

ORIGINAL ARTICLE

Plasma sphingolipids are biomarkers of metabolic syndrome in non-human primates maintained on a Western-style diet

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BACKGROUND: The intake of a Western diet enriched in animal fat has been shown to be a major risk factor for Type 2 diabetes and obesity. Previous rodent studies have indicated that these conditions may be triggered by the accumulation of the sphingolipid ceramide in insulin-sensitive tissues. However, data are lacking in this regard from both humans and non-human primates.

OBJECTIVE: Here we have investigated the relationship between plasma ceramides and metabolic syndrome in Rhesus macaques fed a high-fat and high-fructose (HFFD) 'western' diet.

METHODS: We investigated this relationship in cohorts of monkeys fed a HFFD for a period of 8 months to 5 years. Animals were classified as control, pre-diabetic or diabetic based on fasting plasma parameters and insulin sensitivity.

RESULTS: HFFD treatment produced significant increases in body weight and body fat and also resulted in a decline in insulin sensitivity. In parallel to the reduction in insulin sensitivity, significant increases in both plasma ceramide and dihydroceramide levels were observed, which further increased as animals progressed to the diabetic state. Plasma levels of the rare sphingolipid C18:0 deoxysphinganine, a marker of increased metabolic flux through serine palmitoyl transferase (SPT), were also elevated in both pre- and diabetic animals. Furthermore, plasma serine levels were significantly elevated in diabetic monkeys, which may indicate a shift in SPT substrate selectivity from serine to alanine or glycine. In contrast, branch chain amino acids were unchanged in pre-diabetic non-human primates, and only plasma valine levels were elevated in diabetic animals.

CONCLUSION: Together, these data indicate that HFFD induces *de novo* synthesis of ceramides in non-human primates, and that increased production of plasma ceramides is significantly correlated with the decline in insulin sensitivity.

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INTRODUCTION

Type 2 diabetes mellitus is a major health concern in the world today and is predicted to reach epidemic proportions within the next decade.¹ Although genetic predisposition plays a part in the etiology of this disease, environmental influences such as diet and physical activity both play large roles. In particular, consumption of diets enriched in saturated animal fats has been shown to be causative in initiating insulin resistance and the associated syndromes of dyslipidemia and obesity (collectively referred to as Metabolic Syndrome).^{1,2} This idea has led to the hypothesis that intake of a nutrient-rich diet high in fat initiates accumulation of toxic lipid species that results in insulin resistance, also referred to as 'lipotoxicity'.^{3,4}

Although many hypotheses have been put forth on the identity of the 'toxic' lipid species involved in the generation of insulin resistance due to nutrient influx, a great deal of recent data suggest that the sphingolipid ceramide is the causative agent in this process.^{2,3,5} *De novo* production of ceramides occurs in the endoplasmic reticulum and is catalyzed by serine palmitoyl transferase (SPT), which is the rate-limiting enzyme for *de novo* synthesis of sphingolipids.^{6–8} This enzyme condenses L-serine and palmitoyl-coenzyme A (CoA) to form 3-ketosphinganine that is then converted into ceramides of various chain lengths by a series of enzymatic reactions (Figure 1).⁹ Ceramides are subsequently transported to the plasma membrane where they are

concentrated in 'lipid raft' microdomains that are enriched in both sphingolipids and cholesterol, which serve as platforms for signaling molecule complexes, including the insulin receptor.^{10–13} It is thought that increased accumulation of ceramide in these domains has a deleterious effect on insulin signaling either via activation of protein phosphatase (PP2A) that blocks insulin mediated activation of Akt or by disruption of lipid raft-associated signaling complexes, both of which are crucial for insulin-stimulated glucose uptake.^{14–17}

Although there are a considerable amount of data indicating that intracellular ceramides have deleterious effects on insulin signaling, the vast majority of these studies come from *in vitro* experiments utilizing incubations with supraphysiological concentrations of fatty acids.^{3,5,18} Although these studies are useful for eliciting the mechanism of ceramide-induced insulin resistance, they lack physiological context. Although there are a few human studies correlating elevated levels of plasma ceramides with insulin resistance,^{19–23} there is an absence of longitudinal *in vivo* data showing that dietary-induced elevations in plasma ceramides track with deterioration of insulin sensitivity.

