of the HCAs screened compared to the control group (Fig. 16). This variety of results led us to believe that metabolic processes (glucose and lipid production) are dysregulated by HCAs, but it is currently unclear how and what is causing the differences in dysregulation among varying HCAs. Additionally, HepG2 cells may already exhibit irregular metabolic function because they are a transformed cell line [124]. This led to further investigation of MeIQ and MeIQ-like compounds in primary human hepatocytes, discussed later in this text.

Figure 16. Gluconeogenic and lipogenic gene expression in HepG2 cells

following HCA treatment. HepG2 cells were cultured in low glucose (5.5 mM) DMEM and DMSO or varying concentrations (5 or 50 μ M) of MelQ, MelQx, IQ, or IQx for 3 days and then harvested for RNA. Samples were analyzed with qRT-PCR. Statistical significance was determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test; *, $p < 0.05$, **, $p < 0.01$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Dysregulation of gluconeogenic and lipogenic gene expression in HepG2 cells in low glucose conditions following MeIQ exposure is further

analyzed. Since the previous experiment screening several HCAs had high variability within experimental groups, we repeated the experiment with additional concentrations of MeIQ to analyze if exposure to MeIQ altered transcripts of G6PC, PCK1, FASN, and SREBF1. We cultured HepG2 cells in low glucose DMEM containing MeIQ at varying concentrations (0 – 100 μ M) for 3 days and then harvested for RNA. The samples were analyzed by qRT-PCR to measure transcripts of genes involved in gluconeogenesis and lipogenesis. *G6PC*, *PCK1*, and *FASN* were significantly upregulated following MeIQ exposure. In contrast, *SREBF1* was not significantly altered by MeIQ exposure. These results suggest MeIQ may directly impact steady-state glucose and lipid production in hepatocytes, although insulin treatment must be incorporated into the experimental design to measure alterations in insulin sensitivity. The statistical significance found in this experiment that was not apparent in the results from Figure 17 can likely be accredited to reduced variability within groups in the current experiment.

Figure 17. Gluconeogenic and lipogenic gene expression in HepG2 cells following MelQ treatment. HepG2 cells were cultured in low glucose (5.5 mM) DMEM and DMSO or varying concentrations (0, 1, 10 or 100 μ M) of MelQ for 3 days and then harvested for RNA. Samples were analyzed with qRT-PCR. Statistical significance was determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test; *, $p < 0.05$, **, $p < 0.01$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Changes in gluconeogenic and lipogenic gene expression in HepG2 cells following MeIQ exposure is not observed in the high glucose model. To

investigate effects of MeIQ exposure on gluconeogenic and lipogenic gene expression in the presence of excess nutrients, we cultured HepG2 cells in high glucose DMEM containing MeIQ at varying concentrations (0 – 100 μ M) for 3 days and then harvested for RNA. The samples were analyzed by qRT-PCR to measure transcripts of genes involved in gluconeogenesis and lipogenesis. The changes in gene expression previously observed are no longer present in the high glucose model. The excess nutrients appear to overcome the previously observed effects of MeIQ. These results (Fig. 18) suggest that dysregulation of glucose and lipid production resulting from excess glucose supersede the effects of MeIQ observed in normal nutrient conditions, resulting in no significant changes among varying MeIQ treatment concentrations.

Figure 18. Gluconeogenic and lipogenic gene expression in HepG2 cells following MelQ treatment. HepG2 cells were cultured in high glucose (25 mM) DMEM and DMSO or varying concentrations (1, 10 or 100 μ M) of MelQ for 3 days and then harvested for RNA. Samples were analyzed with qRT-PCR. Statistical significance was determined by one-way ANOVA. Bars represent mean $\pm$ standard error of mean (SEM); n=4.

