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Breath isoprene: Muscle dystrophy patients support the concept of a pool of isoprene in the periphery of the human body

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Abstract

Breath isoprene accounts for most of the hydrocarbon removal via exhalation and is thought to serve as a non-invasive indicator for assaying several metabolic effects in the human body. The primary objective of this paper is to introduce a novel working hypothesis with respect to the endogenous source of this compound in humans: the idea that muscle tissue acts as an extrahepatic production site of substantial amounts of isoprene. This new perspective has its roots in quantitative modeling studies of breath isoprene dynamics under exercise conditions and is further investigated here by presenting pilot data from a small cohort of late stage Duchenne muscle dystrophy patients (median age 21, 4 male, 1 female). For these prototypic test subjects isoprene concentrations in end-tidal breath and peripheral venous blood range between 0.09–0.47 and 0.11–0.72 nmol/l, respectively, amounting to a reduction by a factor of 8 and more as compared to established nominal levels in normal healthy adults. While it remains unclear whether isoprene can be ascribed a direct physiological mechanism of action, some indications are given as to why isoprene production might have evolved in muscle.

Keywords

Exhaled breath analysis; Isoprene; Muscle; Duchenne muscular dystrophy

1 Introduction

Due to its broad scope and applicability, exhaled breath analysis holds great promise as a versatile framework for general biomonitoring applications. As a biochemical probe, volatile

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organic compounds (VOCs) in breath are unique in the sense that they can provide both *non-invasive* and *continuous* information on the metabolic/physiological state of an individual. Apart from diagnostics and therapy control [1], this information might potentially be used for dynamic assessments of normal physiological function (e.g., by a stress test on a stationary bicycle [2–4], in an intra-operative setting [5,6], or in a sleep lab [7]), pharmacodynamics [8] or for quantifying environmental exposure [9].

A prototypic example in this regard is isoprene (CAS 78-79-5), which due to its high volatility and low affinity for blood is particularly abundant in human breath and accounts for up to 70% of total hydrocarbon removal via exhalation [10,11]. Isoprene's unparalleled attention in the field of exhaled breath analysis mainly stems from the fact that it has been put forward as a non-invasive indicator for assaying several metabolic effects in the body [12]. Surprisingly, despite this enormous popularity the physiological behavior of isoprene as well as its source and function in humans are still a matter of debate.

Current theory holds that isoprene released by living organisms is made from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). So far, two major metabolic pathways leading to DMAPP have been identified: the mevalonic acid (MVA) pathway and the 1-deoxy-D-xylulose-4-phosphate/2-C-methylerythriol 5-phosphate (DOXP/MEP) pathway. The DOXP/MEP pathway was demonstrated to prevail in plants and most bacteria [13], whereas the MVA pathway is mainly present in higher eukaryotes [14]. In plants and bacteria DMAPP is converted into isoprene enzymatically by isoprene synthase [13]. In animals and humans isoprene is thought to be produced non-enzymatically by acid-catalyzed solvolysis of DMAPP occurring in the liver [15]. However, as this reaction is slow and may be insignificant at physiological pH values it is unlikely to completely explain the endogenous isoprene production by animals [16].

Similarly, the physiological function of isoprene remains a controversial issue. While in plants this compound seems to protect the photosynthetic apparatus against heat stress or oxidative radicals (with the exact mechanism of action being unknown) [13,17], studies aiming at demonstrating a similar role in humans are sparse and have failed to deliver definite conclusions [18]. Nevertheless, the fact that breath isoprene output is altered under a number of widely distinct physiological and pathophysiological states (including hemodialysis, general anesthesia, liver disease, and cancer [12,19]) clearly motivates further research in this direction.

Within this context, the primary objective of this paper is to introduce a novel working hypothesis with respect to the source of endogenous isoprene in humans: the idea that a peripheral (muscle) tissue group acts as an extrahepatic production site of substantial amounts of isoprene in the body. This new perspective has its roots in the quantitative modeling of breath isoprene dynamics under exercise conditions and will further be investigated here by presenting pilot data from a small cohort of late stage muscle dystrophy patients for which – by reference to the above-mentioned hypothesis – isoprene levels in breath and blood are anticipated to be drastically reduced as compared to the respective nominal values in normal healthy volunteers.