In the present study we have examined plasma ceramide levels in groups of rhesus macaque monkeys that were placed on a defined high-fat and high-fructose diet (HFFD) for varying lengths of time, classified as pre-diabetic or diabetic, and have then correlated these data with markers of insulin resistance. These

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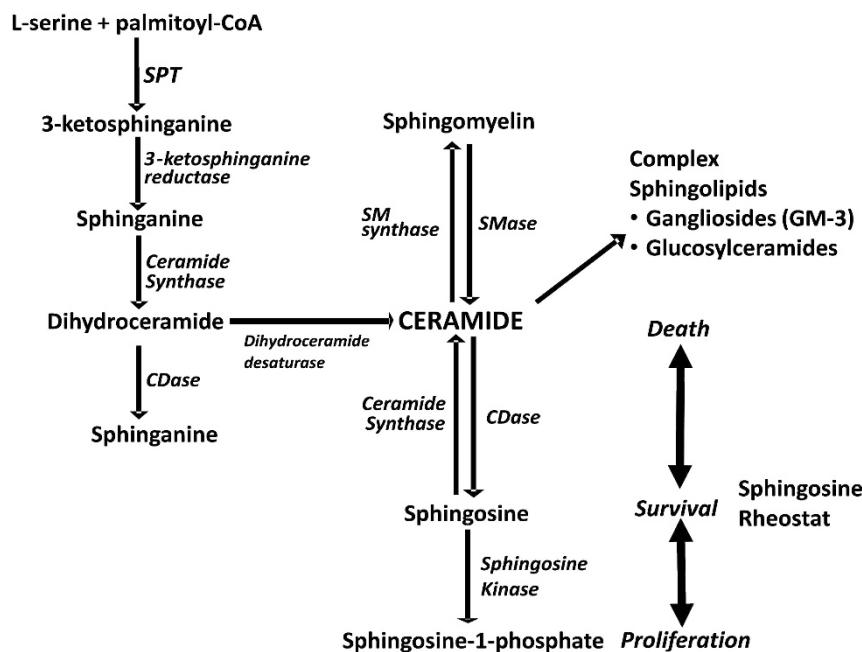


Figure 1. Pathways for *de novo* synthesis of ceramide and ceramide metabolism. Enzymes are shown in italics, whereas arrows indicate direction of reaction. CDase, ceramidase; SMase, sphingomyelinase; SM synthase, sphingomyelin synthase.

data show that elevations in plasma ceramides are markers of a reduction in *in vivo* insulin sensitivity. Additionally, the rare sphingolipid deoxy-sphinganine (deoxySa) is shown to be a potential marker of diabetic progression.

MATERIALS AND METHODS

Animals

All animal care and procedures were done according to the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center at Oregon Health & Science University. Male animals were utilized for all described studies. Animals were fed a diet high in sucrose and fat (HF), manufactured by TestDiet (Test Diet, Richmond, IN, USA) (Diet 5LOP, 36.1% of energy from animal fat, 45.7% from carbohydrates and 18.2% from protein; cholesterol 661 p.p.m.) and food was available *ad libitum* from 1000 h to 1630 h.²⁴ The animals did not have access to food overnight. Animals on the HF diet were supplemented with 500 ml of Kool-Aid (Kraft Foods Group, Glenview, IL, USA) drink mix + 20% fructose three times a week (HFFD). The control animals were fed regular chow (TestDiet 5052, 14.6% of energy from fat, 58.5% from carbohydrates and 26.8% from protein). Water was provided *ad libitum* at all times. Lights were on from 0700 h to 1900 h.

Animals were considered diabetic when sedated fasting glucose levels surpassed 5.5 mmol l⁻¹ or when fasting insulin levels exceeded 2084 pmol l⁻¹ (normal average was 235.4 pmol l⁻¹) and when they demonstrated impaired insulin secretion during the intravenous glucose tolerance test (IVGTT; lack of primary and/or secondary insulin secretion in response to a glucose bolus suggestion β-cell damage).²⁵ Under these parameters, five animals were classified as diabetic and none of the animals displayed β-cell failure.

Body composition

Body composition was determined by dual-energy X-ray absorptiometry (DEXA) scan. Briefly, animals were sedated with Telazol (3 mg kg⁻¹) and the sedation was maintained with ketamine (5–10 mg kg⁻¹). The animals were laid flat on the DEXA (Hologic Discovery A, Bedford, MA, USA) and a whole body scan performed. Data were imported into an excel file and analyzed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Glucose tolerance test and hormone measurements

Glucose homeostasis was determined via IVGTTs, which measure blood glucose clearance after an intravenous bolus infusion of a sterile 50%

dextrose solution (600 mg kg⁻¹), as described in McCurdy *et al.*²⁶ Animals were not fed on the morning of IVGTTs. Blood glucose was measured immediately in whole blood with a glucometer (OneTouch Ultra Blood Glucose Monitor, LifeScan, Milpitas, CA, USA). Plasma was assayed for insulin and leptin by the ONPRC/OHSU Endocrine Services Laboratory using an Immulite 2000 (Siemens USA, Deerfield, IL, USA).