MelQ exposure significantly upregulates gluconeogenic gene transcripts and significantly downregulates lipogenic gene transcripts in primary human hepatocytes. In addition to HepG2 cells, we investigated the effects of HCAs on gluconeogenic and lipogenic gene transcripts in primary human hepatocytes, as these are more physiologically relevant and have relatively normal metabolic function (compared to HepG2 cells, which are transformed cancer cells). We cultured primary human hepatocytes in high glucose DMEM containing 0, 5, or 50 μ M MelQ for 3 days and then harvested for RNA. These samples were analyzed via qRT-PCR. G6PC and PCK1 gene transcripts were upregulated significantly in 5 and 50 μ M MelQ compared to the control (DMSO). Additionally, FASN and SREBF1 gene transcripts were significantly downregulated in 50 μ M MelQ. These results (Fig. 19) suggest that MelQ exposure affects gluconeogenic and lipogenic gene transcript levels also in primary human hepatocytes. We will need to introduce insulin into the experimental design to examine if MelQ affects sensitivity to insulin.

Figure 19. Gluconeogenic and lipogenic gene expression in primary hepatocytes following MelQ treatment. Primary human hepatocytes were cultured in high glucose (25 mM) DMEM with 0, 5, or 50 μM MelQ for 3 days and then harvested for RNA. Samples were analyzed by qRT-PCR. Statistical significance was determined by one-way ANOVA followed by Tukey's Multiple Comparison Test; *, $p < 0.05$, ***, $p < 0.001$. Bars represent mean $\pm$ standard error of mean (SEM); $n=4$.

Insulin-induced p-AKT is reduced by MelQ exposure in primary human

hepatocytes. Although there was a significant difference in gluconeogenic and lipogenic gene transcription in primary human hepatocytes following MelQ exposure, we wanted to determine if MelQ exposure alters insulin signaling as well. We analyzed the effects of MelQ on primary human hepatocytes via p-AKT induction (by insulin) on Western blot. Primary human hepatocytes were cultured with 0, 5, or 50 μ M MelQ in complete media for 2 days, then serum-starved for 24 hours (still with MelQ treatment in serum-free media). Hepatocytes were then treated with 100 nM insulin for 10 minutes. There was an apparent reduction in p-AKT induction in response to insulin in cells treated with MelQ (Fig. 20). These results suggest that MelQ reduces insulin sensitivity in primary human hepatocytes.

Figure 20. Insulin-induced p-AKT following MelQ treatment in primary hepatocytes. Primary human hepatocytes were cultured with 0, 5, or 50 μ M MelQ in complete media for 2 days. Hepatocytes were serum-starved with MelQ treatment for 24 hours. Hepatocytes were then treated with 100 nM insulin for 10 minutes then harvested for protein. Samples were analyzed via Western blot to measure induction of p-AKT.

No change in glucose production is observed in primary hepatocytes

following MelQ treatment. Since we observed changes in gluconeogenic gene expression and insulin signaling in primary hepatocytes following MelQ treatment, we chose to examine if MelQ treatment altered glucose production in primary hepatocytes. Hepatocytes were treated with complete media containing varying concentrations of MelQ for 2 days. Then, media was replaced with serum-free, glucose-free media containing varying concentrations of MelQ for 6 hours. After completion of this period, media was replaced with serum-free, glucose-free media containing gluconeogenic substrates (2 mM pyruvate, 20 mM lactate, 0.5 mM lysine, 5 mM glycerol) for 24 hours. Finally, the media was collected for extracellular measurement of glucose (via glucose oxidase (GO) assay kit), and cells were harvested for normalization to total protein. There was no detectable change in glucose production following treatment with MelQ (Fig. 21).

Figure 21. Glucose production in primary hepatocytes following MeIQ treatment. Primary human hepatocytes were cultured in complete media containing 0, 5, or 50 µM MeIQ for 2 days. Media was replaced with serum-free, glucose-free media containing respective concentrations of MeIQ for 6 hours. Media was replaced in appropriate wells with serum-free, glucose-free media containing gluconeogenic substrates (2 mM pyruvate, 20 mM lactate, 0.5 mM lysine, 5 mM glycerol) for 24 hours. Media was collected for extracellular glucose measurements via a glucose oxidase (GO) assay kit, and cells were harvested for total protein measurements. Bars represent mean $\pm$ standard error of mean (SEM); n=4.

