Metabolic Fingerprints of Proliferative Diabetic Retinopathy: An ¹H-NMR–Based Metabonomic Approach Using Vitreous Humor

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PURPOSE. To explore the metabolic profile of vitreous fluid of patients with proliferative diabetic retinopathy (PDR) using ¹H-NMR–based metabonomic analysis.

METHODS. ¹H-NMR spectra were acquired from vitreous samples obtained during vitrectomy from 22 patients with type 1 diabetes with PDR and from 22 nondiabetic patients with macular hole (control group). Data analysis included a principal component analysis and partial least squares discriminant analysis (PLS-DA). In addition, ¹H-¹H and ¹H-¹³C HMOC correlation spectra were acquired for the identification of metabolites. Furthermore, the main metabolites accounting for the differences in metabolic profile were assessed by current biochemical methods.

RESULTS. Lactate was the most abundant metabolite, and it was present at higher levels in samples from PDR patients than from nondiabetic patients (P = 0.02). Glucose was significantly higher in samples from PDR patients than nondiabetic patients (P = 0.03). After removing the lactate peak at 1.35 ppm and with the use of PLS-DA, a model was obtained that was able to correctly classify 19 of 22 patients with PDR and 18 of 22 controls, resulting in a sensitivity of 86% and a specificity of 81%. The main metabolites involved in this specific pattern recognition were galactitol and ascorbic acid (AA); levels of both were significantly lower in PDR patients.

CONCLUSIONS. ¹H-NMR–based metabonomic analysis of vitreous fluid permits the obtaining of a metabolic signature of PDR. Apart from the higher abundance of lactate and glucose, significant deficits of galactitol and AA are the main metabolic fingerprints of vitreous fluid from PDR patients. (Invest Ophthal Vis Sci. 2010;51:4416–4421) DOI:10.1167/iovs.10-5348

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D iabetic retinopathy (DR) is the leading cause of blindness and visual impairment among working-age persons in developed countries. Although tight control of both blood glucose levels and hypertension are essential to prevent or arrest progression of the disease, the recommended goals are difficult to achieve in many patients, and, consequently, DR develops during the evolution of the disease. When proliferative DR (PDR) does appear, argon-laser photocoagulation is indicated, but its efficacy is limited and there can be adverse effects. Vitreoretinal surgery is an expensive and complicated treatment that should be carried out only by vitreoretinal specialists experienced in this procedure; it is normally reserved for the ultimate blinding complications of PDR. For all these reasons, new pharmacologic treatments based on the understanding of the pathophysiological mechanisms of DR are needed.¹

Vitreous fluid is used to indirectly explore the retinal production of potential candidates involved in the pathogenesis of DR.² However, the volume of vitreous fluid obtained after vitrectomy is approximately 1 mL. Therefore, only a few peptides can be analyzed simultaneously. The recent development of proteomic analysis has made it feasible to analyze the protein profile of the vitreous fluid with only a small sample.³

Metabonomics is the attempt to measure all the metabolites in a biological system. Because the metabolome is downstream from the proteome and transcriptome, it represents a more sensitive level of organization than the proteome or transcriptome for understanding a complex biological system.⁴⁻⁶

The aim of the present study was to explore for the first time the differential metabolic profile of the vitreous fluid of PDR patients using ¹H-NMR–based metabonomic analysis. ¹H-NMR spectroscopy of biofluids generates comprehensive biochemical profiles of low molecular weight metabolites that can be identified by their chemical shift. With this method, comparative analysis of vitreous fluid obtained from patients with PDR and from patients with MH (control group) has been performed.