Lipid analysis

Reagents. The reagents 14:0, 16:0, 18:0, 20:0, 22:0, 24:0, 24:1 Ceramides (Cer), 16:0, 18:0, 24:0, 24:1 Dihydroceramides (DHCer), 16:0, 18:0, 24:1 Glucosylceramides (GlcCer), 17:0, 18:0 Sphingosine (Sph), 18:0 Dihydro-sphingosine (Sa), 17:0, 18:0 sphingosine-1-phosphate, C18:0 dihydro sphingosine-1-phosphate (SaS1P) and 1-deoxysphinganine (1-deoxySa) were purchased from Avanti Lipids (Avanti Polar Lipids, Alabaster, AL, USA). Stable-isotope labeled ceramides (C16:0, C18:0, C22:0, C24:1 and C24:0) were prepared from [3-¹³C], [¹⁵N]-sphingosine and 17:0 GlcCer that were synthesized internally.

Sphingolipid analysis

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS) analysis of sphingolipids was performed using a TSQ Quantum Ultra-triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray ionization probe and interfaced with an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA). Lipid extracts were separated with a Xbridge C8 (2.1 × 30 mm with 3.5 μm, Waters, Milford, MA, USA). Mobile phase A was MeOH/H₂O/CHCl₃/Formic acid (55:40:5:0.4% by v/v) and mobile phase B was MeOH/Acetonitrile/CHCl₃/Formic acid (48:48:4:0.4% by v/v). Mass spectrometric analyses were performed online using electrospray ionization tandem mass spectrometry in the positive multiple reaction monitoring mode. Samples were extracted using 1 phase extraction method (methanol-dichloromethane) with internal standards. Sphingosines, sphingosine-1-phosphate, sphinganine, dihydro sphingosine-1-phosphate, ceramides, dihydroceramides and glucosylceramides were quantified by the ratio of analyte and internal standard and calibration curves obtained by serial dilution of a mixture of sphingolipids.

Statistical analysis

Data were analyzed for statistical differences by analysis of variance using JMP 9.0 software (SAS Institute Inc., Cary, NC, USA). When a significant F-ratio was found, differences between means were determined by Dunnett's *post hoc* analysis, with a level of significance set at *P* < 0.05. All

Table 1. Body composition and plasma lipid parameters

Group	Age (years)	Time on diet at the time of sampling (months)	Total mass (kg)	% Fat	Cholesterol (mmol l ⁻¹)	TG (mmol l ⁻¹)	HDL (mmol l ⁻¹)	LDL (mmol l ⁻¹)
Control (12)	9.5 ± 0.5	NA	9.5 ± 1.8	22.7 ± 2.6	3.2 ± 0.28	1.2 ± 0.30	1.5 ± 0.17	1.1 ± 0.26
Pre-diabetic (10)	9.7 ± 0.6	31.7 ± 5.9	16.9 ± 9.9*	36.0 ± 2.3*	4.9 ± 0.30*	1.3 ± 0.23	2.9 ± 0.29*	1.6 ± 0.13
Diabetic (5)	12.6 ± 0.8	29.2 ± 8.1	17.4 ± 1.5*	42.3 ± 2.5*	5.0 ± 0.57*	3.2 ± 0.67**	1.9 ± 0.28**	1.7 ± 0.31

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not applicable; TG, triglyceride. Values are expressed as mean ± s.e.m. Numbers in parentheses indicate number of animals. *Statistically different from control value, $P < 0.05$. **Statistically different from corresponding pre-diabetic value, $P < 0.05$.

Table 2. Glucose homeostasis parameters

Group	Fasting glucose (mmol l ⁻¹)	Fasting insulin (pmol l ⁻¹)	HOMA-IR (mmol l ⁻¹ × μU ml ⁻¹)	Glucose AUC	Insulin AUC
Control (12)	3.2 ± 0.18	235.4 ± 74.3	4.5 ± 1.5	7446.9 ± 582.3	8930.7 ± 2364.6
Pre-diabetic (10)	3.4 ± 0.14	510.5 ± 107.0	10.7 ± 1.9	9084.5 ± 625.0	21 208.2 ± 6734.5
Diabetic (5)	5.4 ± 0.48***	3664.2 ± 1515.4***	112.4 ± 36.4***	11 422.5 ± 1436.8*	33 273.1 ± 16 891.1

Abbreviations: AUC, area under the curve; HOMA-IR, homeostasis model assessment for insulin resistance. Values are expressed as mean ± s.e.m. Numbers in parentheses indicate number of animals. *Statistically different from control value, $P < 0.05$. **Statistically different from corresponding pre-diabetic value, $P < 0.05$.

data are reported as mean ± s.e.m. Spearman's rank correlations and partial correlation analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA), with a level of significance set at $P < 0.05$. Partial correlations between sphingolipids and homeostasis model assessment for insulin resistance (HOMA-IR), glucose area under the curve (AUC), insulin AUC and fasting glucose or insulin were calculated with the effect of length of time on diet or percentage body fat (%BF) removed.

