76316_Auto_Edited.docx

WORD COUNT	6016	TIME SUBMITTED	01-JUL-2022 01:09AM
		PAPER ID	87885152

Name of Journal: *World Journal of Diabetes*

Manuscript NO: 76316

Manuscript Type: ORIGINAL ARTICLE

Basic Study

Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation

Carpéné C *et al*. Benzylamine and glucose handling in lipoatrophic diabetic mice

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Abstract

BACKGROUND

Benzylamine oral administration delays the onset of hyperglycemia in insulin-resistant *db*$^{-/-}$ mice, a genetic model of obesity and type 2 diabetes.

AIM

To extend the antihyperglycemic properties of oral benzylamine to a model of insulin-deficient type 1 diabetes.

METHODS

Male Swiss mice were rendered diabetic by streptozotocin treatment (STZ) and divided in two groups: One received 0.5% benzylamine as drinking solution for 24 d (STZ Bza-

drinking) while the other was drinking water *ad libitum*. Similar groups were constituted in age-matched, non-diabetic mice. Food intake, liquid intake, body weight gain and non-fasting blood glucose levels were followed during treatment. At the end of treatment, fasted glycemia, liver and white adipose tissue (WAT) mass were measured, while glucose uptake assays were performed in adipocytes.

RESULTS

STZ diabetic mice presented typical features of insulin-deficient diabetes: reduced body mass and increased blood glucose levels. These altered parameters were not normalized in Bza-drinking group in spite of restored food and water intake. Benzylamine consumption could not reverse the severe fat depot atrophy of STZ diabetic mice. In the non-diabetic mice, no difference was found between control and Bza-drinking mice for any parameter. In isolated adipocytes, hexose uptake was partially activated by 0.1 mmol/L benzylamine in a manner that was obliterated *in vitro* by the amine oxidase inhibitor phenelzine and that remained unchanged after Bza supplementation. Oxidation of 0.1 mmol/L benzylamine in WAT was lower in STZ diabetic than in normoglycemic mice.

CONCLUSION

Bza supplementation could not normalize the altered glucose handling of STZ diabetic mice with severe WAT atrophy. Consequently, its antidiabetic potential observed in obese and diabetic rodents does not apply for lipoatrophic type 1 diabetic mice.

Key Words: Diabetes; Adipocytes; Amine oxidases; Insulin-like agents; Glucose transport; polydipsia

Carpéné C, Stiliyanov Atanasov K, Les F, Mercader Barcelo J. Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation. *World J Diabetes* 2022; In press

Core Tip: Benzylamine is oxidized by amine oxidases highly expressed in adipose tissue, and stimulates glucose uptake in adipocytes. Accordingly, benzylamine oral administration alleviates insulin-resistant diabetes in obese and diabetic mice, at the expense of adipose tissue enlargement. The current study aimed at verifying whether benzylamine could also exhibit antihyperglycemic action in insulin-deficient type 1 diabetes. To this aim, non-obese diabetic mice were subjected to a 0.5% benzylamine drinking solution. The results show that lipoatrophic diabetic mice drinking benzylamine did not reduce their elevated blood glucose and did not recover their reduced adiposity. Remarkably, oral benzylamine limited the increased food and water intake of diabetic mice, without modifying that of non-diabetic. These results indicate that a minimal level of adiposity might be required to support benzylamine oxidation and to improve glucose utilization, while the antipolydipsic effects of the dietary amine appear to be centrally mediated. Consequently, our previously proposed therapeutic interest of benzylamine-based antidiabetic approaches, established in obese and diabetic models, does not apply for diabetic states with severe lipoatrophy.

INTRODUCTION

A recent study indicates that orally given benzylamine delays the onset of diabetes in obese and insulin-resistant *db*$^{-/-}$ mice[1]. In fact, a supplementation with 0.5% benzylamine (5 g/L) in the drinking water impaired the increase in blood glucose, water intake and urine emission that occurs after weaning in this mouse model of insulin-resistant type 2 diabetes. The proposed mechanism of action for ingested benzylamine, which is naturally present in vegetables and edible plants, relies on its oxidation by an amine oxidase, which is a copper-containing enzyme highly expressed in fat cells[2,3]: The semicarbazide-sensitive amine oxidase (SSAO)[4] also known as amine oxidase copper containing 3[5] and identical to vascular adhesion protein 1 (VAP-1)[6]. More precisely, it is hydrogen peroxide, one of the products of amine oxidation, and known from decades to stimulate glucose uptake in fat cells[7], that supports the

insulin-mimetic actions of benzylamine in adipocytes, either in rodents[8] or in humans[9]. The *in vitro* insulin-like actions of benzylamine encompass activation of glucose uptake[10], induction of adipogenesis[11] , stimulation of lipogenesis[12], and inhibition of lipolysis. They occur even in the absence of insulin[8]. It was therefore of interest to investigate whether an oral treatment with benzylamine is capable of alleviating the impaired glucose handling of insulin-deficient, type 1 diabetic states.

