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Increased breath biomarkers of oxidative stress in diabetes mellitus

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Abstract

Background: Oxidative stress has been implicated in the major complications of diabetes mellitus, including retinopathy, nephropathy, neuropathy and accelerated coronary artery disease. There is a clinical need for a marker of oxidative stress which could potentially identify diabetic patients at increased risk for these complications. We measured oxidative age, a new breath marker of oxidative stress, in diabetic patients. *Methods*: Three groups were studied: type 1 diabetes mellitus ($n=9$), type 2 diabetes mellitus ($n=53$) and non-diabetic normals ($n=39$). Volatile organic compounds (VOCs) in breath were assayed by gas chromatography and mass spectroscopy to construct the breath methylated alkane contour (BMAC), a three-dimensional display of oxidative stress markers, C4–C20 alkanes and monomethylated alkanes. The collective abundance of these VOCs was reduced to a single value, the oxidative age, comprising the volume under the curve of the BMAC corrected for chronological age. *Results*: Oxidative age was significantly increased in type 1 diabetes (mean = 0.103, S.E.M. = 0.119, $p<0.01$) and type 2 diabetes (mean = 0.103, S.E.M. = 0.047, $p<0.05$) compared to age-matched normals (mean = – 0.248, S.E.M. = 0.079). No significant correlation between oxidative age and blood glucose or hemoglobin A_1C was observed in either group. *Conclusions*: Oxidative age, a marker of oxidative stress, was significantly increased in both type 1 and type 2 diabetes mellitus. Oxidative age merits further study as a candidate marker of risk for the complications of diabetes mellitus.

Keywords: Diabetes mellitus; Oxidative stress; Breath; Alkanes; Methylated alkanes

1. Introduction

Oxidative stress plays a central role in the onset of diabetes mellitus as well as in the development of vascular and neurologic complications of the disease [1]. The source of oxidative stress is a cascade of reactive oxygen species (ROS) leaking from the mitochondria [2], and this process has been associated with the onset of type 1 diabetes via the apoptosis of pancreatic beta-cells, and the onset of type 2 diabetes via insulin resistance [3]. The underlying mechanisms in the onset of diabetes are complex because hyperglycemia may be both cause and effect of increased oxidative stress [4].

These findings have stimulated the search for an objective biomarker of oxidative stress which might be clinically useful in patients with diabetes mellitus. Such a biomarker could potentially indicate the severity of disease, identify those at increased risk of

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complications and monitor the response to treatment. A variety of candidate markers of oxidative stress have been studied in diabetics, including hydrogen peroxide, malonyldialdehyde, thiobarbituric acid-reacting substances, superoxide dismutase, glutathione peroxidase and carbon monoxide [5–7].

Breath microassays have opened a new window on to the detection of oxidative stress because ROS oxidize polyunsaturated fatty acids in membranes to alkanes such as ethane and pentane which are excreted in the breath as volatile organic compounds (VOCs) [8,9]. We have reported a highly sensitive test for the detection of breath VOCs in picomolar concentrations [10,11], and have employed this test to identify a new set of markers of oxidative stress, the breath methylated alkane contour (BMAC), comprising a three-dimensional display of the abundance of C4–C20 alkanes and monomethylated alkanes [12]. This breath test demonstrated that the intensity of oxidative stress varied with age [13], and was significantly increased by breathing oxygen [14], normal pregnancy and preeclampsia of pregnancy [15].

We report here a new breath biomarker of oxidative stress: oxidative age. It was derived from comparing the observed abundance of alkanes and monomethylated alkanes in an individual's BMAC to the expected abundance in a normal subject of the same age. We evaluated this biomarker in patients with type 1 and type 2 diabetes mellitus.

