Long-Term Activation of Semicarbazide-Sensitive Amine Oxidase Lowers Circulating Levels of Uric Acid in Diabetic Conditions

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Received April 13, 2011
Accepted October 5, 2011
On-line April 5, 2012

Summary

Uric acid is involved in nitrogenous waste in animals, together with ammonia and urea. Uric acid has also antioxidant properties and is a surrogate marker of metabolic syndrome. We observed that the elevated plasma uric acid of high-fat fed mice was normalized by benzylamine treatment. Indeed, benzylamine is the reference substrate of semicarbazide-sensitive amine oxidase (SSAO), an enzyme highly expressed in fat depots and vessels, which generates ammonia when catalysing oxidative deamination. Ammonia interferes with uric acid metabolism/solubility. Our aim was therefore to investigate whether the lowering action of benzylamine on uric acid was related to an improvement of diabetic complications, or was connected with SSAO-dependent ammonia production. First, we observed that benzylamine administration lowered plasma uric acid in diabetic db/db mice while it did not modify uric acid levels in normoglycemic and lean mice. In parallel, benzylamine improved the glycemic control in diabetic but not in normoglycemic mice, while plasma urea remained unaltered. Then, uric acid plasma levels were measured in mice invalidated for AOC3 gene, encoding for SSAO. These mice were unable to oxidize benzylamine but were not diabetic and exhibited unaltered plasma uric levels. Therefore, activated or abolished ammonia production by SSAO was without influence on uric acid in the context of normoglycemia. Our observations confirm that plasma uric acid increases with diabetes and can be normalized when glucose tolerance is improved. They also show that uric acid, a multifunctional metabolite at the crossroads of nitrogen waste and of antioxidant defences, can be influenced by SSAO, in a manner apparently related to changes in glucose homeostasis.

Key words

Diabetes • Adipose tissue • Primary amine oxidase • Urea • Ammonia

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Introduction

Uric acid is one of the most important molecules of nitrogenous waste in animals, together with ammonia and urea. In man, uric acid is sadly known for causing gout attack (arthritis) when its circulating values exceed the normal range 3–7 mg/dl and when it crystallizes between bone joints. For this reason the dietary income of uric acid, mainly due to foods that contain proteins and purines, has been recommended to be limited. However, uric acid has antioxidant properties (Becker et al. 1991), and has also been considered as a surrogate marker of metabolic syndrome since its circulating levels have been repeatedly reported to increase with cardiovascular diseases, diabetes and obesity (Yoo et al. 2005). These multiple aspects of uric acid complicate our understanding of its physiological role, together with the fact that kidney, which eliminates this metabolite is also actively reclaiming it. Therefore uric acid cannot be merely considered as a nitrogen waste. Similarly, it is not the sole trigger of human kidney pathologies. Once its renal filtration/reabsorption (clearance) is altered, uric
acid, which is slightly soluble in water, precipitates in urine and generates kidney stones. However, only approx. 10 % of the kidney stones are constituted by uric acid – or more precisely urate – crystals, while calcium oxalate crystals are found in most of the nephropathies (Coe et al. 2005). Accordingly, not all the kidney stones are accompanying metabolic diseases such as diabetes or obesity (Maalouf 2011), while many of them coexist with inflammatory, irritating or infectious diseases (Soble et al. 1999). Nevertheless, overproduction of uric acid in blood is probably more responsible than its decreased clearance in the appearance of troubles in its circulating levels during the onset of metabolic syndrome (Reaven 1999).

We have recently observed that long-term oral treatment with benzylamine decreased plasma uric acid in mice fed a high-fat diet (HFD) (Iffiú-Soltész et al. 2010). Indeed, benzylamine is the reference substrate of semicarbazide-sensitive amine oxidase (SSAO), an enzyme highly expressed in fat depots (Bour et al. 2007) and in vessels (Nunes et al. 2011). This enzyme mainly located at the plasma membrane exhibits its catalytic site at the cell surface, i.e. in the interstitial milieu (for adipocytes or vascular smooth muscle cells) or in the blood (for endothelial cells). SSAO, also called vascular adhesion protein-1, is currently being renamed primary amine oxidase (PAO) because there are now many inhibitors that exhibit higher affinity and selectivity than semicarbazide itself (O’Rourke et al. 2008), and therefore will be referred to as SSAO/PAO throughout this report. SSAO/PAO, such as many other amine oxidases, including the widely known monoamine oxidases (MAO-A and MAO-B), generates ammonia when catalysing the oxidative deamination of any soluble amine substrate of endogenous or exogenous origin (methylamine, tryptamine, benzylamine, polyamines …).