RESULTS

HFFD induced alterations in plasma chemistry profiles

We examined a range of physiological parameters in young adult Rhesus monkeys placed on HFFD for periods of time that varied from 8 to 66 months and then classified them as pre-diabetic or non-insulinopenic diabetic based on the parameters described in the Materials and methods section. When compared with control animals on normal chow, the HFFD significantly increased both body weight as well as %BF in both pre-diabetic and diabetic animals (Table 1). Various plasma lipid parameters were also significantly altered because of HFFD feeding in both pre-diabetic and diabetic animals. Total cholesterol levels were significantly increased in both pre-diabetic and diabetic monkeys, whereas low-density lipoprotein was unchanged. Plasma high-density lipoprotein levels were transiently elevated in pre-diabetic monkeys, and were not significantly different from control in diabetic animals. In agreement with the other changes in plasma lipids, plasma triglyceride levels were not altered in pre-diabetic animals, whereas they were significantly elevated in diabetics. These data indicate that the HFFD induced changes in plasma lipids indicative of development of metabolic syndrome.

The HFFD also had profound effects on insulin sensitivity (Table 2). As shown in Table 2, fasting glucose and insulin were unchanged in pre-diabetics but significantly elevated in diabetic animals, which resulted in a significant increase in HOMA-IR in these animals alone. The IVGTT data paralleled the changes in fasting glucose and insulin (Table 2) in that both glucose and insulin AUC trended upward in both groups; however, glucose AUC alone was significantly different in diabetic animals, indicative of a decrease in glucose clearance. These data signify marked declines in insulin sensitivity due to the HFFD, and

together with the alterations in plasma lipids are consistent with induction of metabolic syndrome in these animals.

Alterations in plasma sphingolipid levels due to HFFD

To explore the link between plasma ceramides and HFFD-induced insulin resistance, we measured plasma sphingolipid levels in control, pre-diabetic and diabetic animals via liquid chromatography/mass spectrometry. Both total plasma dihydroceramides and ceramides were significantly elevated in pre-diabetic and diabetic animals, with diabetic animals showing higher levels for both species (Figure 2 and Supplementary Figure 1). This contrasted with the observed changes in the species of each individual lipid. Although, in general, increases were noted in all species of both lipids in pre-diabetic and diabetic animals, ceramide species C14:0, C16:0, C22:0 and C24:0 showed the largest changes in diabetics as compared with the controls, whereas the C24:1 species was unchanged in either condition. Similarly, dihydroceramides C16:0, C22:0 and C24:0 showed the largest changes in diabetics as compared with controls, whereas C14:0 showed smaller increases.

De novo synthesis of ceramides occurs via SPT that condenses serine and palmitoyl-CoA to form 3-ketosphinganine, which is ultimately converted to ceramide via a series of enzymatic steps (Figure 1).^{9,27} Ceramides are in turn metabolized to a number of bioactive lipids, including sphingomyelin (SM) and sphingosine (Sph) or sphinganine (Sa) (Figure 1). Sph and to a lesser degree Sa are also acted upon by sphingosine kinase to form sphingosine-1-phosphate (S-1-P) and sphinganine-1-phosphate (Sa-1-P), which are agonists at the EDG (endothelial differentiation gene 1) family of receptors.^{28,29} As shown in Supplementary Table 1, plasma Sa and Sph levels were unchanged in pre-diabetic animals, but were significantly elevated in diabetics. In contrast, S-1-P was significantly elevated in both groups of animals as compared with controls, whereas Sa-1-P was unchanged. SM is another sphingolipid in equilibrium with ceramides. However, SM levels were unchanged in either pre-diabetic or diabetic animals, indicating that the increases in ceramides were due to *de novo* synthesis and that the major flux through this pathway occurs through ceramidase to generate Sph, and subsequently S-1-P. Phosphatidylcholine (PC), a lipid species involved in SM and diacylglycerol