METHODS

Patients and Sample Selection

Vitreous samples from 22 patients with type 1 diabetes (12 men, 10 women; diabetes duration, 14.2 ± 6.7 years; HbA1c, 8.1% ± 1.8%) with PDR and from 22 nondiabetic patients with macular hole (MH) (control group; 14 men, 8 women) closely matched in terms of age (46.1 ± 9.2 vs. 45.3 ± 11.5 years) were selected from our vitreous bank. Exclusion criteria were as follows: previous vitreoretinal surgery; photocoagulation in the preceding 6 months; recent vitreous hemorrhage (<3 months before vitrectomy), macroscopic hemorrhage, or intravitreal hemorrhage (>5 mg/mL; history of glaucoma; renal failure (plasma creatinine ≥120 μM); and other chronic diseases apart from...
diabetes. Details of vitrectomy and sample collection have been described previously elsewhere. The samples were stored at −80°C until analyzed.

The research followed the tenets of the Declaration of Helsinki. The protocol for sample collection was approved by the hospital ethics committee, and informed consent was obtained from patients.

NMR Spectroscopy and Pattern Recognition

NMR spectra were acquired on a 400 MHz (9.4 T) magnet interfaced to a spectrometer (Avance 400; Bruker, Rheinstetten, Germany). Samples (200 μL) were diluted in D2O (400 μL) and kept at 30°C throughout the experiment, as described previously. In the NMR spectra, each peak corresponded to a chemical moiety that could be identified by its chemical shift. Before pattern recognition, spectra were digitized in 1000 bins of equal length so that all peaks in the spectra were taken into account.

Once digitized, the spectra were fed into a software package (SIMCA-P; Umetrics, Umeå, Sweden) with which a reduction in the variable numbers using principal component analysis or partial least squares was performed. After that, discriminant analysis was used to predict how well a given sample could be classified between two previously defined populations (PDR and MH). When a discriminant model was created, we were able to inquire which NMR peaks (i.e., metabolites) were responsible for the differences.

RESULTS

Visual Inspection

1H-NMR spectra of vitreous fluid can be seen in Figure 1. The spectra were dominated by a high presence of lactate and, to a lesser extent, glucose. Other metabolites present were alanine, valine, glutamine, acetate, leucine, isoleucine, and succinate. Slow tumbling macromolecules, such as lipids and proteins, did not contribute significantly to the NMR spectra.

The peak areas of 1H-NMR spectra, corresponding to lactate, glucose, alanine, and acetate, were significantly higher in PDR patients than in nondiabetic controls (P = 0.02, P = 0.03, P = 0.03, and P = 0.05, respectively) By contrast, the succinate NMR peak area was lower in PDR patients than in controls (P = 0.04).

Lactate was the most abundant metabolite, and its methyl peak at 1.35 ppm dominated the spectra. The lactate peak area was found to be variable between samples but was always the largest peak in the spectra, and it was significantly higher in samples from PDR patients than from nondiabetic patients (mean ± SEM: 149.24 ± 7.17 vs. 127.26 ± 4.91 AU; P = 0.02). Lactate levels assessed by NMR were 6.17 ± 0.97 mM for PDR patients and 5.28 ± 0.99 for controls (P = 0.05). These results were in agreement with those obtained using a commercial kit (Lactate Assay Kit II; BioVision Research Products, Mountain View, CA).
View, CA)—8.77 ± 2.39 mM vs. 5.96 ± 1.74 mM (P = 0.054)—and they were similar to those previously reported with the use of NMR in rabbits.9

Glucose peaks found between 3.2 and 3.9 ppm were also clearly present in all samples with varying intensities between samples and were significantly higher in samples from PDR patients than from nondiabetic patients (273.42 ± 40.57 vs. 148.24 ± 18.99; P = 0.027). This finding was further supported by measuring glucose by the classical method of gluco-oxidase that also revealed higher levels of glucose in the vitreous fluid of PDR patients than in nondiabetic controls (6.5 ± 4.0 vs. 3.8 ± 0.9 mM; P = 0.016).

The peak corresponding to acetate at 1.93 ppm was also conspicuous in all spectra and was higher in the PDR group than in the control group (7.84 ± 3.11 vs. 1.93 ± 0.45; P = 0.49). However, this difference was primarily accounted for by four diabetic patients. In these patients, ketone bodies were also found in blood serum.