Type 1 diabetes is characterized by a deficiency in insulin resulting from endocrine pancreas injury. To treat this disease, it is necessary to permanently normalize the altered blood glucose homeostasis. Since insulin is the major regulator of blood glucose levels, many therapeutic beneficial approaches have consisted in providing this pancreatic hormone, *via* repeated injections, or even by more sophisticated administration modes using biotechnologies, islet transplants or cell therapies[13]. Whatever the mode of supply, insulin overdose has to be avoided to prevent the risk of fatal hypoglycemia and to limit the onset of insulin resistance. Of note, various pharmacological agents or naturally occurring molecules can act as insulin-like factors on the glucose utilization by peripheral tissues[14]. In this view, testing the putative antihyperglycemic effect of benzylamine in type 1 diabetic rodents remained a preclinical step that deserves descriptive studies.

Alongside its great capacity to oxidize benzylamine[1], fat tissue is not quantitatively but qualitatively of paramount importance in the regulation of glucose disposal. Not only adipose tissue uses glucose for accumulating lipid stores, it also act as an endocrine organ secreting a variety of adipokines with hyperglycemic or hypoglycemic properties, even in the absence of exogenous insulin[15]. In this view, the lack of adipose tissue (lipoatrophy), as that obtained in several genetically modified mice, is accompanied with altered glucose homeostasis[16,17]. Similarly, diabetic type 1 models, such as the "streptozotocin (STZ) diabetic" rodents challenged with STZ, which destroys endocrine pancreas, exhibit very reduced fat stores[18,19]. In humans, successful treatment of type 1 diabetes is concomitant with both restoration of normal glucose levels and adipose tissue recovery[20].

More importantly, diabetic phenotypes of diverse animal models have been ameliorated when white adipose tissue (WAT) or brown adipose tissue (BAT) has been reintroduced in these models, irrespective of the method used. Nowadays, it is suggested that adipose tissue contributes to the correction of type 1 diabetes, since hyperglycemia was lowered in diabetic mice treated by conditioned media from adipose-derived stem cells[21], and since mitigation of diabetes was observed in STZ diabetic mice receiving BAT transplant[19]. To date, the beneficial effects of ingested benzylamine on glucose and lipid handling have been studied in obese rodents only[1,22]. These studies suggested that enhanced fat deposition contributed to the insulin-like effects observed *in vivo*. Again, these considerations reinforced our interest in investigating the effects of benzylamine in a lipoatrophic model of type 1 diabetes.

In fact, the capacity of benzylamine to activate glucose transport in rat or mouse adipocytes is potentiated by the presence of vanadium[10,23], a widely recognized insulin-like agent[24,25]. Accordingly, it has been already demonstrated that *in vivo* treatments with a combination of amine oxidase substrates and vanadium exert antidiabetic effects in diverse diabetic rodents, including the STZ diabetic rats[10,26]. However, we demonstrated in recent studies that benzylamine[9] or catecholamines[27] are capable of activating glucose transport in human adipocytes, even in the absence of vanadium, and that the synergism vanadate/amine is much more weak in human adipocytes than in the murine ones. All these observations prompted us to examine for the first time the influence of prolonged oral administration of benzylamine alone—without any added vanadate—in a model of type 1 diabetes, which is non-obese and insulin-deficient: the STZ-induced diabetic mouse.

To this aim, we investigated in the current study whether benzylamine alone was able, *via* oral consumption, to improve glucose handling in insulin-deficient STZ mice. Clearly, the following results will not confirm our assumption, while they suggest that benzylamine action on glucose disposal requires a minimal amount of adipocytes prone to increase their glucose consumption when oxidizing this SSAO substrate.

MATERIALS AND METHODS

Chemicals

Benzylamine hydrochloride, STZ, bovine insulin, phenelzine, collagenase A, and most of the other reagents were from Sigma-Aldrich-Merck (Saint Quentin Fallavier, France). [^{3}H]-2-deoxyglucose (2-DG) was from Perkin Elmer (Boston, MA, United States). The glucometers and consumables for follow up of fed blood glucose were provided by Pr. Valet P. (Univ Toulouse, France), and used as previously described[28].