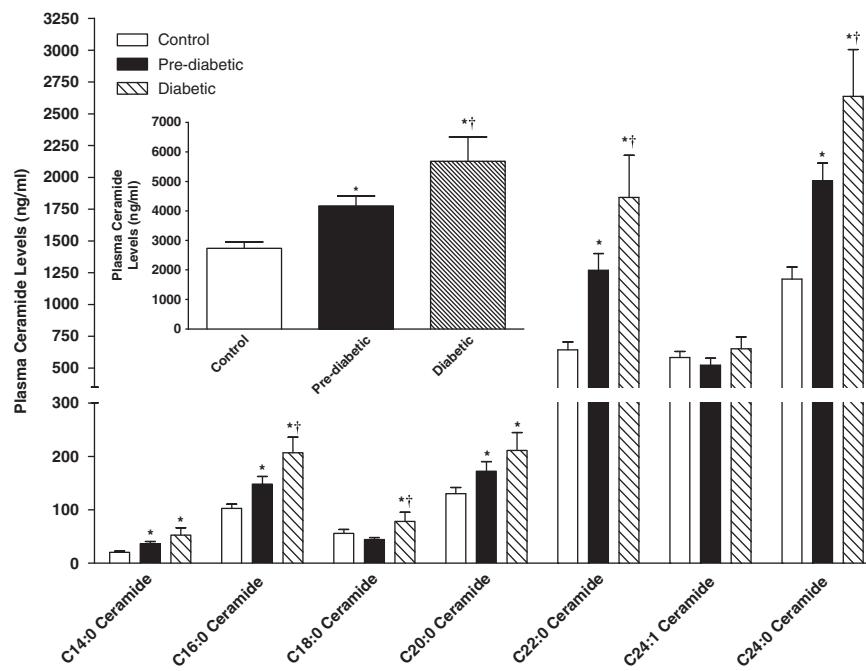


Figure 2. Plasma ceramide levels from control, pre-diabetic and diabetic Rhesus Macaque monkeys. Various ceramide species were separated and detected via liquid chromatography/mass spectrometry (LC/MS), and are expressed as ng ml^{-1} of plasma. Inset figure shows total ceramide levels. Error bars indicate s.e.m. *Statistically significant difference from corresponding control value, $P < 0.05$. **Statistically significant difference from corresponding pre-diabetic value, $P < 0.05$.

synthesis, was also measured, as a decline in the PC to SM ratio indicates an increase in the enrichment of SM in lipoprotein particles.³⁰ In the present data, however, we observed that this ratio was actually increased in both pre-diabetic and diabetic animals, but only reached statistical significance in the diabetic group. As SM is largely carried in low-density lipoprotein particles,³⁰ this may reflect the observed small increase in low-density lipoprotein (Table 1), or the transient increase in high-density lipoprotein, as a smaller fraction of SM is also carried in this particle.

In addition to ceramide, gangliosides are another class of sphingolipids, derived from ceramides using glucosylceramide as a precursor, that have been implicated as a modulator of lipotoxicity-induced insulin resistance.^{31–34} As shown in Supplementary Figures 2 and 3, the plasma levels of C16:0 as well as C18:0 species of ganglioside GM-3 were significantly elevated in both pre-diabetic and diabetic monkeys, as well as the levels of four of the six measured species of glucosylceramide (C16:0, C22:0, C24:0 and C24:1). However, unlike what was observed with the ceramides, only C16:0 GM-3 was significantly elevated (Supplementary Figure 3) in the diabetics, whereas C18:0 were not different from control values. These data indicate that upregulation of other reactions are utilizing glucosylceramides to form additional lipid species that prevent their conversion to GM-3, but do in part support previous observations that GM-3 levels are elevated in insulin resistance and diabetes.

HFFD induces production of deoxySa

As previously noted, SPT catalyzes the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine. Recently published data have shown that SPT mutations causing hereditary sensory neuropathies alter the substrate selectivity of SPT from serine to alanine and glycine.^{30,35–38} Condensation of these amino acids with palmitoyl-CoA produces deoxySa and deoxy-methylsphinganine, respectively, and it is hypothesized that concentration of nonmetabolizable products of these lipids in peripheral nerves are responsible for these neuropathies.^{35,37} It

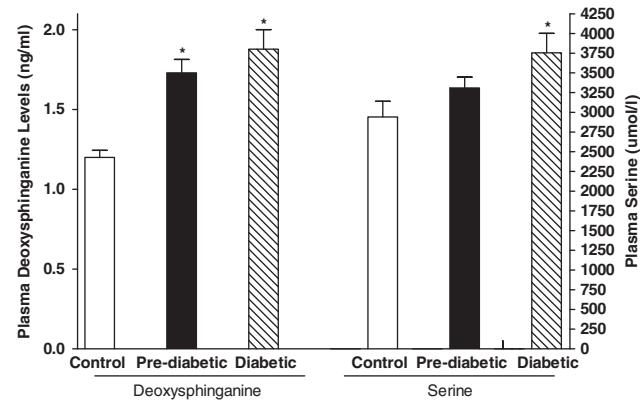


Figure 3. Plasma deoxySa and serine levels from control, pre-diabetic and diabetic Rhesus Macaque monkeys. C18:0 deoxySa and serine were separated and detected via liquid chromatography/mass spectrometry (LC/MS), and expressed as ng ml^{-1} of plasma. Error bars indicate s.e.m. *Statistically significant difference from corresponding control value, $P < 0.05$.

has been shown that levels of deoxy-sphingoid lipids are elevated in plasma of human diabetics, indicating an increase in the frequency of the utilization of alanine or glycine over serine by SPT in diabetes.^{30,38} To investigate this hypothesis, we measured the levels of deoxySas in plasma from the monkeys in the present study (Figure 3). Although deoxy-methylsphinganine levels were beneath the level of detection, significant elevations in deoxySa were detected in the pre-diabetic and diabetic groups as compared with control animals. These data support a hypothesis that HFFD feeding increases the frequency of utilization of alanine by the SPT reaction and production of deoxySa.