2 Recent experimental findings with respect to breath isoprene output

Physical activity causes marked changes in the exhaled breath isoprene concentrations of humans. Real-time measurements during moderate workload ergometer challenges consistently show an exercise-induced peak-shaped increase of breath isoprene concentrations by a factor of 2–3 in mixed exhaled breath [20], and by a factor of 3–4 in end-tidal breath [2,3]. In a first ad hoc model by Karl et al. [20] this behavior was explained by reference to a delayed rise of ventilation as compared to an instantaneous increase in perfusion at the start of exercise (i.e., by assuming a temporary drop in the average pulmonary ventilation–perfusion ratio, implying an increase in isoprene concentrations according to classical pulmonary gas exchange theory). However, recent evidence questions this view and points towards a *substance-specific* wash-out from an isoprene buffer. The main experimental findings in this context can be summarized as follows:

- (a) Exerting effort at constant workload interrupted by breaks of variable duration lead to markedly different heights of the above-mentioned peak of breath isoprene concentration at the onset of pedaling, despite an almost identical behavior of cardiac output and alveolar ventilation throughout all workload segments [2].
- (b) During one-legged ergometer experiments at moderate workloads a switch of the working leg after a short break following an ergometer exercise segment at 50 W results in an immediate recovery of the initial peak height, whereas continuing the exercise with the same leg leads to a washout effect similar to the two-legged case [21].
- (c) Compared to two-legged ergometer exercise, arm-crank exercise induces much lower peak isoprene concentrations, despite similar time courses of the ventilation–perfusion ratio within both exercise regimes [22].
- (d) Breath isoprene bursts during sleep are highly specific for leg movements as scored by tibial electromyography [7].
- (e) Blood-borne VOCs anticipated to exhibit exhalation kinetics similar to isoprene (i.e., having a comparable blood:gas partition coefficient, such as butane for instance) show a distinct breath behavior in response to exercise [3].

Taken together these findings provide strong experimental evidence in favor of the hypothesis that breath isoprene dynamics during physical activity reflect an increased stimulus of a peripheral isoprene source. Indeed, this concept has been backed quantitatively by a mathematical modeling study in Ref. [21], postulating muscle tissue as an active production site of endogenous isoprene in the human body.

3 Materials and methods

Motivated by the background material presented in the previous paragraphs, this section focuses on the determination of breath and blood isoprene levels for a prototypic group of patients suffering from advanced stage Duchenne muscular dystrophy (DMD). Duchenne muscular dystrophy is an inherited muscle wasting disease caused by an absence of or defect

in the protein dystrophin. Females are very rarely affected, while its incidence in male infants is around 1 in 3600–6000 births. We recruited 5 patients (4 male, 1 female) in the most severe stages of the disease (Swinyard classification 7–8, i.e., all subjects were dependent on an electrical wheel-chair and unable to perform any activity of daily life without assistance [23]). The female patient had a balanced X-autosome translocation leading to the disruption of the dystrophin gene on Xp21 and inactivation of the normal X chromosome, thus giving rise to DMD. For demographic data we refer to Table 1. Given the low prevalence of DMD and considering a typical life expectancy of 25 years, the exclusive selection of late stage patients inevitably results in a drastically reduced number of potential volunteers. However, the progressive nature of the disease as well as the pronounced age-dependency of breath isoprene concentrations during childhood and adolescence (see, e.g., [24]) justify this restriction. None of the test subjects were receiving statins, whereas two of the volunteers had been prescribed nitrogen-containing bisphosphonates for osteoporosis treatment. While the latter are known to affect the mevalonate pathway, evidence indicates that their exclusive molecular target of action is farnesyl pyrophosphate synthase [25]. Hence a direct influence on *in vivo* isoprene production from DMAPP/IPP in response to N-containing bisphosphonate medication appears unlikely. Moreover, where obtainable, the serum cholesterol levels of the patients investigated fall within normal ranges, so that a general distortion of cholesterol biosynthesis with potential effects on systemic isoprene production can largely be excluded (see Table 1).

Measurements were approved by the Ethics Commission of Innsbruck Medical University and carried out in conformity with the Declaration of Helsinki. All volunteers gave written informed consent to provide two breath samples and, in the case of adult test subjects, an additional peripheral blood sample for the determination of the corresponding isoprene content.

3.1 Measurement of breath isoprene

Depending on the volunteer's ability, one or two end-exhaled breath samples were collected from each test subject into 3-l Tedlar bags (SKC Inc., USA) by means of a CO₂ triggered breath sampler developed at Innsbruck Medical University, Austria [26]. Briefly, this device selectively extracts the last segments of each exhalation (i.e., the portion of exhaled breath characterized by a carbon dioxide content higher than a predefined threshold of 3% in this case) and automatically directs them from a mouthpiece into the sampling bag via a heated Teflon transfer line. Around 10 breaths were provided for filling each bag, usually resulting in a sample volume of a few hundred milliliters. Additionally, a separate room air sample was taken for determining the background level of isoprene.