Insulin-deficient type 1 diabetic mice

Male Swiss mice obtained from Charles River Laboratories (L'arbresle, France) were housed at constant temperature (20-22 °C) and with a 12h light-dark cycle. At the age of 2 mo, they received an intraperitoneal injection of streptozocin (40 mg/kg) diluted in citrate buffer (0.05 mmol/L, pH 4.5) for 4 consecutive days, as in[21]. A week later, mice receiving only citrate buffer (non-diabetic) and treated mice exhibiting blood glucose ≥ 300 mg/100 mL (STZ diabetic) were subdivided into four groups of 8 males, with either free access to water (control) or having a 0.5 % benzylamine solution as drinking liquid (Bza-drinking) for 24 d. To measure plasma insulin levels at the beginning of treatment, blood samples were withdrawn from tail vein then centrifuged and analyzed using Ultrasensitive insulin-ELISA kit from Mercodia (Uppsala, Sweden). All the mice had free access to food and water and were treated in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)[29]. During this period, non-fasting blood glucose levels were determined every three days at 12:00 (equivalent in the used circadian rhythm to four hours after lights turned on) using an Accu-Check glucometer (Roche Diagnostics) on a blood drop withdrawn from the tail vein. Mice were euthanized after overnight fasting at the end of treatment and organs were collected and weighed.

Adipocyte preparations

Adipocyte preparations were obtained by collagenase digestion of a pool of WAT immediately after removal from the epididymal, intra-abdominal and inguinal anatomical locations. WAT was cut into small pieces, then digested at 37 °C by collagenase under agitation in Krebs–Ringer buffered at pH 7.5 with 15 mmol/L sodium bicarbonate, 10 mmol/L HEPES, supplemented with 3.5% of bovine serum albumin, as previously described[1]. Preparations of buoyant adipocytes were isolated from the digested WAT by filtration through nylon stockings and two gentle buffer washes as in[10]. In our digestion process, approximately one gram of WAT was necessary to obtain sufficient functional adipocytes for the subsequent hexose uptake assays. When total amount of dissected WAT exceeded 1 g, excess samples were snap-frozen at -80 °C. This occurred for each of the normoglycaemic mice but not for the lipoatrophic STZ-treated mice. In this case, pools of two mice were used to freeze c.a. 200 mg WAT.

Glucose transport assays

The non-metabolizable analogue [^{3}H]-2-DG was the only source of hexose for the cell preparations during glucose transport assays. It was added at a final concentration of 0.1 mmol/L after 45 min incubation of the fat cell suspension with the tested agents, as previously detailed[10]. 2 mmol/L pyruvate was also present in the medium throughout the experiments for energy supply. 100 µL of the radioactive 2-DG (approximately 1300000 dpm/vial) were added to 400 µL of fat cell suspension and hexose uptake assays were stopped 10 min later with 100 µL of 100 µmol/L cytochalasin B. Then, 200 µL of cell suspension were immediately transferred to plastic centrifugation microtubes prefilled with dinonyl-phthalate (density 0.98 g/mL), then subjected to a 30 s spin. The upper part of the tubes, containing radiolabelled hexose internalized in intact fat cells floating above the silicon layer was then counted in scintillation vials as described in[10]. The extracellular [^{3}H]-2-DG present in the upper part of the tubes was determined in tubes receiving cytochalasin B prior to 2-DG. It averaged 1%-5% of the radioactivity found in control uptake, and was subtracted from all assays, as in[9].

Determination of benzylamine oxidation

Amine oxidase activity was determined at 37 °C using [^{14}C]-benzylamine as substrate, in homogenates of thawed WAT samples as previously described[10]. Isotopic dilution of [^{14}C]-benzylamine (final concentration: 0.1 mM) was incubated for 30 min in 200 µL of 200 mmol/L phosphate buffer with approximately 50 µg proteins, then the radiolabeled oxidation products were immediately extracted in toluene/ethyl acetate and counted as previously specified[9]. Results were expressed as nmol of deamination products/mg protein/min.

Statistical analysis

Results are presented as means ± SEM of (n) observations. All the statistical analyses for comparisons between parameters used ANOVA followed by post-hoc Dunnett's multiple comparisons test, analyzed with Prism 6 for Mac OS X (from GraphPad software, Inc). Relative EC_{50} values were calculated by nonlinear regression. NS means non-significant difference.

RESULTS

Benzylamine supplementation normalizes the increased food and water consumption of STZ-induced diabetic mice without restoring body weight gain

At the start of the experiment, the STZ-induced diabetic mice exhibited a slightly lower body weight (bw) when compared to age-matched normoglycemic mice (Figure 1). The bw gain of the insulin-deficient mice was also very limited during the treatment period and was not corrected by Bza supplementation: at the end of the experiment, the mean bw of STZ mice remained lower than that of normoglycemic mice. Hence, Bza supplementation tended to limit bw gain in both groups, but this trend did not reach the limit of significance (Figure 1A). No significant decrease in food consumption was found in the Bza-drinking normoglycemic mice. By contrast, the hyperphagic status of the STZ mice was alleviated by Bza supplementation (Figure 1B). A similar influence of

Bza supplementation was found for water consumption: an almost normalization of the elevated daily water intake of STZ diabetic mice occurred in the group subjected to Bza-drinking (Figure 1B).