As deoxySa levels are directly related to serine metabolism, we also analyzed plasma serine as well as the plasma levels of the branch chain amino acids (isoleucine, leucine and valine) (Figure 3 and Supplementary Table 2), as elevated levels of branch chain

Table 3. Spearman's rank correlations of sphingolipids with various *in vivo* parameters

Lipid parameter	Time on diet		%BF		Fasting glucose		Fasting insulin		HOMA-IR		Glucose AUC		Insulin AUC	
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value
DeoxySa C18:0	0.812	<0.0001	0.545	0.003	0.263	0.184	0.660	0.0002	0.664	0.0002	0.335	0.095	0.534	0.005
GM-3 (16:0)	0.444	0.016	0.402	0.038	0.037	0.109	0.315	0.272	0.235	0.127	0.224	0.373	-0.206	0.264
GM-3 (18:0)	0.269	0.158	0.307	0.856	-0.152	0.237	0.182	0.312	-0.003	0.460	0.227	0.988	-0.138	0.502
Ceramide (14:0)	0.607	0.0005	0.557	0.003	0.504	0.007	0.448	0.022	0.532	0.005	0.123	0.322	0.311	0.122
Ceramide (16:0)	0.490	0.007	0.390	0.044	0.541	0.004	0.295	0.143	0.383	0.053	0.202	0.550	0.106	0.605
Ceramide (18:0)	0.167	0.388	0.040	0.842	0.452	0.018	-0.075	0.716	0.020	0.925	-0.005	0.980	-0.253	0.212
Ceramide (20:0)	0.468	0.011	0.404	0.037	0.493	0.009	0.265	0.191	0.363	0.068	0.112	0.587	0.041	0.844
Ceramide (22:0)	0.654	0.0001	0.540	0.004	0.463	0.015	0.573	0.002	0.666	0.0002	0.162	0.428	0.296	0.142
Ceramide (24:0)	0.656	0.0001	0.559	0.003	0.490	0.010	0.597	0.001	0.687	0.0001	0.252	0.214	0.293	0.146
Ceramide (24:1)	-0.169	0.380	-0.017	0.933	0.165	0.411	0.013	0.949	0.074	0.721	-0.126	0.539	0.006	0.978
Total ceramides	0.568	0.0013	0.465	0.015	0.480	0.011	0.472	0.015	0.577	0.002	0.111	0.589	0.219	0.282

Abbreviations: AUC, area under the curve; %BF, percentage body fat; DeoxySa, deoxy-sphinganine; HOMA-IR, homeostasis model assessment for insulin resistance. Bold indicates correlations that are statistically significant.

amino acids have been implicated in insulin resistance and diabetes.³⁹ Although plasma serine levels were significantly elevated in the diabetics, the levels of branch amino acids were unchanged in pre-diabetics, with only plasma valine levels showing an increase in diabetics. Therefore, the increases in plasma serine noted in the diabetics may be indicative of a shift in substrate utilization by SPT.

Plasma sphingolipids correlate with measures of insulin sensitivity and obesity

Spearman's correlation analysis of the relationship between the sphingolipid data and insulin sensitivity parameters are shown in Table 3. This analysis indicated that C14:0, C16:0, C22:0, C24:0 and total ceramides as well as C18:0 deoxySa showed significant correlations with HOMA-IR, indicating that elevated plasma sphingolipids negatively correlate with insulin sensitivity. Plasma sphingolipid levels also showed significant correlation with %BF, showing that obesity plays a part in increasing plasma ceramides. In addition to %BF, the length of time on diet could also contribute to increases in ceramide. To investigate this, Spearman's correlations using residuals were calculated with the effect of each variable independently removed (Supplementary Tables 3 and 4). When the effect of either variable was removed, the correlations between short chain (C14:0, 16:0 and 20:0) and HOMA-IR was lost, whereas the correlations with longer chain ceramides (C22:0 and 24:0) remained significant. An exception was C18:0 deoxySa, which lost significance only when the effect of time on diet was removed. Thus, the length of exposure to HFFD seems to play a larger role in the rise in plasma C18:0 deoxySa, than does obesity.