Breath isoprene levels were obtained by means of gas chromatography mass spectrometry coupled with solid phase microextraction (SPME GC–MS). For this purpose, a volume of 18 ml of gas from the Tedlar bag was transferred into an evacuated glass vial (20 ml in volume, Gerstel, Germany) sealed with a 1.3 mm butyl/PTFE septum (Macherey–Nagel, Germany). Finally, the pressure in the vial was balanced with isoprene-free nitrogen. The measurement procedure (instrument settings, SPME extraction conditions and calibration method) was analogous to the one described in Ref. [3], whereas the PoraBond Q column (25 m × 0.32

mm, film thickness 5 μm , Varian, USA) temperature program was chosen as follows: 40 $^{\circ}\text{C}$ for 1 min, increase to 260 $^{\circ}\text{C}$ at a rate of 7 $^{\circ}\text{C}/\text{min}$ and 260 $^{\circ}\text{C}$ for 5 min. For reasons of quality control we additionally determined sample CO_2 content by means of a separate sensor (AirSense Model 400, Digital Control Systems, USA).

Since the sampling procedure was quite fatiguing for all DMD patients, the relative standard deviation (RSD) for the above measurements had to be determined by analyzing five consecutive breath samples taken from a normal healthy volunteer in an identical manner. However, an effort was made to collect a similar breath sample volume (100–200 ml) into each Tedlar bag as in case of DMD patients. For this test subject, the breath isoprene levels varied around a mean value of 4.5 nmol/l, yielding an RSD of 7%.

3.2 Measurement of blood isoprene

For verification purposes three adult volunteers agreed to provide 2.7 ml of peripheral venous blood drawn from the cephalic vein, which was collected using heparinized monovettes previously purged with high purity nitrogen to eliminate any material-related isoprene contamination. Subsequently, both blood and headspace gas in the monovette were transferred into an evacuated glass vial (20 ml, Gerstel, Germany) containing 0.3 ml of Dulbecco's PBS (PAA Laboratories, Austria) and blood isoprene content was determined by a head-space solid phase microextraction (HS-SPME) GC–MS technique analogous to the one described by Miekisch et al. [27].

Due to the above-mentioned sampling restrictions, the RSD for these measurements had to be determined by analyzing five consecutive blood samples taken from a normal healthy volunteer in an identical manner. For this test subject, the blood isoprene levels varied around a mean value of 15.5 nmol/l, yielding an RSD of 5.5%.

4 Results and discussion

Table 1 summarizes the observed isoprene levels as determined above. The sampling protocol was well tolerated by all volunteers (except volunteer 4 for whom breath sampling was too exhaustive), however, the rapid and shallow breathing pattern typically adopted in neuromuscular diseases [28] resulted in relatively small sampling volumes of breath (usually only around a few hundred milliliters per bag). Nevertheless, the associated CO_2 levels confirm that all patients were capable of providing alveolar samples, so that a possible influence of higher dead space ventilation or ventilation–perfusion mismatch on the measured breath isoprene concentrations can largely be excluded.

End-tidal isoprene concentrations in normal healthy adults under resting conditions have been shown to follow a log-normal distribution with a relatively gender- and age-invariant geometric mean of about 100 ppb (approximately 4 nmol/l at standard ambient pressure and temperature) and an associated geometric standard deviation of 1.65 ppb [14,24]. Note that with these parameters the breath levels for all DMD patients as reported here fall well below the 1-percentile of this distribution (calculated as 1.26 nmol/l), taking values between 0.09 and 0.47 nmol/l. Similarly, peripheral venous blood isoprene levels in this patient group spread between 0.11 and 0.72 nmol/l which is drastically lower than typical values in

healthy adult volunteers shown to vary between 4.5–38 nmol/l (mean 14 nmol/l) [29] and 15–70 nmol/l (mean 37 nmol/l) [30]. In particular, this parallel reduction in breath and peripheral blood concentrations appears to rule out impaired pulmonary gas exchange as a primary cause for the observed isoprene decrease. While correlating isoprene levels with disease status is inherently difficult in the present context due to the small number of possible patients available and the purely descriptive nature of the disease stage, it is worth noting that those two volunteers with higher isoprene levels were also less affected in the sense that they could still actively move their torso and arms.