Figure 1 also shows that the characteristic polydipsic feature that takes place in the STZ-induced type 1 diabetes was of greater magnitude than the hyperphagy triggered by the noxious diabetogenic agent. The exaggerated liquid consumption of the diabetic group was increased by 5.7 times when compared to normoglycemic control while this increase only reached 1.7 times for food intake. The former defect was expected to traduce glycosuria[19,30], while the second likely corresponded to a lowered efficiency of the ingested carbohydrates that accompanies insulin deficiency[31].

In view of these alterations of food and water intake in STZ diabetic mice and their recovery after Bza-drinking, the influence of Bza supplementation on blood glucose levels was examined in both fed and fasted conditions.

Influence of oral supplementation of benzylamine on blood glucose in non-diabetic and diabetic mice

Figure 2 shows the pattern of non-fasting glycemia during the treatment period for the four experimental groups. The unfasted blood glucose levels of the mice previously challenged with STZ were at least twice higher than those of control throughout the study (Figure 2A). Such strong hyperglycemia was mainly a consequence of the low circulating levels of insulin found at the start of treatment in the two groups of STZ diabetic mice (0.40 ± 0.04 and 0.38 ± 0.05 ng/mL) when compared to the non-diabetic ones (1.26 ± 0.14 and 1.35 ± 0.09 ng/mL, $n = 8$; $P < 0.001$). In the STZ diabetic mice, the blood glucose levels remained elevated in both Bza-drinking and water drinking groups (Figure 2A). In the normoglycemic mice, the non-fasting blood glucose was superimposed in control and Bza-drinking groups and remained below 200 mg/100 mL. Thus, blood glucose levels were not significantly influenced by repeated Bza consumption.

To avoid any alteration in body weight gain and in glucose handling, the mice were subjected to overnight fasting only once, at the end of experiment. Figure 2B shows that fasting blood levels were expectedly lower than non-fasting blood glucose. Again, the fasting values were superimposable in Bza-drinking mice and their respective control while the fasting blood glucose of STZ diabetic mice was much higher than the values found in non-diabetic groups (Figure 2B). Thus, Bza supplementation did not exhibit any hypoglycemic or anti-hyperglycemic action in this animal model of severe type 1 diabetes.

These findings contrasted with the capacity of benzylamine to delay the onset of diabetes in the genetically obese and diabetic *db*$^{-/-}$ mouse[1]. Given the unexpected lack of efficiency of Bza consumption on glucose handling, it was poorly appropriate to delineate its putative mechanisms of action or to further examine other surrogate makers of diabetic state, as reported in[1]. Instead, it was verified whether the dose of Bza ingested was similar in the two diabetic models. Considering the daily liquid intake and the body mass of the STZ mice, it was calculated that these type 1 diabetic mice ingested 10850 ± 598 µmoles of benzylamine/kg bw/d throughout the treatment. This dose was similar to that used for Bza supplementation in the young type 2 diabetic *db*$^{-/-}$ mice studied in[1], which ranged between 9300 and 10100 µmol/kg bw/d. However, another difference between type 2 (insulin-resistant) and type 1 (insulin-deficient) diabetic mouse models lays in the occurrence of excessive fat depots in the former and a clearly emaciated state in the latter. Therefore, attention was focused on WAT in the STZ mice and their controls.

Comparison of fat stores between normoglycemic and STZ-induced diabetic mice

Smaller mass of subcutaneous and visceral WAT was a typical feature of STZ-induced diabetic mice when compared to normoglycemic control (Figure 3). In the STZ diabetic mice, the very low mass of fat pads was not modified by Bza-drinking, whatever their anatomical location. Similarly, the normal adiposity of the non-diabetic mice was not modified after oral Bza supplementation.

When the mass of the dissected fat depots was normalized as percentage of bw, such "adiposomatic index"[22] was significantly lower in diabetic than in non-diabetic mice (1.2 ± 0.4% *vs* 3.7 ± 0.6%, $P < 0.001$). Again, Bza supplementation did not modify adiposomatic index: 1.3 ± 0.4% and 3.9 ± 0.4%, in Bza-drinking diabetic and non-diabetic groups, respectively.