**Pattern Recognition**

Using partial least squares-discriminant analysis (PLS-DA), we obtained models able to differentiate between control and PDR samples (Fig. 2A). Similar models were obtained when using the whole spectra (between 0.5 and 9 ppm, except for the water peak) or only the aliphatic portion between 0.5 and 4.25 ppm, possibly because few metabolites are present in the aromatic part of the spectra (between 5.25 and 9 ppm) and they do not play an important role in the differentiation of the samples.

Very large peaks may have a disproportionate influence on normalization.10 Thus, though it would have been possible to obtain a model using the entire aliphatic portion of the spectra, it was further improved by removing the lactate doublet centered at 1.35 ppm. The model obtained without the lactate doublet was able to correctly classify 19 of 22 patients with PDR and 18 of 22 controls, resulting in a sensitivity of 86% and a specificity of 81%.

Analysis of the loading plots (Fig. 2B) showed that the main difference in the spectra of control and PDR samples was a peak that appeared at 3.76 ppm and that was relatively elevated in control samples. PDR samples also had higher levels of acetate; the peak appeared at 1.93 ppm, but, because of its higher variability, it was not as relevant as the peak at 3.76 ppm in differentiating the two groups.

Average reconstructed spectra were similar between groups except for the 3.76- and 1.93-ppm peaks, thus reinforcing the concept that peaks other than these do not play an important role in differentiating between control and PDR samples.

**Metabolite Identification**

The area around 3.76 ppm was a crowded region of the spectra, where it was possible to find peaks corresponding to different sugars. Therefore, to identify the particular molecule responsible for the increase of the 3.76-ppm peak in control samples, we obtained spectra of glucose, sorbitol, mannitol, and galactitol standards with the same NMR acquisition parameters as vitreous fluid (Fig. 3). In addition, when galactitol was spiked into some samples, the increase in peak resonances correlated with the peak at 3.76 ppm. When glucose, sorbitol, or mannitol was spiked into vitreous fluid, the signal increase was in peaks other than the one at 3.76 ppm. In fact, most of the resonances from vitreous humor can be explained by the addition of the spectra of glucose and galactitol (data not shown), thus demonstrating that both metabolites accounted for most of the NMR peaks. The peak corresponding to galactitol was significantly lower in vitreous samples of PDR patients than of nondiabetic controls (386.68 ± 17.53 vs. 183.70 ± 21.18 AU; P = 0.043).

Two dimensional 1H-13C correlated spectra showed cross-peaks at 1.93 to 24.6 ppm and 3.76 to 65.4 ppm that were unambiguously assigned to acetate and ascorbic acid (AA), respectively (Fig. 4A).

**Ascorbic Acid Assessment**

To verify the results of 1H-NMR regarding AA, we determined AA using a modification of the ferric reducing antioxidant power assay, known as FRASC (ferric reducing/antioxidant and ascorbic acid concentration)11 in 20 additional vitreous and serum samples (10 from PDR, 10 from MH).

Figure 4A shows a 1H-13C heteronuclear multiple quantum coherence (HMQC) spectra with the AA peak (marked with an arrow) among larger glucose and lactate peaks. Lower AA levels were found in the vitreous fluid of PDR patients than of nondiabetic controls (386.68 ± 366.56 vs. 1676.96 ± 564.80 μM; P < 0.001) and of serum samples (22.68 ± 20.12 vs. 53.64 ± 36.27 μM; P = 0.09) (Fig. 4B).

**DISCUSSION**

In this article we have shown for the first time a comprehensive characterization of the metabolic phenotype of vitreous humor of patients with PDR. In addition, the main metabolic fingerprints that permit us to differentiate it from that of nondiabetic patients have been also identified.