The above-mentioned reduction of endogenous isoprene levels in patients suffering from a late stage muscle wasting disease is well in line with our original hypothesis proposing a muscular production of this compound. Furthermore, our findings are consistent with previous reports suggesting an isoprenoid deficiency in muscular dystrophy [31,32]. Although the investigation of muscle dystrophy patients does not provide a direct proof that the associated low levels of endogenous isoprene can be explained by the degeneration of skeletal muscle alone, the experimental results discussed in Section 2 suggest that a potential contribution of different muscle sources (e.g., cardiac or diaphragmatic muscle, both of which are affected in DMD) to overall systemic isoprene content is probably small. Moreover, focusing on skeletal muscle has substantial explanatory power with respect to some major characteristics of breath isoprene output that go well beyond the dynamic response observed during exercise. For instance, the pronounced age dependent increase of breath isoprene concentrations during childhood and adolescence (with undetectable levels in the breath of neonates and a strong increase during puberty [24]) may be a direct consequence of skeletal muscle growth and maturation during that time. Furthermore, considering the fact that human skeletal muscle cells were found to be substantially more sensitive to statin-mediated inhibition of the mevalonate pathway than hepatocytes [33], it cannot be excluded that the observed decrease of breath isoprene levels during statin treatment is reflective of a medication-based depletion of muscle isoprenoid pools. Indeed, statin-induced myopathy has been linked to a reduced availability of isoprenoid co-metabolites (particularly geranylgeranyl pyrophosphate and farnesyl pyrophosphate) [34], which in turn is thought to disturb the (dis-)prenylation of proteins crucial to the functional and mechanical integrity of skeletal muscle (e.g., lamins [35]). It would be intriguing to investigate if endogenous isoprene can serve as a marker substance for monitoring such processes *in vivo*.

While it remains essentially unclear whether isoprene might itself be ascribed a direct physiological mechanism of action, by drawing analogies with plant physiology it is tempting to speculate about two possible effects.

4.1 Thermoprotection

The widely held thermotolerance hypothesis states that plants synthesize isoprene in order to maintain a constant level of cell membrane cohesiveness, thereby protecting plants against heat stress [13]. Molecular dynamics simulations of phospholipid bilayers suggest that isoprene enhances the packing of lipid tails and show that a moderate amount of isoprene dissolved in a model membrane can mediate an increase in membrane order equivalent to a

temperature reduction of 10 K [36]. This effect is similar to cholesterol, which is well known to attenuate the effect of temperature on phospholipid fluidity [37]. Note that both isoprene and cholesterol are derived from the mevalonate pathway, the activity of which is elevated in human cells exposed to heat stress [38]. Hence, it is conceivable that cells experiencing regular thermal disturbance (e.g., in response to locomotion as in the case of skeletal myocytes) may have upregulated this pathway, eventually leading to an increased isoprene production.

4.2 Oxidative protection

Another hypothesized effect of isoprene derived from plant physiology is that it offers protection against reactive oxygen species by reactions through the conjugated double bond system [17]. Similarly, increased isoprene production may result from an elevated synthesis of polyisoprene antioxidants (e.g., squalene, coenzyme Q [39]), their degradation [40], or both. Again, this rationale is particularly applicable to skeletal muscle tissue, which is especially prone to oxidative stress as a result of normal physiological function [41].

While both modes of action can provide a first tentative answer as to why (skeletal) muscle tissue would be characterized by high isoprene levels, further verification will certainly have to await additional experimental efforts. We are confident that focusing on the hypothesis of a substantial muscular isoprene source as discussed here will allow for designing more targeted experimental tests in this context. We also hope that the present contribution will stimulate future efforts in isoprene research and can help to identify potential confounding factors affecting the interpretation of breath tests involving this prominent VOC.

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Table 1

Characteristics of the DMD cohort under study. Isoprene levels in breath and blood are given in nmol/l. Normal values for healthy adult volunteers spread around 14 nmol/l in blood and 4 nmol/l in breath. Cholesterol levels were determined on the same day as isoprene concentrations (volunteer 1 and 5) or during routine clinical visits falling within 4 months of the respective date.

Volunteer	1	2	3	4	5
Gender	f	m	m	m	m
Age	24	21	25	19	15
Swinyard-Deaver stage	8	7	8	8	7
Cholesterol level (mg/dl)	178	126	–	144	145
Breath CO ₂ sample 1 (%)	4.83	4.22	5.14	–	3.90
Breath CO ₂ sample 2 (%)	–	2.68	–	–	3.60
<i>SPME GC-MS data</i>					
Breath isoprene sample 1	0.10	0.45	0.09	–	0.47
Breath isoprene sample 2	–	0.45	–	–	0.39
Isoprene level room air	0.05	0.06	0.09	–	0.06
Blood isoprene level	0.11	0.72	–	0.12	–