In contrast, the weight of the liver was identical in the four experimental groups (Figure 3). However, when liver mass was expressed as ratio to bw, the difference that appeared between diabetic and non-diabetic animals was opposed to that of adiposomatic index. The liver represented 5.3 ± 0.2 % of body mass in both STZ diabetic and STZ diabetic Bza-drinking mice, respectively (NS, $n = 8$). This proportion was smaller in non-diabetic mice (4.2 ± 0.1 %, $P < 0.001$), even after Bza-drinking (4.5 ± 0.2 %).

Taken together, these observations indicated that the STZ-induced diabetic mice did not normalize their reduced fat deposition and bw gain after Bza supplementation, in spite of a partial recovery of their altered food intake. Moreover, Bza supplementation was not efficient in normalizing the altered blood glucose control or relative hepatomegaly of the STZ mice, although limiting polydipsia. Since we have previously proposed that the Bza oxidation occurring in the hypertrophied WAT of obese and diabetic $db^{-/-}$ mice supports its insulin-like *in vitro* effects by facilitating glucose utilization in adipocytes and contributes to its antihyperglycemic action[1], such *in vitro* effects were examined.

Effects of insulin and benzylamine on glucose transport in mouse adipocytes

Unfortunately, the WAT atrophy of the STZ diabetic mice did not allow the preparation of sufficient biological resource for exploring the activation of 2-DG uptake in functional adipocytes from diabetic and Bza-drinking diabetic mice. There was only a pool of around 400 mg of WAT dissected from different anatomical locations in each STZ mouse while there was between 1 and 2 g removed from each non-diabetic mouse. Consequently, sufficient adipocytes could be isolated from the latter samples only, and

the subsequent hexose uptake assays were performed with adipocyte preparations that contained 18.0 ± 2.8 and 19.0 ± 2.5 mg lipid/400 µL in normoglycemic Bza-drinking and control mice, respectively. Thus, Figure 4A shows insulin stimulation of 2-DG uptake in non-diabetic mice only. As expected, insulin dose-dependently activated hexose uptake in adipocytes from control mice and a tendency to improve insulin maximal effect was detected in Bza-drinking mice. EC_{50} values of insulin were 0.4 and 2.3 nmol/L for Bza-drinking and control mice groups, respectively, without showing significant difference between them. Figure 4B indicates that 0.1 mmol/L benzylamine was capable of reproducing one-third of the maximal insulin stimulation, in a manner that was blunted by the amine oxidase inhibitor phenelzine, which was inactive on basal or insulin-stimulated hexose uptake. Moreover, the amine oxidase-dependent insulin-like effect of 0.1 mmol/L benzylamine was similar in control and Bza-drinking non-diabetic mice. Finally no influence of oral benzylamine supplementation was found on the capacity of phenelzine to inhibit *in vitro* the insulin-like action of the amine (Figure 4B).

Oxidation of benzylamine in thawed preparations of adipose tissues

Amine oxidase activity was determined in homogenates from thawed WAT samples by measuring their capacity to oxidize 0.1 mmol/L [^{14}C]-benzylamine. When expressed as nmoles of amine oxidized/mg protein/min, the activity was limited in WAT from STZ diabetic mice compared to normoglycemic ones, whatever the group considered, control or Bza-drinking (Figure 5). The reduced amount of WAT and its limited amine oxidase activity did not argue for a strong contribution of fat stores in the biotransformation of the Bza ingested by STZ diabetic mice.

DISCUSSION

At the first glance, the lack of antihyperglycemic effect of Bza-drinking described here in STZ diabetic mice contrasts with its antidiabetic action observed in obese and diabetic *db*$^{-/-}$ mice[1]. Indeed, and as discussed below, all these findings converge to propose that the difference in Bza-drinking efficiency between the models of type 1 and

type 2 diabetes is not related to insulin deficiency *vs* resistance, but rather to a difference in adiposity between the murine models.

Alongside bearing dramatically larger fat depots than their lean counterparts, the obese and diabetic *db*$^{-/-}$ mice also possess higher levels of SSAO activity in their fat cells[1,32]. Thus, the antihyperglycemic effect of oral Bza reported for *db*$^{-/-}$ mice, and not for their lean littermates, could be related to the elevated amine oxidase activity found in the hypertrophied WAT of obese and diabetic animals [1]. In contrast, STZ diabetic rats exhibit lower monoamine oxidase (MAO) and SSAO activities in WAT than their normoglycemic controls[18]. The lack of antihyperglycemic effect of Bza supplementation in STZ mice reported here resembles to the weak antidiabetic effect of prolonged administration of tyramine in STZ rats[18]. Tyramine, which is a substrate of both MAO and SSAO, was capable of limiting the hyperglycemic responses to a glucose load during a glucose tolerance test but was unable to normalize the elevated fasting blood levels of these insulin-deficient rats. Tyramine or benzylamine could lower the elevated blood glucose of STZ-induced diabetic rats only when combined with vanadium[10,18,33].