DISCUSSION

There have been recently documented associations between dietary heterocyclic amine (HCA) intake and insulin resistance and between *N*-acetyltransferase 2 (NAT2) polymorphisms and insulin resistance [111, 112]. However, the association between dietary HCA intake and pathogenesis of insulin resistance has yet to be confirmed or directly investigated, and it is currently unclear the role of NAT2-mediated metabolism in this process. The current study investigates the effects of HCA and NAT2 phenotypes on insulin sensitivity and expression levels of genes involved in glucose and lipid metabolism in hepatocytes of human origin, including HepG2 (hepatocellular carcinoma) cells and cryopreserved human primary hepatocytes. Dysregulation of glucose and lipid production was observed in HepG2 NAT2 shRNA cell lines in both normal and excess nutrient conditions (Figs. 10 – 13), suggesting that NAT2 activity levels may directly play a role in glucose and lipid homeostasis in hepatocytes. MeIQ, a common HCA found in cooked meat, also altered levels of mRNA gene transcripts of genes involved in gluconeogenesis and lipogenesis and reduced insulin signaling, suggesting that MeIQ may directly impact steady-state glucose and lipid production and reduce insulin sensitivity in hepatocytes, although no changes in glucose production were observed in response to MeIQ treatment.

HepG2 NAT2 shRNA cell lines and measuring transcripts of insulin-target genes

Recently, certain genetic polymorphisms of NAT2 have been linked to insulin resistance, high serum triglyceride, coronary artery disease, and high fasting plasma glucose level [112 – 116]. Analysis of *Nat1*-knockout (homolog of human *NAT2*) mice revealed that *Nat1* deficiency leads to development of insulin resistance and metabolic defects at the systemic level, supporting the previously unrecognized role of NAT2 in regulating metabolism and insulin sensitivity [117 – 118]. However, it is currently unknown how NAT2 influences cellular metabolism and insulin sensitivity.

In the current study, dysregulation of genes involved in glucose and lipid metabolism was observed in HepG2 NAT2 shRNA cell lines in both normal and excess nutrient conditions (high glucose and high free fatty acid) (Figs. 10-13). A significant reduction ($p < 0.001$) in transcripts of genes involved in gluconeogenesis (*G6PC*, *PCK1*) and lipogenesis (*FASN*, *SREBF1*) was detected in both NAT2 shRNA cell lines (shRNA4 and shRNA7) in low and high glucose conditions (Fig. 10, Fig. 11), indicating that the presence of excess nutrients was not required for alterations in mRNA transcript levels, but rather reduced NAT2 activity caused the difference observed.

The high FFA treatment, in contrast, downregulated *FASN* and *SREBF1* transcripts in control cells. NAT2 shRNA cells, however, showed increases in expression of the lipogenic genes under the high FFA condition. In addition, *PCK1* mRNA was significantly upregulated in high FFA-treated NAT2 shRNA

cells, while the treatment did not affect *PCK1* expression in control cells. These results (Figs. 12 – 13) suggest that depletion of NAT2 leads to reduction in glucose and lipid metabolism in HepG2 cells at both normal and excessive levels of nutrients and that hepatocytes expressing low NAT2 activity may be less able to effectively respond to the switch between fed and fasted states. Further investigation of the dysregulation of gluconeogenic and lipogenic genes in NAT2 knockdown cells will aid in understanding how NAT2 influences hepatic glucose and lipid homeostasis. Incorporating insulin into the experimental design will aid in understanding how changes in NAT2 activity affect insulin sensitivity. Additionally, utilizing cryopreserved primary human hepatocytes with naturally occurring slow, intermediate, or rapid NAT2 acetylator phenotypes will provide a more physiologically relevant model for these experiments and translation of the results to human populations.

PhIP treatment on HepG2 cells and measuring transcripts of insulin-target genes

HCAs are produced when cooking meats at high temperatures or for long periods of time. Cooking method, temperature, type of meat, and length of cooking time impact the type of HCA and level present in the cooked meat [46 – 48]. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a common HCA found in cooked meats, though it is less genotoxic compared to other HCAs [44, 109]. Though we do not know if genotoxicity of HCAs is linked to their

hypothesized effect on insulin sensitivity, we chose to utilize PhIP for initial experiments since it is the most common HCA found in cooked meat.