Vitreous fluid obtained from patients undergoing vitrectomy surgery is used as a mirror of the in vivo metabolic events that are taking place in the retina. However, there are two
important confounding factors that could lead to misinterpretation of the results.\(^2\) First, vitreous hemorrhage, which often occurs in PDR, can produce a massive influx of plasma metabolites into the vitreous fluid, thus precluding the usefulness of vitreous fluid when studying intraocular metabolite production. To minimize this problem, blood contamination was ruled out by selecting vitreous samples with hemoglobin levels lower than 5 mg/mL. Second, laser photocoagulation can modify transcriptional activity in the retina, and, for this reason, samples from patients in whom laser photocoagulation had been performed in the preceding 6 months were excluded. In addition, a high level of homogeneity was further achieved by exclusively analyzing vitreous samples from PDR patients with type 1 diabetes without nephropathy and closely matched by age with the control group.

On these bases, metabolomic analysis revealed that the concentrations of several retinal metabolites involved in different pathways varied between PDR patients and nondiabetic controls. These biochemical processes included glucose levels, glucoalytic products (lactate), \(\beta\)-oxidation (ketone body-derived metabolite/acetate) aldose reductase or polyol pathway (galactitol), and AA defense against oxidative stress.

The presence of lactate in human vitreous has been reported using in vivo\(^1\) and in vitro NMR spectroscopy.\(^5\) In the present study, we found that lactate was the most abundant metabolite, and it was found at higher levels in the vitreous fluid of PDR patients. There are several reasons for this finding.

Lactate is a major product of glucose metabolism, and elevated lactate levels reflect increased tissue acidosis and anaerobic glycolysis. In addition, the retina is the part of the body that proportionally consumes more oxygen. Therefore, altered microcirculation in conjunction with hypoxia (hallmarks of PDR) can trigger lactate production by anaerobic glycolysis to compensate for reduced oxidative ATP production. Furthermore, inflammation, which is also involved in the pathogenesis of DR,\(^15\) can stimulate lactate production.

Acetate is a metabolite of N-acetylaspartate and the neurotransmitter, acetylcholine, but it is also involved, as acetyl-coA, in lipid and carbohydrate metabolism (entry into the citric acid cycle). In patients with type 1 PDR, acetate accumulation in the vitreous fluid might be interpreted as a result of ketone body formation by \(\beta\)-oxidation. In fact, the differences between PDR patients and controls were accounted for in the four instances of PDR. It was precisely in these instances that we also found ketone bodies in serum.

After removing the lactate peak at 1.35 ppm, we were able to obtain a model that differentiates control and PDR samples with 86% sensitivity and 81% specificity. The primary metabolites involved in this specific pattern recognition of NMR spectra were galactitol (also named dulcitol) and AA, both located in a peak at 3.76 ppm and significantly lower in PDR patients.

Galactitol, the metabolite of galactose, has not previously been identified in the \(^1\)H-NMR spectra of biofluids or biological tissues. The lower levels of galactitol detected in the vitreous fluid of PDR patients can be attributed to the activation of the polyol pathway, a metabolic pathway involved in the pathogenesis of DR.\(^16\) Aldose reductase (AR) is the first and rate-limiting enzyme in this pathway, and both glucose and galactose are substrates that compete for this enzyme while being reduced to sorbitol and galactitol, respectively. Because AR has a higher affinity for galactose than for glucose, under physiological conditions (normoglycemia) glucose is poorly reduced by AR to sorbitol; therefore, galactitol production is favored. By contrast, when intracellular glucose levels are elevated, the polyol pathway of glucose metabolism becomes active and sorbitol rather than galactitol is produced.\(^16\)\(^17\) The reason we did not find higher levels of sorbitol in the vitreous of PDR patients could be that it is metabolized to fructose; fructose-3-
phosphate, and 3-deoxyglucosone. By contrast, galactitol is not further metabolized or transported and thus accumulates inside AR-expressing cells.