Particular attention has been paid to studying the potential antidiabetic effects of amines alone since the synergism between vanadium and biogenic amines on the activation of glucose transport does not work well in human adipocytes[9,27]. Moreover, the potential antidiabetic use of vanadium derivatives is still limited by toxicological aspects. In this view, several observations suggest that the beneficial effects of dietary amines on glucose handling in diabetic rodents (even when not combined with vanadium) rely with the amount of SSAO present in WAT. The supplementation of drinking water with 0.4 % methylamine (another SSAO substrate) has been reported to increase epididymal WAT mass and to improve glucose tolerance in transgenic mice overexpressing a human form of SSAO/VAP-1, while it was inefficient in non-transgenic mice[34]. Oral benzylamine also improves glucose handling in high-fat diet fed mice, characterized by increased adiposity[22]. Here, we suppose that it is the

lipoatrophy of STZ diabetic mice (and not their lack of insulin) that prevented the occurrence of an antihyperglycemic action of benzylamine.

The sole beneficial effect of Bza-drinking evidenced in the STZ diabetic mice was an almost total recovery of their characteristic hyperphagic and polydipsic behavior[31]. Thereby, it could be supposed that urinary glucose leak of STZ mice was partially rescued by Bza-drinking. Unfortunately, individual metabolic cages were not available for this study and we could not determine daily urine emission or glucosuria. However, water intake reduction occurred without correction of hyperglycemia. This tended to indicate that renal glucose leak, if any, was not sufficiently rescued by Bza-drinking to influence the overall glucose homeostasis, while this was the case for *db*$^{-/-}$ mice[1]. Food intake was also reduced in Bza-drinking STZ diabetic mice, but without notable decrease in body weight gain. Thus, food efficiency was increased by Bza-drinking. However, we cannot propose any underlying mechanism for this effect.

Indeed, it cannot be excluded that mechanisms other than oxidation by amine oxidases might be involved in the *in vivo* effect of benzylamine on food and water intake. The group of Raimondi and coworkers has already reported that benzylamine, like methylamine, rapidly induces hypophagia in mice *via* a modulation of neuronal channels, which is reinforced by SSAO inhibition[35,36]. This suggests that adipose SSAO is likely not the sole target of ingested benzylamine. Regarding activation of glucose uptake in adipocytes, the effect of benzylamine is impaired when its oxidation by SSAO is blocked. Surprisingly, the opposite occurred regarding its central effects on food and water intake. In fact, when benzylamine degradation by SSAO is blocked, its half-life is increased and its capacity to modulate the neuronal channels depicted by the group of Raimondi is improved[35,36]. Since there is practically no WAT in the STZ-diabetic mice, and since they have little adipose SSAO, we propose that the limitation of hyperphagia and polydipsia observed in these animals is likely due to a central effect distinct from oxidation by peripheral tissues.

Although liver is another of the organs reached by ingested benzylamine, it is not a major site for its biotransformation or detoxification since benzylamine is metabolized

to only a small extent by hepatic subcellular fractions, as observed by Mutlib *et al* [37]. By contrast, these authors reported that, when orally given to rats, benzylamine undergoes oxidative deamination and generates benzaldehyde, then hippuric acid, which is the major metabolite. These authors also observed that benzylamine was fairly stable in rat plasma in spite of the presence of a soluble form of SSAO. In fact, although known to increase with diabetes[18,38-40], the circulating SSAO activity is very feeble when compared to the levels of SSAO found in WAT[1]. A putative mediation of the amine effects *via* modulation of insulin secretion can be ruled out since, in another model of insulin-deficient diabetes, the alloxan-injected rat, oral administration of tyramine was able to reduce the hyperglycemia by 35%-43% in a manner that was more dependent on insulin-like than on insulin-releasing actions[41].

A limitation of the study could be that insulin plasma levels were not determined throughout the treatment since such measurements were performed only at the beginning. However, since circulating insulin was dramatically decreased by STZ challenge, and since the overt hyperglycaemia was not corrected by Bza-drinking, it was hypothesized that pancreas injury was not recovered. In this view, the hyperinsulinemic levels of the insulin-resistant $db^{-/-}$ mice remained unchanged after Bza supplementation[1]. Similarly, no change in plasma insulin was found in the $db^{+/+}$ lean control after Bza-drinking. Nonetheless, it has been reported that methylamine (another SSAO substrate) limits the insulin degradation by adipocytes[42]. If one supposes that increasing insulin's ability to stimulate glucose transport is one of the mechanisms involved in the anti-diabetic effect of benzylamine, this can explain why benzylamine was active in insulin-resistant diabetes but not in insulin-deficient models. Such paradigm of “insulin-sensitizer” capacity might provide an alternative to our interpretations based on the necessary abundance of SSAO and WAT to support peripheral glucose disposal. However, it requires to be demonstrated by further investigations, while we report in the current study that benzylamine alone activates 2-DG uptake in adipocytes, being therefore able to act as an “insulin mimicker” even in the absence of insulin.