2. Materials and methods

2.1. Human subjects

Characteristics of human subjects are shown in Table 1. The results of the breath tests in the control

Table 1
Human subjects

	Type 1 diabetes	Type 2 diabetes	Normals
Number	9	53	39
Age (years) (NS)	47.7 (15.1)	55.9 (13.9)	50.9 (18.5)
Blood glucose	206.6 (132.)	196.6 (115.7) (NS)	
Hemoglobin A_1C	8.7 (1.5)	9.1 (2.3) (NS)	

Mean values are shown, with standard deviations (S.D.).
NS = not significant.

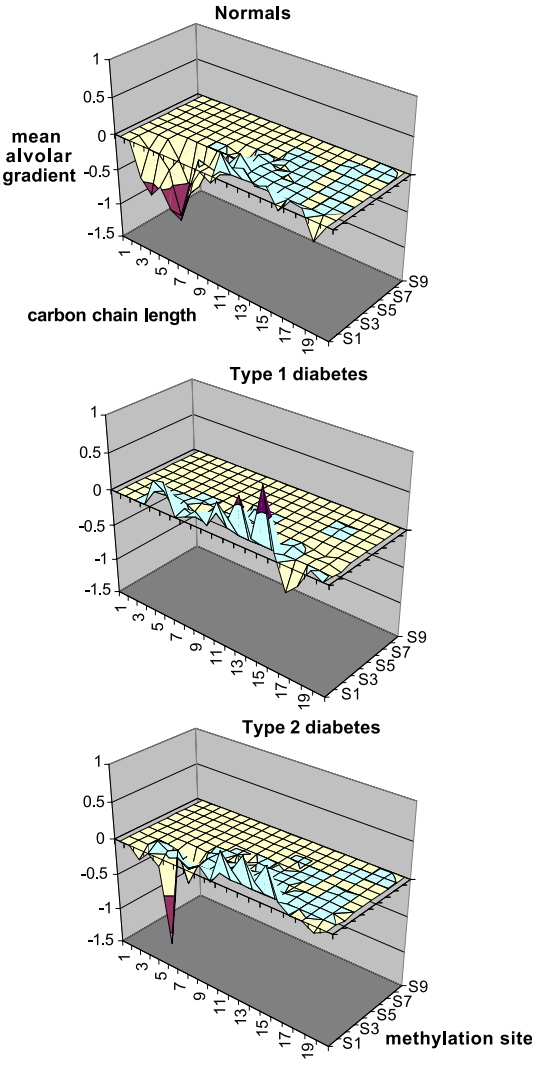

Fig. 1. Mean breath methylated alkane contour (BMAC) in normals, type 1 and type 2 diabetes mellitus. These figures display the abundance of alkanes and monomethylated alkanes in breath. The carbon chain length is shown on the *x*-axis, ranging from C4 (butane) to C20 (eicosane), and the methylation site is shown on the *z*-axis. The alveolar gradient is shown on the *y*-axis, i.e., the abundance of a VOC in breath minus its abundance in room air. Alveolar gradient varies with rate of synthesis minus rate of clearance of a VOC, and increases with the intensity of oxidative stress. Peaks are predominantly negative in the normals but predominantly positive in type 1 diabetes mellitus, indicating a generalized increase in the synthesis of alkanes and monomethylated alkanes resulting from oxidative stress. Peaks in type 2 diabetes mellitus are either increased or decreased, intermediate between normals and Type 1 diabetes mellitus.

group of normal volunteers have been previously reported [13]. Subjects sat for approximately 20 min prior to the collections of breath and air in order to allow time for equilibration between VOCs in room air and in the blood. Non-fasting breath samples were collected between 7:00 am and 12:00 noon from diabetic patients attending an outpatient clinic at Bayley Seton Hospital, Staten Island, NY. Blood was drawn for estimation of hemoglobin A_1C, and glucose was estimated with a fingerstick method (Glucometer Elite, Bayer, Elkhart, IN). The presence of complications of diabetes mellitus was not recorded. The institutional review board of the Sisters of Charity Medical Center, St. Vincent's Campus, Staten Island, NY, approved the human research.