The administration of benzylamine supplies more substrate to SSAO/PAO and to MAO-B and increases ammonia formation in a way that was suspected to modify the nitrogen waste and to influence the circulating levels of uric acid, by modifying its production or by reducing its solubility (Bowyer et al. 1979). Alternatively, the SSAO/PAO activation, alongside its production of ammonia, generates other end-products of oxidative deamination: the aldehyde corresponding to the oxidized primary amine and hydrogen peroxide. Excess of the latter product is known to be involved in oxidative stress but hydrogen peroxide has also been demonstrated to mimic several insulin actions and is involved in the antidiabetic properties of benzylamine (Carpéné 2009, Iffiú-Soltész et al. 2010, Mercader et al. 2010).

Our aim was therefore to investigate whether the lowering effect of benzylamine treatment on circulating uric acid was a direct consequence of amine oxidase-related ammonia production or was more related to an improvement of the glucose handling in the treated diabetic mice.

The following data bring evidence that activation of SSAO/PAO in normoglycemic mice does not reduce uric acid circulating levels and that the invalidation of SSAO/PAO does not influence its levels, either.

**Methods**

**Animals and treatments**

All mice were handled in accordance with the guidelines of the French National Institute of Medical Research (INSERM) for experimental animal care. They were housed under specific pathogen-free conditions, at 20 to 22 °C, with a 12-hour light-dark cycle and free access to food and water. Male db-/- and their heterozygotes (db-/+) for the naturally occurring mutation of the db gene, encoding for leptin receptor, were offspring of genitors from Charles River Laboratories France. After weaning (4-week old), the males were separated in two groups, one receiving benzylamine in the drinking water at 0.5 % (changed weekly) while the other group of littermates remained untreated. At the age of 6 weeks, the db-/- animals, phenotyped on their overweight were separated from their lean db-/+ littermates to facilitate the weekly follow-up of growth and food consumption. At the end of treatment (11 weeks of age), body mass and unfasted blood glucose were measured in all the groups as well as urea, uric acid and various metabolic circulating parameters.

AOC3-KO mice (knock out for SSAO/PAO) generously given by Prof. S. Jalkanen and colleagues (Turku, Finland) on C57Bl6 background were crossed with FVB/N mice for two generations in our breeding center (Service Zootechnie, UMS 006, Anexplo, Toulouse, France). AOC3 gene invalidation was confirmed by RT PCR as previously reported (Stolen et al. 2005). Homozygotes for the invalidated gene and homozygotes for the wild type gene were used as AOC3-KO and WT control, respectively with heterozygoteous littermates excluded from the study. Mice were sacrificed...
at 26-28 weeks of age after overnight fasting and subcutaneous white adipose (SCWAT) was frozen for subsequent determination of benzylamine oxidation.

**Determination of circulating parameters and of tissue-bound amine oxidase activity**

Assays of circulating uric acid were performed with a kit from Horiba ABX Medical (Montpellier, France) using an uricase-peroxidase method and a spectrophotometric detection at 550 nm of the hydrogen peroxide generated during the catalysis of uric acid into allantoin, based on the use of peroxidase and amino-4-antipyrine / N-ethyl-N-(2-hydroxy-3-sulfopropyl) n-toluidine.

Urea was determined with a kit from Horiba ABX Medical (Montpellier, France) using an urease-glutamate dehydrogenase method. Urea is hydrolysed in presence of urease to produce ammonia, which combines with 2-oxoglutarate and NADH and is converted by glutamate dehydrogenase to glutamate and NAD. The decrease of NADH was spectrophotometrically detected at 340 nm. The volumes of samples and reactives were adapted to 96-well microplates for use in a multi-analyser ABX Pentra 400. Blood glucose levels were determined using an Accu-Chek glucose monitor (Roche Diagnostic, Meylan, France).

Amine oxidase activity was measured on homogenates of thawed SCWAT with 0.1 mM [14C]-benzylamine in 200 mM phosphate buffer and in the presence of an antiprotease cocktail from Sigma as previously described (Visentin *et al.* 2005). SSAO/PAO activity was sensitive to semicarbazide 1 mM as previously documented (Prévot *et al.* 2007).

**Chemicals**

Benzylamine hydrochloride, semicarbazide, and other reagents were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). [14C]-benzylamine (57 mCi/mmol) came from Amersham Biosciences (Buckinghamshire, UK).