Whether the *in vitro* SSAO-mediated insulin-like effect of benzylamine is the sole responsible of the anti-hyperglycemic effect of Bza-drinking is far from being demonstrated here. However, this assumption agrees with the conclusions of independent studies showing that treatment of diabetic rodents with SSAO inhibitors prevents from diabetic complications but is not antihyperglycemic at all[43-45]. Whatsoever, all these observations bring evidence that adipose cells are predominantly involved in benzylamine oxidation, as a consequence of their high SSAO expression[3], though they do not rule out other concomitant mechanisms.

We designed the current study to reach a similar daily amount of Bza ingested by the STZ diabetic mice than that ingested by the obese and type 2 diabetic *db*$^{-/-}$ mice[1]. The results show that such objective was reached. However, similar amine intake did not result into similar beneficial influence on glucose disposal in the two models. In the STZ diabetic mice, the lipoatrophy and the lower richness of WAT in amine oxidase activity gave less probability for an adipocyte-dependent metabolism of the ingested amine and subsequent insulin-like actions. Another apparent weakness of the present study is that the non-diabetic Swiss mice did not ingest the same daily amount of benzylamine than those subjected to the STZ diabetogenic challenge. Indeed, our experiments showed that the polydipsia of the STZ diabetic mice was early rescued, after the first week of Bza supplementation. They also showed that, among the Bza-drinking groups, the accumulated fluid intake of the STZ diabetic mice was about twice more than that of the normoglycemic mice. Then, it could be easily justified *post hoc* that, considering the initial polydipsia of diabetic mice, it would have been preferable to double the benzylamine concentration in the solution given to the Bza-drinking non-diabetic group. Hence, it cannot be excluded that such higher dose of benzylamine would have reduced liquid consumption in the non-diabetic mice also. By assumption, a disgusting adverse effect on liquid consumption remains unlikely since, as other organic amines, benzylamine has a taste varying from almond to fish waste[46], which is not supposed to be reluctant for rodents. In reality, achieving exactly the same oral dose of benzylamine for diabetic and non-diabetic animals would have required weekly pair-adjustments,

relatively difficult to settle, and would not have yielded more information about the mechanisms of action. The unchanged lipoatrophy together with the early recovery of polydipsia in the Bza-drinking group, converge to indicate that the antipolydipsic effect of the amine is mediated by a central effect, distinct from that observed in adipocytes.

The *in vitro* insulin-like effect of submillimolar dose of benzylamine on glucose transport in adipocytes - and its blockade by phenelzine - reinforced our hypothesis of an enhancement of peripheral glucose disposal, although it could not be evidenced in lipoatrophic Bza-drinking STZ mice. Phenelzine, which is a combined MAO and SSAO inhibitor, was used since both MAO and SSAO substrates mimic insulin-like effects in adipocytes[33]. It blocked the benzylamine-stimulated hexose uptake, but not the basal or the insulin-stimulated hexose uptake. No resistance to the selective blockade by phenelzine appeared in the fat cells from Bza-drinking non-diabetic mice, indicating that continuous supplementation with the substrate did not dramatically down-regulate the amine oxidase activities. These hexose uptake assays, which could be performed on non-diabetic mice only, confirmed that, even in the absence of insulin, benzylamine oxidation activates hexose uptake in adipocytes from Swiss white mice as well as in the case of various other rodents[43]. According to the literature, the increase of glucose transport by SSAO activation is rather limited to adipocytes, and only scarce reports have extended this hydrogen peroxide-dependent insulin-like action to other cell types[47]. Unfortunately, an insufficient amount of adipocytes isolated from the atrophied WAT of STZ mice hampered the verification of glucose transport responsiveness to insulin and benzylamine in the type 1 diabetic state. Even if such insulin mimicry occurred also in adipocytes from insulin-deficient mice, it was too limited to modify the glucose handling by the organism, when considering the low mass of WAT, as attested by the significantly lower adiposomatic index found in STZ-treated mice. Moreover, the limited oxidative metabolism of benzylamine found in WAT of STZ mice was likely unable to contribute to a replenishment of the atrophied fat depots *via* the increase of glucose utilization evidenced in adipocytes of normoglycemic control.