2.2. Breath collection and assay

The method has been described [10,11]. In summary, a portable breath collection apparatus was employed to capture the VOCs in 1.0 l breath and in 1.0 l room air on to separate sorbent traps. Each subject wore a nose clip while breathing in and out of the disposable mouthpiece of the apparatus for 2.0 min. Breath samples could be collected without discomfort because light flap valves in the mouthpiece presented low resistance to respiration. VOCs captured in sorbent traps were analyzed by automated thermal desorption, gas chromatography and mass spectroscopy.

2.3. Derivation of BMACs

The method has been described [12]. Data from the assays of breath and air VOCs was employed to generate three-dimensional surface plots of C4–C20 *n*-alkanes and their monomethylated derivatives. For each breath VOC, V_b denotes the area under the curve associated with the chromatogram peak, and I_b denotes the analogous area associated with the

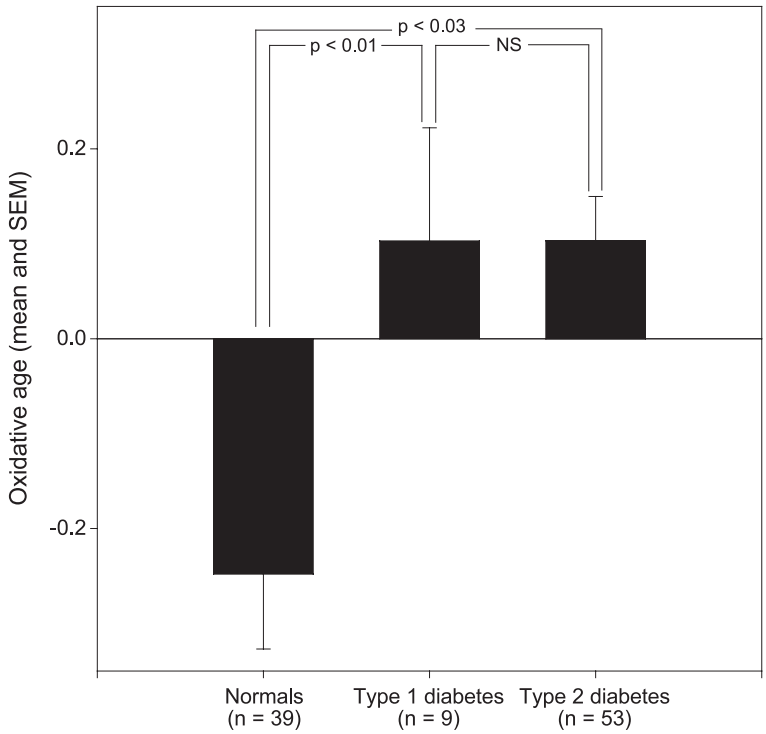

Fig. 2. Oxidative age in normals, type 1 and type 2 diabetes mellitus. The oxidative age of a subject was derived from their BMAC as the volume under the three-dimensional curve, corrected for the subject's age. Oxidative age was significantly increased in both type 1 and type 2 diabetes mellitus compared to age-matched non-diabetic normals.

internal standard used to calibrate the instrument (0.25 ml 2 ppm 1-bromo-4-fluoro-benzene (Supelco, Bellefonte, PA). V_a and I_a denote corresponding areas derived from the associated sample of room air. The alveolar gradient [11] of each VOC was then determined as:

$$\text{alveolar gradient} = V_b/I_b - V_a/I_a.$$

The mean alveolar gradients of these VOCs were computed for all subjects and displayed in surface plots showing the carbon chain length on the x-axis, the methylation site on the z-axis and the mean alveolar gradient on the y-axis.

2.4. Derivation of oxidative age

The value of oxidative age was determined in all subjects as $(O-E)/S$ where O = observed VUC of BMAC in the study subject (VUC = volume under the curve of BMAC), E = expected VUC of BMAC in a normal subject of the same age = $33.7 - 3.29x + 0.072x^2 - 0.0004x^3$ (x = age in years) [13] and S = standard deviation of $O-E$ in all normal subjects.