In the current study, there were no statistically significant changes observed in gluconeogenic (*G6PC*, *PCK1*) or lipogenic (*FASN*, *SREBF1*) gene expression following exposure to PhIP at any concentration in either low glucose or high glucose conditions. These results (Fig. 15) suggest that PhIP may have little impact on regulation of insulin-target genes in hepatocytes. Additionally, previous studies investigating carcinogenic potential of HCAs largely focused on MeIQ and MeIQ-like compounds because they are more genotoxic in mammalian cells [38]. Thus, we chose to focus future experiments in this study on MeIQ and MeIQ-like compounds.

Screening of treatment with different HCAs on HepG2 cells and measuring transcripts of insulin-target genes

Several different HCAs are found in cooked meats, and the type of HCA and their concentrations vary depending on cooking method, temperature, type of meat, and length of cooking time [46 – 48]. Since no changes in transcript levels of insulin-target genes were observed with PhIP treatment in HepG2 cells, we aimed to screen additional HCAs for future experiments. 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ); and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ); and their structurally related compounds 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx); and 2-amino-3,8-dimethylimidazo[4,5-

f]quinoxaline (8-MeIQx) were screened next, as these are additional HCAs frequently found in cooked meat [49 – 53].

In this experiment, we observed a variety of results among the different HCAs (Fig. 16). *G6PC* was upregulated in MeIQx, while *G6PC* and *SREBF1* were downregulated in IQ and IQx, compared to control treatment. There were no statistically significant changes in *FASN* levels among any of the HCAs screened compared to the control group. This variety of results led us to believe that metabolic processes (glucose and lipid production) are dysregulated by HCAs, but it is currently unclear how and what is causing the differences in dysregulation among varying HCAs.

As the mechanisms by which HCAs alter insulin-target gene transcription remains unknown, structural differences among different HCAs may account for differences observed in this study [123]. Additionally, HepG2 cells may already exhibit irregular metabolic function because they are an immortalized cell line [124], and thus it is unclear if some differences seen in this study are the result of HepG2 model system. Moreover, we conducted experiments with MeIQ and MeIQ-like compounds in primary human hepatocytes to account for potential irregularities in HepG2 cellular metabolic function.

MelQ treatment on HepG2 cells and measuring transcripts of insulin target genes

As previously mentioned, MelQ and MelQ-like compounds are HCAs frequently found in cooked meat [49 – 53]. Our previous study (Fig. 16) indicated that gluconeogenic and lipogenic gene transcripts were dysregulated with some of the compounds in this structural group of HCAs, so we investigated this phenomenon further.

In this study, *G6PC*, *PCK1*, and *FASN* transcripts were significantly upregulated following MelQ exposure. In contrast, *SREBF1* was not significantly altered by MelQ exposure (Fig. 17). These results suggest MelQ may impact steady-state glucose and lipid production in hepatocytes, although insulin treatment must be incorporated into the experimental design to measure alterations in insulin sensitivity. The statistical significance found in this experiment that was not apparent in the results from Figure 16 can likely be accredited to reduced variability within experimental groups in the current experiment.

We additionally incorporated our previously characterized high glucose model (Fig. 5) into our experiment with MelQ treatment on HepG2 cells to evaluate if excess nutrient conditions further exacerbated the effects observed in normal nutrient conditions. In this study, the changes in gene expression previously observed (Fig. 17) are no longer present in the high glucose model conditions. The excess glucose appears to abolish the previously observed effects of MelQ, resulting in no change between the MelQ-treated groups and

control groups. Another consideration is that if both high glucose and MelQ use the same pathway to induce gluconeogenic genes, then additive or synergistic effects may not be observed. These results (Fig. 18) suggest that dysregulation of glucose and lipid metabolism resulting from excess glucose supersede the effects of MelQ treatment observed in normal nutrient conditions, resulting in no significant changes among varying MelQ treatment concentrations, compared to control. It is unclear at this time why effects of MelQ treatment observed in low glucose conditions are not present in high glucose conditions. However, we need to incorporate insulin treatment into our experimental design to measure changes in mRNA transcripts in response to insulin to further investigate this observation.