Being poorly biotransformed by the limited fat stores of STZ diabetic mice, the ingested benzylamine could not increase glucose entry in adipocytes and thereby did not contribute to glucose disposal. We presume that such lack of benzylamine action explains how its consumption did not decrease elevated blood glucose. Such inefficiency does not preclude future improvements of the antidiabetic therapeutic applications of other amine substrates. However, our findings limit the relevance of Bza consumption to alleviate the complications of type 1 diabetes, especially when accompanied with lipoatrophy. Nevertheless, benzylamine and its derivatives remain potential anti-hyperglycemic agents since a recent integrated network pharmacology analysis has revealed that benzylamine derivatives contribute to the anti-insulin resistance effects of *Moringa oleifera*[48], one of the most potent antidiabetic medicinal plants[30,49,50].

CONCLUSION

Although Bza-drinking is devoid of beneficial *in vivo* effect on the type 1 diabetes at doses at which limit the onset of type 2 diabetes in the genetically obese $db^{-/-}$ mice, the present findings reinforce the hypothesis that oxidation of the amine at the level of adipocytes contributes to peripheral glucose uptake and improves glucose homeostasis. When no sufficient WAT is present in the organism (current case of STZ diabetic mice), the antihyperglycemic effect of benylamine is hampered. In contrast, when benzylamine can be readily oxidized in WAT, it improves glucose tolerance at the expense of an enlargement of fat stores (case of the $db^{-/-}$ mice). The *in vitro* experiments of the current study confirm the capacity of submillimolar doses of benzylamine to activate glucose transport in adipocytes. They also show that such SSAO-dependent insulin mimicry is not altered by chronic administration of the substrate.

ARTICLE HIGHLIGHTS

Research background

Oral administration of benzylamine (Bza) exerts antihyperglycemic effects in obese and diabetic rodent models. This effect has been proposed to depend on the insulin-like action of benzylamine in adipose cells. The amine oxidation catalyzed by amine oxidases abundantly present in adipocytes generates hydrogen peroxide, which activates glucose transport.

Research motivation

To extrapolate the potential antihyperglycemic properties of benzylamine found in obese and diabetic models to the treatment of insulin-deficient type 1 diabetic states. Benzylamine administration might facilitate glucose utilization to increase lipogenic and adipogenic activities in the adipose tissue and thereby improve glucose disposal even in the absence of insulin.

Research objectives

To evaluate the impact of benzylamine supplementation on hyperglycaemia, polydipsia and hyperphagia in type 1 diabetic mouse, and to demonstrate that benzylamine metabolism by adipose tissue supports these antidiabetic effects.

Research methods

Benzylamine solution (5 g/L, Bza-drinking) replaced drinking water in streptozotocin (STZ)-induced, insulin-deficient diabetic mice. Similar comparison between control and Bza-drinking groups was performed in normoglycemic mice. Non-fasting blood glucose, water and food intake were periodically recorded in the four groups. Adiposity was determined at the end of a 24-day treatment. Glucose transport in freshly isolated adipocytes was assessed *ex vivo* by determining the uptake of the non-metabolizable radiolabeled 2-deoxyglucose.

Research results

Chronic benzylamine intake did not normalize hyperglycaemia in STZ diabetic mice, despite it alleviated their excessive water and food consumption. Benzylamine intake had no effect on the limited body weight of the STZ diabetic mice and could not restore their dramatically reduced adipose tissue mass. In normoglycemic mice, Bza-drinking group did not show altered body weight, food or water consumption. However, when directly given *in vitro* to adipocytes isolated from non-diabetic mice, benzylamine was efficient in activating glucose uptake in both control and Bza-drinking groups.

Research conclusions

The capacity of benzylamine supplementation to reduce hyperglycaemia, previously reported in obese and diabetic rodents, was not detectable in the emaciated and insulin-deficient STZ diabetic mice. However, the capacity of benzylamine to activate glucose transport in adipocytes was confirmed in non-obese, non-diabetic mice. It is likely the adipose tissue atrophy induced by STZ challenge that hampered the lipogenic and adipogenic action of benzylamine in this severe model of lipoatrophic, insulin-deficient diabetes.

Research perspectives

The current findings and their interpretations considerably limit the field of applications of oral benzylamine since this molecule does not work as an antidiabetic agent in rodents with reduced adiposity as it is the case of type 1 STZ diabetic and lipoatrophic mice. Nevertheless, since SSAO substrates exhibit a direct action on glucose handling by fat cells, they still represent potential interest for therapeutic use to combat other diabetic states.

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