DISCUSSION

It has been hypothesized that consumption of a diet rich in saturated animal fats leads to generation of a lipid metabolite that in turn triggers insulin resistance associated with the increasing prevalence of obesity and Type 2 diabetes (noninsulin-dependent diabetes mellitus).^{1,4,40–42} The nature of this lipid species has been extensively investigated over the past decade, and considerable recent focus has been placed on the sphingolipid ceramide.^{2,18,21,41,43} However, there are little data linking elevations in plasma levels of ceramides to the development of insulin resistance in humans or non-human primates. Here we have performed an extensive analysis of various plasma sphingolipids in rhesus monkeys, which we utilized because of the closer similarity in their sphingolipid profile to humans than in cynomolgus monkeys, another extensively studied species.^{19,20} This analysis was performed on animals placed on normal chow or on a defined HFFD 'western' diet for various periods of time and

characterized as pre-diabetic or diabetic based on measures of insulin sensitivity.

A primary finding of the present investigation is the significant increase in plasma ceramides due to HFFD feeding, and its correlation with markers of *in vivo* insulin sensitivity. Total ceramide mass as well as a number of individual ceramide species were increased in both pre-diabetic and diabetics as compared with control animals. In parallel with these changes, reductions in insulin sensitivity were also noted, as fasting insulin, HOMA-IR, glucose AUC and insulin AUC were all increased in animals from these groups. Indicative of induction of metabolic syndrome by the HFFD, %BF, body mass as well as plasma triglycerides and cholesterol were all also increased in these animals. It is also noteworthy that the levels of four of the seven ceramide species measured, as well as total ceramide levels, were further increased in the diabetic animals as compared with pre-diabetics, indicating that the decline in markers of insulin sensitivity correlates with further increase in plasma ceramides. Spearman's correlation analysis (Table 3) indicated that four of the species of ceramide (14:0, 16:0, 22:0 and 24:0) as well as total ceramides were significantly correlated with HOMA-IR. This relationship is also driven by both obesity (%BF) and the length of time the animals were on the diet, as removal of either variable from the analysis resulted in the loss of correlation between HOMA-IR and the shorter chain ceramide species (C14:0, C16:0 and C20:0), which are believed to have a larger role in disruption of insulin signaling.^{17,41,43,44}

These results differ from a previously published report in spontaneously diabetic cynomolgus monkeys that indicated that plasma ceramides in diabetic monkeys declined when compared with control animals.²² However, we believe that both the HFFD feeding paradigm utilized in the present study and the similarity between the sphingolipid profiles of rhesus and humans make these results more relevant to the etiology of human disease.

It is also significant that differences exist in the responses of the various ceramide species to the HFFD. Plasma levels of two of the shorter ceramide species (C14:0 and C16:0) showed some the largest changes (2.6- and 2.0-fold, respectively) compared with control animals, likely because of these animals consuming a diet enriched in animal fat and comprising largely C16:0 fatty acids (palmitic acid).²⁴ Additionally, these two species of ceramide were significantly correlated with the largest number of *in vivo* parameters (%BF, fasting insulin, fasting glucose and HOMA-IR; Table 3). Ceramides are concentrated in microdomains within the plasma membrane (lipid rafts) that serve as platforms for complexes of signaling molecules.^{13,45,46} Interestingly, it has been shown that shorter chain ceramide species are more disruptive to membrane architecture and organization of signaling complexes within these domains.^{17,41,43,44} Therefore, although the shorter chain ceramides

are less abundant based on their total mass, the increases in these species are especially significant in that they play a larger role in disruption of insulin signaling.

Ceramides are also further metabolized to create additional biologically active sphingolipids (Figure 1).²⁸ These include ceramide-1-phosphate, SM, Sph, Sa and S-1-P, a potent agonist at EDG1 receptors that is involved in atherosclerosis.^{47,48} In the present results, SM levels were not altered in HFFD animals, indicating that pathway flux through SM synthase does not appear to be a major disposal pathway for ceramide in primates. This is further supported by the elevation in plasma PC levels, which indicates a reduction in PC utilization by SM synthase. Together with the elevations in Sph (diabetics only) and S-1-P, these data indicate that the major pathway for metabolism of *de novo* generated ceramides is through ceramidase. We hypothesize that this is a compensatory reaction in order to minimize the apoptotic effects of an increase in intracellular ceramide levels (Figure 1, 'Sphingosine Rheostat'); however, it will require analysis of tissues from these animals to determine if this hypothesis is correct.