MelQ treatment on cryopreserved human primary hepatocytes and measuring transcripts of insulin-target genes

MelQ treatment on HepG2 cells significantly changed mRNA levels of insulin target gene transcripts (Fig. 17). However, as previously mentioned, HepG2 cells are immortalized and thus may exhibit irregularities in metabolic function and glucose and lipid homeostasis [124]. Thus, we chose to additionally investigate effects of MelQ treatment in cryopreserved human primary hepatocytes.

In this study, *G6PC* and *PCK1* gene transcripts were upregulated significantly following treatment with 5 and 50 μ M MelQ compared to the control (DMSO). Additionally, *FASN* and *SREBF1* gene transcripts were significantly

downregulated following treatment with 50 μ M MeIQ. These results (Fig. 19) suggest that MeIQ alters gluconeogenic and lipogenic gene transcription in primary human hepatocytes, similarly to the effects observed in HepG2 cells. Again, we will need to introduce insulin into the experimental design to examine if MeIQ affects sensitivity to insulin. However, these results indicate a change in glucose and lipid homeostasis induced by treatment of MeIQ, further validating the previously observed phenomenon.

MeIQ treatment on cryopreserved human primary hepatocytes and measuring p-AKT induction as an indicator of insulin sensitivity

MeIQ treatment significantly altered gluconeogenic and lipogenic gene transcript levels in human primary hepatocytes (Fig. 19). However, changes in mRNA transcripts of gluconeogenic and lipogenic genes is not adequate to determine insulin sensitivity. We followed up by incorporating insulin into the experimental design and measuring insulin-mediated induction of p-AKT, a key component of the insulin cascade, which is discussed in detail in the Background portion of this text (Fig.4).

In this study, there was an apparent reduction in p-AKT induction in response to insulin in cells treated with MeIQ (Fig. 20). These results suggest that MeIQ induces insulin resistance in primary human hepatocytes. However, these results do not indicate if there is a change in glucose production in response to insulin following treatment of MeIQ. Thus, a glucose production

assay is needed to measure the ultimate physiological effect of insulin resistance.

MelQ treatment on cryopreserved human primary hepatocytes and measuring glucose production

MelQ treatment on HepG2 cells significantly changed mRNA levels of gene transcripts involved in insulin signaling (Fig. 17), and there was an apparent reduction in p-AKT induction in response to insulin in cells treated with MelQ (Fig. 20). However, neither of these endpoint measurements indicate if MelQ treatment results in a change of glucose production in response to insulin. Thus, we utilized a glucose production assay to measure extracellular glucose concentrations in response to insulin following treatment with MelQ.

In this study, there was no detectable change in glucose production following treatment with MelQ (Fig. 21). It is currently unclear as to why changes in gluconeogenic gene transcripts verified by a reduction in insulin signaling did not translate into changes in glucose production. However, this experiment still needs to be repeated to ensure reproducibility. Additionally, insulin must be incorporated into the experimental design to determine changes in glucose production in response to insulin treatment following MelQ treatment.

CONCLUSIONS AND SUMMARY

Strengths of work

Recently, NAT2 polymorphisms have been linked to insulin resistance, high serum triglyceride levels, coronary artery disease, and high fasting plasma glucose level [112 – 116]. A deficiency of mouse *Nat1* leads to development of insulin resistance and metabolic defects in *Nat1*-knockout mice [117, 118]. However, thus far, all studies on this link have been conducted in murine systems. Using *Nat1* KO mice as a surrogate for studying the effects of human NAT2 presents major limitations. As previously reviewed [125], the mouse *Nat1* coding sequence is only slightly more homologous to human NAT2 than to human NAT1, and for amino acid homology, mouse Nat1 is actually more homologous to human NAT1 (73%) than human NAT2 (72%). Mouse Nat1 is much less stable than human NAT2 [125]. Mouse Nat1 is expressed across every tissue examined [126], whereas human NAT2 expression is largely limited to liver and GI tract. Additionally, the substrate profiles of mouse Nat1 and human NAT2 are less similar than previously believed [127]. Therefore, it is likely that mouse Nat1 and human NAT2 do not share identical roles. Also, the findings obtained from *Nat1* KO mice are limited by the fact that human NAT2 alleles that were reported to be linked to insulin resistance, as well as coronary artery disease and high triglyceride levels, are not null but *functional* alleles [116].