AA, which is an essential substance in humans, acts as a cofactor in the enzymatic biosynthesis of collagen, catecholamines, and peptide neurohormones. However, one of its most important functions is to act as an antioxidant or free radical scavenger. Although most animals can synthesize vitamin C from glucose, humans can only acquire the vitamin from dietary sources because they lack gluconolactone oxidase, the enzyme required for AA biosynthesis. Vitamin C exists in two major forms. The charged form, AA, is taken into cells by way of sodium-dependent facilitated transport. The uncharged form, dehydroascorbate (DHA), enters cells by way of glucose transporters (GLUTs) and is then converted back to AA within these cells. Retinal cells appear to be dependent on GLUT-1 transport of DHA rather than sodium-dependent AA uptake. DHA uptake through facilitative glucose transport is competitively inhibited by D-glucose. In fact, the molecule of AA is similar to D-glucose. Therefore, chronic hyperglycemia of long-standing diabetes reduces DHA transport through GLUT1 at the blood retinal barrier (BRB). Exclusion of DHA from cells by hyperglycemia deprives the cells of the central antioxidant action of AA, thus favoring accumulation of reactive oxygen species. It should be noted that the retina is the only neural tissue that has direct and frequent exposure to light, thus leading to free radical production because of photo-oxidation, which becomes extremely toxic to retinal cells. AA is present in the retina at a high concentration compared with its presence in other organs in humans, and it is able to protect the retina against oxidative damage. In fact, we have found approximately 20-fold higher levels of AA in the vitreous fluid than in serum. Given that oxidative stress is a key factor in the pathogenesis of DR, the significantly lower levels of AA detected in the vitreous fluid of PDR patients point to this deficit as a crucial factor in determining DR development. AA is a required cofactor for several intracellular hydroxylases, including proline hydroxylase and dopamine hydroxylase. Therefore, AA deficit could also participate in the impairment of neuropeptide production and, therefore, in neurodegeneration, a key element in the pathogenesis of DR. Consequently, the lower levels that exist in diabetic patients can contribute to neovascularization, the hallmark of PDR. There are several explanations for the lower levels of AA detected in the vitreous fluid of PDR patients: the competitive inhibition mediated by hyperglycemia in AA transport from systemic circulation to the retina, the AA consumption that exists in the diabetic retina to compensate for elevated degree of oxidative stress, and the lower serum levels of AA in PDR patients in comparison with control group. Our results agree with those of a previous report showing that diabetic patients...
with microangiopathy had lower AA serum concentrations than those without microangiopathy and control subjects, probably because of a high metabolic turnover reflecting increased consumption of AA by oxidation to DHA. Taken together, AA can be thought of as a new therapeutic target. Prospective trials using diet supplements of vitamin C for preventing or arresting DR and experimental studies addressed to increasing AA transport across BRB in the presence of hyperglycemia are needed.

There are three main limitations of the present study. First, vitreous fluid obtained from nondiabetic subjects without any retinal disease would be a more appropriate control than that obtained from subjects with MH. However, nondiabetic subjects without ocular disease are not submitted to vitrectomy and, consequently, vitreous fluid from nondiabetic subjects without retinal disease can only be obtained from autopsy eyes or eye bank eyes. However, as soon as blood circulation stops, ischemic or postmortem changes start to take place in the ocular tissues of the cadaver. These changes can extensively alter the metabolic composition in the vitreous humor, making these eyes no longer representative of normal controls. For this reason, we used vitreous from nondiabetic patients with MH as a “control group” or at least as a valid comparator with those obtained from PDR patients. Another limitation of our study is that NMR spectroscopy is less sensitive than other analytical methods, such as mass spectroscopy. However, NMR spectroscopy has the advantage of being applicable in vivo in the eye, thus allowing a new strategy for monitoring DR. Finally, though the contribution of the retina is essential as a source of free AA, increased consumption of AA by oxidation to DHA. Taken to-gether, AA can be thought of as a new therapeutic target.

In summary, 1H-NMR-based metabolomic analysis of vitreous fluid permits the detection of the characteristic fingerprints of PDR. This, in turn, provides a sound basis not only for further mechanistic studies but also for the identification of molecular targets for a more efficient pharmacologic treatment of this devastating complication of diabetes.

References