It has been proposed that a ceramide metabolite rather than ceramide itself is the causative agent in lipid-induced insulin resistance. Most notably, previously published animal, human and *in vitro* studies have implicated increases in GM-3 rather than ceramide in insulin resistance.^{31,34,49,50} Here we show that although both C16:0 and C18:0 GM-3 were elevated in pre-diabetics, neither of these lipids showed a significant correlation with any insulin sensitivity parameter (Table 3). Although it is possible that GM-3 participates in HFFD-induced insulin resistance, ceramides appear to be a more robust biomarker of this condition.

A second key finding of the present paper is the increase in deoxySa, which occurs because of the condensation of alanine rather than serine with palmitoyl-CoA by SPT. This metabolite lacks the C1 hydroxyl group of Sa and cannot be further metabolized to form complex sphingolipids.³⁷ This misincorporation occurs infrequently *in vivo*; however, the frequency of this event is increased by mutations in the SPT1 subunit of SPT that cause hereditary sensory neuropathies (HSAN1),^{30,35,36} a disease characterized by loss of sensory neurons and chronic ulceration in the lower extremities.⁵¹ Furthermore, the etiology and symptoms of this disease are similar to those of diabetic neuropathy and human diabetics show significant increases in C18:1 deoxySa.^{30,38} Here we show for the first time that increased consumption of a HFFD increases plasma C18:0 deoxySa. Although a direct causative role for these lipids in insulin resistance is unclear, we hypothesize that they are a biomarker of insulin resistance and diabetic complications, such as neuropathy. This is supported by the fact that when length of time on diet is removed from the analysis, the correlation between C18:0 deoxySa and HOMA-IR is lost (Supplementary Table 3). In addition, this lipid species was significantly correlated with fasting insulin, insulin AUC and HOMA-IR (Table 3) as well as %BF, providing further support for the value of deoxySa as a biomarker of insulin resistance.³⁸

It was previously assumed that the increases in deoxySa resulted from an elevation in SPT activity due to increased dietary substrate provision. However, recent studies have suggested that SPT exists in a complex with other proteins that regulate activity of the enzyme.^{52,53} Thus, an alternative hypothesis is that intake of a HFDD diet causes deregulation of the SPT enzyme that consequently increases the frequency of alanine incorporation. This hypothesis is further supported by the increases in dihydroceramides (Supplementary Figure 1), also indicative of increased SPT activity and substrate flux.

The increase in plasma C18:0 deoxySa is paralleled by an increase in plasma serine levels in the diabetic animals. Serine is synthesized from the glycolytic precursor 3-phosphoglycerate, and therefore this could be attributed to increased synthesis via glycolytic metabolism, but may also reflect a reduction in serine catabolism. *De novo* sphingolipid synthesis via SPT represents

an intersection between lipid (palmitoyl-CoA) and amino acid (serine) metabolism, and is a major catabolic sink for serine. We hypothesize that the increase in plasma serine represents a reduction in catabolism of serine by SPT because of increased alanine utilization.³⁰ Moreover, this may also indicate that plasma serine levels are potentially an additional biomarker of lipotoxic insulin resistance.

A potential caveat of the present work is that we are measuring plasma ceramides as surrogate biomarkers of tissue insulin resistance, but have not measured ceramides in insulin-sensitive tissues. Although it is assumed that liver is responsible for generation of the majority of plasma ceramides,^{30,54} other cell types can contribute significant amounts of ceramide to the plasma in the form of exosomes, vesicles secreted from cells in response to the fusion of multivesicular bodies with the plasma membrane.^{55,56} These vesicles are purported to function in cell-cell communication by merging with and releasing their contents into other cells.^{56,57} This represents a mechanism whereby *de novo* ceramide production in one tissue can induce insulin resistance in other peripheral tissues. Nevertheless, the differential biological effects of plasma versus intracellular ceramide on insulin-sensitive tissues is an area that will require further investigation.^{17,46,58}

In conclusion, these data have shown that provision of a HFFD 'Western' diet to nonhuman primates increased *de novo* ceramide biosynthesis and resulted in elevated plasma levels of ceramides. Although these data do not provide a direct causal relationship between ceramides and insulin resistance, the increases in ceramides significantly correlated with reductions in insulin sensitivity in the pre-diabetic and diabetic animals. Furthermore, the increases in deoxySa support that SPT activity is deregulated, and indicate that these sphingolipids are an additional marker of insulin resistance. Although the identity of the tissue(s) responsible for the increase in plasma ceramides remains unknown, this will require further analysis of sphingolipid levels in insulin-sensitive tissues from these animals, and perhaps analysis of the sphingolipid content of the raft domain to definitively answer this question. Clearly, this should remain an area of vigorous research for the foreseeable future.

CONFLICT OF INTEREST

Joseph Brozinick, Eric Hawkins, Hai-Hoang Bui, Ming-Shang Kuo and Bo Tan are employees of Eli Lilly and Company. Kevin Grove is a paid consultant of Eli Lilly and Company.

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