**Statistical analysis**

Results are given as means ± S.E.M. Differences between BZA-treated or Knock out and respective control were determined by Student’s *t* test.

**Results**

**In vivo activation of SSAO/PAO and uric acid levels**

In a previous experiment, oral administration of benzylamine at 510 mg/kg bw/day resulted in a decrease in plasma uric acid in mice fed a high-fat diet (HFD) (Iffiú-Soltész *et al.* 2010). We observed that plasma uric acid fell from 365.9 ± 32.4 in HFD mice to 234.1 ± 13.6 µmol/l in the group subjected to HFD + benzylamine (n = 12, *p*<0.001). Our first aim was to confirm whether such changes induced by continual administration of benzylamine could be reproduced in db-/- mice, a model of obesity and diabetes that is more drastic than the HFD-induced ones, and which offers the advantage to occur with the same standard chow given to lean control littermates.

Table 1 shows that administration of benzylamine in the drinking water, leading to a oral spontaneous ingestion of approximately 1000 mg/kg bw/day during 8 weeks, lowered the plasma uric acid in the obese and diabetic db-/- mice. Such reduction was concomitant with a reduction of the dramatically elevated blood glucose, a typical characteristic of this genetic model of diabetes associated to obesity. To distinguish whether the involved mechanism was dependent on the
ammonia generation or on the mitigation of insulin resistance and/or improvement of glucose tolerance, the same treatment was performed in non-diabetic, non-obese mice. By contrast, this treatment did not modify the blood glucose or the plasma uric acid of the lean heterozygous control mice. Indeed, the db-/+ mice were normoglycemic and non-obese and they did not exhibit any detectable change in response to benzylamine treatment. Lastly, the db-/- mice did not exhibit any significant change in their circulating urea, and benzylamine was without any influence on this parameter in both genotypes. Therefore, benzylamine administration was able to modify the circulating levels of uric acid only when they were elevated and associated to a diabetic state or to metabolic syndrome.

Moreover, the change of ammonia production suspected to occur upon SSAO/PAO activation by increasing substrate availability was unable to alter the plasma urea in diabetic as in non-diabetic conditions, while it was associated with a clear decrease in uric acid only when there was a normalisation of the hyperglycemia. This latter beneficial issue was likely the consequence of the benzylamine oxidation, since it has been reported in both in vitro and in vivo models that the insulin-like effects of SSAO/PAO substrates are mediated by one of the end products of amine oxidation: hydrogen peroxide.

Invalidation of SSAO/PAO and uric acid levels

Thus, it was decided to determine the uric acid levels in mice invalidated for the AOC3 gene, which encodes for SSAO/PAO. These mice were totally unable to oxidize benzylamine (Table 2) or any of the other SSAO substrates (not shown). Under standard conditions of breeding, they appeared overall healthy and their plasma uric levels were unchanged compared to their respective controls (Table 2). Therefore, the complete obliteration of the ammonia production mediated by the SSAO-dependent oxidation of endogenous or exogenous amines was not sufficient to alter uric acid production/elimination in such mice. Similarly, the complete lack of SSAO activity was without any consequence on the fasting glycemia (Table 2).

To summarize our observations of the diverse mouse models studied, it can be suggested that SSAO/PAO activation by prolonged challenge to one of its substrates, or SSAO/PAO complete blockade, are not able on their own to modify uric acid levels when they are considered as being in the normal range. The decrease in uric acid seems to accompany the normalization of blood glucose that can be observed with SSAO substrate administration.

Discussion

The lowering effect of benzylamine on circulating uric acid in db-/- diabetic mice is in perfect agreement with the observed reduction in the levels of this metabolite in HFD-fed mice drinking benzylamine (Hfflú-Soltész et al. 2010). In this former report, it was suggested that the oxidation of benzylamine was required to observe such effects. In fact, the acute effect of benzylamine on glucose tolerance, which leads to a reduction in the hyperglycemic excursion after a glucose load, has been reported to be abolished by previous treatment with semicarbazide, an inhibitor of SSAO/PAO (Iglesias-Osma et al. 2004). Such a hypothesis of the involvement of SSAO/PAO activity in the antidiabetic action of prolonged benzylamine treatment totally fits with the fact that benzylamine is also active in db-/- mice.
regarding to the higher SSAO/PAO activity we found in the fat depots of db/-/- when compared to their lean littermates (Ifiú-Soltész et al. 2011). Moreover, our data confirm that plasma uric acid is a surrogate marker of metabolic syndrome, which increases in type 2 diabetic conditions, and which can be almost normalized once the hyperglycemia is alleviated. It was the case here for the db/-/- mice in untreated and benzylamine-treated conditions, respectively.