3. Results

Table 1 displays the characteristics of the study subjects, as well as mean blood glucose and hemoglobin A_1C levels in the diabetic patients. Fig. 1 shows the mean BMACS in age-matched normals, and subjects with type 1 and type 2 diabetes mellitus. Fig. 2 shows the mean oxidative age in these three groups. Differences in oxidative age were significant in a one-way ANOVA ($p<0.001$). When the three groups were compared with a Newman–Keuls tests, oxidative age was significantly increased in type 1 diabetes (mean = 0.103, S.E.M. = 0.119, $p<0.01$) and type 2 diabetes (mean = 0.103, S.E.M. = 0.047, $p<0.05$) compared to age-matched normals (mean = −0.248, S.E.M. = 0.079). Neither blood glucose nor hemoglobin A_1C demonstrated any significant correlation with oxidative age in type 1 or type 2 diabetes mellitus.

4. Discussion

This study demonstrated two main findings: First, oxidative stress was significantly and similarly increased in type 1 and type 2 diabetes mellitus. Second, this increase appeared to be independent of glycemic control; oxidative stress did not vary with blood sugar concentration or with hemoglobin A_1C levels in either group.

Several different mechanisms have been proposed to explain why oxidative stress is increased in diabetes mellitus. These mechanisms fall into two general categories: increased production of ROS and decreased antioxidant defenses. Hyperglycemia in diabetes mellitus may increase ROS production via changes in the redox potential of glutathione [4], though non-hyperglycemic mechanisms have also been reported, e.g., increased activity of xanthine oxidase, a superoxide-generating enzyme [16]. Decreased antioxidant defenses have also been observed in diabetes mellitus, including reductions in serum paraoxonase [17] and in total antioxidant capacity in plasma [18]. Some of these mechanisms may possibly operate simultaneously in a synergistic fashion.

The rationale for oxidative age as a new biomarker of oxidative stress was derived from three sets of observations. First, we identified the BMAC as a rational set of markers of oxidative stress, based on evidence that breath alkanes are products of ROS-induced lipid peroxidation of polyunsaturated fatty acids [8,9,12]. Second, we confirmed that the BMAC was a marker of increased oxidative stress by demonstrating increased abundance of its component VOCs in normal humans breathing 28% oxygen [14], as well as in pregnancy and preeclampsia [15]. Third, we found that oxidative stress measured with the BMAC varied with age in a non-linear fashion in a group of normal humans aged from 9 to 89 years [13]. Consequently, in order to employ the abundance of BMAC VOCs as a biomarker of oxidative stress, this value must first be corrected for a subject's age. We expressed this corrected value as the oxidative age which incorporates two variables: polarity and magnitude. Positive or negative polarity indicates whether oxidative stress was increased or decreased in an individual compared to normal controls of the same age, and the magnitude of the change was expressed as

the number of standard deviations from the mean expected value.

Oxidative age could potentially provide a new tool in clinical practice because it demonstrated an increase in oxidative stress in diabetic patients that was not related to blood glucose concentration or hemoglobin A_1C levels. Major complications of diabetes mellitus have been associated with increased oxidative stress, including retinopathy [7,19], nephropathy [20,21], neuropathy [22,23] and accelerated coronary artery disease [24,25]. A breath test for oxidative age could potentially identify diabetic patients at increased risk for these complications who might benefit from intensive treatment with antioxidants and other prophylactic drugs. Subsequent measurements of oxidative age could determine the effectiveness of these interventions, and potentially predict the prognosis of complications.

Oxidative age is a rational and non-invasive marker of oxidative stress that was significantly increased in patients with type 1 and type 2 diabetes mellitus. Oxidative age merits further study as a candidate marker which could potentially identify diabetic patients at increased risk for complications, measure the impact of therapy and predict prognosis.

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