Therefore, murine models of Nat1 biology may *not* accurately reflect the role of human NAT2 in insulin resistance. The present study utilizes *human* cell systems (HepG2 NAT2 shRNA cell lines) that mimic the conditions created by different NAT2 alleles. Thus, this study may provide a more clinically relevant depiction of the effects of NAT2 activity on insulin sensitivity in humans.

Additionally, a significant association was recently reported between HCA exposure via cooked meat consumption and insulin resistance after the multivariate analysis was adjusted for saturated fatty acid and cholesterol intake [111]. However, this association has yet to be confirmed in cell or animal models. There was a lack of laboratory studies entirely on this alleged link. Thus, this study reports the first findings in associations between HCA exposure and insulin resistance in a cell culture model.

Our results indicate that both level of NAT2 activity and exposure to certain HCAs may alter cellular glucose and lipid homeostasis. Additionally, these results demonstrate changes in sensitivity to insulin following exposure to some HCAs. These findings are significant because they indicate a genetic component (NAT2 polymorphism) and an environmental component (dietary HCA exposure via consumption in cooked meat) in the pathogenesis of insulin resistance in hepatocytes of human origin. As insulin resistance is a key component of type II diabetes mellitus and metabolic syndrome [71 – 73], these findings pose a significant contribution to public health.

Limitations of work

The present study utilizes human cell systems (HepG2 NAT2 shRNA cell lines) that mimic slow acetylator NAT2 phenotype. However, additional studies must be conducted using HepG2 cells overexpressing NAT2, which serve to mimic rapid acetylator NAT2 phenotype. Moreover, as previously mentioned, HepG2 cells are immortalized and may not represent a physiologically accurate model of “normal” energy homeostasis. HepG2 cells also have naturally low *NAT2* expression. To account for this, cryopreserved human primary hepatocytes that have been genotyped and grouped by rapid, intermediate, or slow NAT2 acetylator phenotype should be used to confirm findings from experiments conducted with HepG2 cells.

Additionally, this study reports this first findings in associations between HCA exposure and insulin resistance in a cell culture model. However, several experiments presented here need to be repeated with insulin treatment incorporated into the experimental design so that changes in response to insulin may be measured. Furthermore, associations between HCA exposure and insulin resistance have yet to be investigated in animal models. MeIQx, the second most abundant HCA found in cooked meat [47], has been shown to cause liver tumors in animal models [128]. A previous study reported that oral administration of MeIQx (0.06% in food pellet) for 84 weeks in CDF1 mice resulted in increased frequency of hepatocellular carcinoma in male and female mice [128]. Similar animal models to those investigating carcinogenesis and HCA

exposure in mice can be applied to the studies here to analyze endpoints relating to insulin signaling and insulin sensitivity.

Lastly, and most importantly, these studies have not yet incorporated both NAT2 polymorphism and HCA treatment into one experimental design. The major metabolic pathway of HCAs includes hepatic *N*-hydroxylation by CYP1A2 followed by *O*-acetylation by NAT2 to form reactive oxygen species (ROS) leading to oxidation of lipids, proteins, and nucleic acids [129, 130]. Future studies should investigate both the novel link between HCA exposure to the development of insulin resistance and the role of NAT2-mediated metabolism in this process to consider a gene-environmental interaction.