Indeed, it has already been proposed that a portion of the ingested benzylamine can be taken up through the intestinal barrier and can be oxidized in peripheral tissues (Ifiú-Soltész et al. 2010). Such peripheral oxidation by SSAO/PAO and MAO-B facilitated glucose utilization, especially in fat cells (Ifiú-Soltész et al. 2007, Mercader et al. 2010) while SSAO substrates have been described also to increase glucose consumption by cultured smooth muscle cells (El Hadri et al. 2002) and by immune cells (Lin et al. 2011). As a consequence, it appeared that the chronic benzylamine administration improves glucose disposal in diabetic mice by facilitating its use in peripheral tissues (Ifiú-Soltész et al. 2007). However it remains unclear how this resulted in a decrease of uric acid once diabetes was partially or entirely prevented. Benzylamine oxidation was supposed to occur mainly in the WAT of the genetically obese/diabetic treated mice (owing to the elevated SSAO/PAO expression in the enlarged fat depots) (Ifiú-Soltész et al. 2011), and by consequence, was improving one major sign of the metabolic syndrome: the elevated blood glucose. Then, others components of the metabolic syndrome of the db/-/- model should have been normalized either, including the elevated levels of uric acid. The fact that benzylamine participated in lowering uricemia, without modifying urea levels may indicate that uric acid was more concerned as a metabolic syndrome marker than as a nitrogen catabolism end-product. While further studies on this model can pour more light on this issue, the verification we made in the non-diabetic, non-obese mice showed that benzylamine ingestion/oxidation on its own was unable to modify the plasma uric acid levels. This definitively indicated that the changes induced by daily ingestion of benzylamine on uric acid turnover were more related to the observed mitigation of glucose tolerance and insulin sensitivity than to an alteration in the control of waste products of nitrogen metabolism.

To further test whether ammonia production by SSAO/PAO could influence uric acid metabolism/solubility, it was feasible to verify the consequences of the complete invalidation of this amine oxidase since the mice KO-AOC3 are viable (Stolen et al. 2005) and devoid of capacity to oxidize benzylamine (Bour et al. 2009). Our results show that the lack of SSAO/PAO is without noticeable influence on uric acid levels, at least in the context of normoglycemia. Therefore, the complete obliteration of the ammonia production mediated by the SSAO-dependent oxidation of endogenous or exogenous amines was not sufficient to alter uric acid production/elimination in the KO-AOC3 mice.

Taken together, our observations of long-term activation of SSAO/PAO by benzylamine, (a protoalkaloid found in several medicinal plants and present at low amount in edible vegetables) showed that the levels of uric acid can be modified only with the occurrence of an improvement of glucose intolerance and insulin resistance. It remains to be determined whether the role of SSAO/PAO in inflammatory process (O’Rourke et al. 2008) (via its action on leukocyte adhesion (Jalkanen et al. 2007)) is involved in such beneficial effect for reducing hyperuricemia. Nonetheless, when glycemic control is unaltered, any increase or blockade of SSAO/PAO activity has no detectable influence on uric acid production/elimination. Our supposed link of compensatory mechanism between ammonia production and nitrogen waste production was far from being evidenced, at least when SSAO/PAO is considered as instrumental to alter ammonia production in the organism. Similarly, our investigations did not allow to precise whether hyperuricemia is a causative process or a protective feedback in the metabolic syndrome.

Thus, uric acid, a multifunctional metabolite which is a the crossroads of nitrogen waste excretion and of antioxidant defences or glucose homeostasis, can be influenced by SSAO/PAO activity in an indirect manner that depends more on an overall improvement of glucose disposal in diabetic states than on nitrogen catabolism.

Conflict of Interest
There is no conflict of interest.

Acknowledgements
This work was partly granted by INTERREG IVB-SUDEO-FEDER (DIOMED, SOE1/P1/E178, http://diomed-sudoe.eu) and partly supported by “Communauté de Travail des Pyrénées” and by Balaton
Programme for French-Hungarian exchanges. The authors express gratitude to Prof. Philippe Valet (UMR1048, Toulouse) and all the staff of their animal unit for facilitating the breeding of the offsprings of the KO-AOC3 mice generously given by Prof. Sirpa Jalkanen (Turku Univ, Finland), and to Sandy Bour for invaluable help.

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