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CURRICULUM VITAE

Kennedy M. Walls

University of Louisville School of Medicine

Department of Pharmacology & Toxicology

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Louisville, KY 40202

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EDUCATION

BS	University of Louisville, Chemistry Focus in Biochemistry	May 2019
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HONORS, AWARDS, CERTIFICATIONS

IPIBS Fellowship (full tuition and stipend)	2019 - 2021
Awarded by the University of Louisville upon admittance as a graduate student to the Department of Pharmacology and Toxicology	

Rachel Davis Memorial Scholarship Recipient	2019
Awarded by the Rachel Davis Memorial Scholarship Endowment Chair for displaying exceptional stewardship in environmental extracurriculars; monetary award	

University of Louisville Cancer Education Program Trainee	2018
Participant in the 10-week summer Cancer Education Program, a competitive program funded by the National Institute of Health; aims to train the next generation of cancer researchers	

University of Louisville Undergraduate Research Grant	2018
Awarded by the University of Louisville to sponsor undergraduate research and poster presentation	

Pharmacy Technician Certification 2017
Awarded by the National Healthcare Association upon passing the Exam of the Certified Pharmacy Technicians (ExCPT)

Speed School Academic Affairs Scholarship 2016
Awarded by the J.B. Speed School of Engineering Academic Affairs Committee for displaying academic excellence; monetary award to assist in paying for study abroad

P.E.O. STAR Scholarship Recipient 2015
Awarded by the national P.E.O chapter for exhibiting excellence in leadership, academics, extracurricular activities, community service and potential for future success; monetary award for higher education

Kentucky Governor's Scholar 2014
Participant in the Kentucky Governor's Scholars Program, a highly competitive summer program which provides opportunity for academic and personal growth

RESEARCH EXPERIENCE

University of Louisville, Department of Pharmacology and Toxicology 2019 – present

Graduate fellow, Hein Lab

- Determining effects of NAT2 genetic polymorphism on insulin sensitivity
- Evaluating impacts of HCAs on insulin resistance
- Investigating the role of NAT2-mediated metabolism of HCAs in pathogenesis of insulin resistance

University of Louisville, Department of Microbiology and Immunology 2019
Research Scientist, Sokoloski Lab

- Selecting for viral revertants by serial passaging the existing SINV nsP1 mutants until genetic reversion was observed via a change in viral kinetics or an oblique alteration of virus phenotype
- Identifying the genetic basis of the reversion event(s) using next-generation sequencing technologies to map the amino acid changes with respect to time
- Biochemically and biologically assessing the revertants by using site-directed mutagenesis to recapitulate the observed amino acid changes in a clean genetic background to evaluate the impact of the reversion events on viral RNA synthesis and viral capping kinetics in tissue culture models of infection

Cancer Education Program, Department of Pharmacology and Toxicology 2018
Research Scientist, Siskind Lab

- Determining the effects on renal function of using erlotinib as an injury-ameliorating agent following the completion of the clinically relevant CDDP repeated dosing model
- Executing lab techniques including PCR, Western Blot analysis, IHC to collect data in an independent study

University of Louisville, Department of Pharmacology and Toxicology 2018
Research Scientist, Siskind Lab

- Utilizing a clinically relevant mouse model of cisplatin-induced kidney injury
- Determining the effects on renal function of using erlotinib, an EGFR inhibitor, in combination with cisplatin

PRESENTATIONS

American Society of Pharmacology and Experimental Therapeutics

April 2021

Department of Pharmacology and Toxicology

“Changes in Insulin Signaling and Gluconeogenic Gene Expression in Human Hepatocytes Following Exposure to Heterocyclic Amines”

Graduate Student Regional Research Conference, virtual

February 2021

Department of Pharmacology and Toxicology

“Changes in Insulin Signaling and Gluconeogenic Gene Expression in Human Hepatocytes Following Heterocyclic Amine Treatment”

Ohio Valley Society of Toxicology Annual Meeting, virtual

November 2020

Department of Pharmacology and Toxicology

“Depletion of arylamine *N*-acetyltransferase 2 results in dysregulation of gluconeogenic and lipogenic genes in HepG2 hepatocellular carcinoma cell line”

UofL Pharmacology and Toxicology Dept. Seminar, virtual

April 2020

Department of Pharmacology and Toxicology

“Heterocyclic Amines and Arylamine *N*-Acetyltransferase 2 Polymorphism in Insulin Resistance”

ACC Meeting of the Minds, Louisville, KY

March 2019

Department of Pharmacology and Toxicology

“Repeated administration of cisplatin increases EGFR/EGFR activation and renal fibrosis in Kras4bG12D lung adenocarcinoma-bearing mice, but kidney injury is further exacerbated with erlotinib/cisplatin combination treatment”

Posters at the Capitol, Frankfort, KY

February 2019

Department of Pharmacology and Toxicology

“Repeated administration of cisplatin increases EGFR/EGFR activation and renal fibrosis in Kras4bG12D lung adenocarcinoma-bearing mice, but kidney injury is further exacerbated with erlotinib/cisplatin combination treatment”

Research!Louisville Poster Presentation, Louisville, KY

October 2018

Department of Pharmacology and Toxicology

“Repeated administration of cisplatin increases EGFR/EGFR activation and renal fibrosis in Kras4bG12D lung adenocarcinoma-bearing mice, but kidney injury is further exacerbated with erlotinib/cisplatin combination treatment”

Cancer Education Program Poster Presentation, Louisville, KY

August 2018

Department of Pharmacology and Toxicology

“Repeated administration of cisplatin increases EGFR/EGFR activation and renal fibrosis in Kras4bG12D lung adenocarcinoma-bearing mice, but kidney injury is further exacerbated with erlotinib/cisplatin combination treatment”

University of Louisville Undergraduate Research Symposium

April 2018

Department of Pharmacology and Toxicology

“Repeated administration of cisplatin increases EGFR/EGFR activation and renal fibrosis in Kras4bG12D lung adenocarcinoma-bearing mice, but kidney injury is further exacerbated with erlotinib/cisplatin combination treatment”

PEER REVIEWED PUBLICATIONS

Hong, K. U., Doll, M. A., Lykoudi, A., Salazar-González, R. A., Habil, M. R., **Walls, K. M.**, . . . Hein, D. W. (2020). Acetylator Genotype-Dependent Dyslipidemia in Rats Congenic for N-Acetyltransferase 2. *Toxicology Reports*. doi:10.1016/j.toxrep.2020.09.011

WORK EXPERIENCE

Walmart Pharmacy
Certified Pharmacy Technician

2016-2019

PROFESSIONAL TRAINING

Graduate Student Regional Research Conference

Virtual, 2021

Attended virtually and presented orally; viewed oral and poster presentations

Ohio Valley Society of Toxicology Annual Meeting

Virtual, 2020

Attended virtually and presented poster; viewed oral and poster presentations

ACC Meeting of the Minds Annual Conference

Louisville, KY 2019

Attended and presented research poster; participated in networking and professional development seminars

Society of Women Engineers Annual Conference

Nashville, TN 2015

Attended and participated in networking and professional development seminars

PROFESSIONAL AFFILIATIONS

American Society of Pharmacology and Experimental Therapeutics, 2020-present

Society of Toxicology, 2020-present

Society of Undergraduate Chemistry Students, 2016-2019

Society of Women Engineers, 2015-2019

PROFESSIONAL SERVICE

University of Louisville Undergraduate Peer Mentor Host 2015-2019
Provide campus tours to prospective chemistry students at the University of Louisville

VESA Student Helper 2018-2019
Assist in distributing material to University of Louisville students about the Volunteer Eco Students Abroad program
Present orally about VESA experience to prospective students

GREEN Program On-Campus Ambassador 2017-2018
Lead a recruitment campaign on the University of Louisville campus for the GREEN study abroad program
Create and distribute marketing materials to provide information to students
Present orally at club meetings and relevant classrooms about the program

SERVICE ABROAD

Volunteer Eco Students Abroad
Volunteer, South Africa, May 2018
Volunteer focus in conservation, education, and construction
Built a watering hole at St. Lucia Crocodile Center, cleaned pens of savannah cats at The Emdoneni Cheetah Project
Taught mathematics/English at local school in the Zulu Village, prepared lunches at local orphanage
Built the foundation of a home gifted to a local family of 9 deemed in dire need of assistance

Elephant Haven
Volunteer, Thailand, July-August 2017
Offered time and compassion to feed and bathe elephants in a sanctuary setting during the rehabilitation process

EDUCATIONAL TRAVEL

Reykjavik University Iceland School of Energy 2016
Studied geothermal energy, hydroelectric energy, energy economics
Toured clean energy facilities