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## Research Article

### INSULIN-LIKE AND MIMETIC MOLECULES FROM LOWER EUKARYOTES

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#### ABSTRACT

Insulin was first discovered in extracts of vertebrate pancreas during a focused search for a therapy for diabetes. Subsequent efforts to discover and isolate a similar active principle from yeast and plants driven by the hope to identify insulin-like/mimetic molecules with critical advantages in the pharmacokinetic profile and expenditure of production compared to authentic human insulin were not successful. As a consequence, it has generally been assumed that hormones evolved exclusively during course of the evolution of vertebrate endocrine organs, implying a rather recent origin. Concomitantly, the existence and physiological role of vertebrate hormones in lower multi- and unicellular eukaryotes have remained a rather controversial subject over decades, albeit there is some evidence that hormones and hormone-binding proteins resembling those of vertebrates are expressed in fungi and yeast. Past and recent findings on the existence of insulin-like and mimetic materials, such as the glucose tolerance factor, in lower eukaryotes, in particular *Neurospora crassa* and yeast, will be presented. These data provide further evidence for the provocative view that the evolutionary roots of the vertebrate endocrine system may be far more ancient than is generally believed and that the identification and characterization of insulin-like/mimetic molecules from lower eukaryotes may be useful for future drug discovery efforts.

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#### INTRODUCTION

A multitude of treatment regimens for the therapy of type 2 diabetes (T2D) have been introduced during the past six decades. Nevertheless despite the administration of anti-diabetic drugs, such as insulin sensitizers (e.g. metformin) and insulin releasers (e.g. sulfonylureas), and (genetically modified) insulins, such as long- or short-acting ones (e.g. insulin glargine or lispro) in various combinations, a continuous and considerable deterioration of the glycemic control has to be diagnosed in the majority of patients. The ongoing loss of functional  $\beta$ -cells and/or impairment of insulin sensitivity of the peripheral insulin target cells, such as adipocytes, myocytes and hepatocytes, are thought to be the underlying pathophysiological mechanisms. Consequently, the identification of novel therapeutics (chemicals and biologicals) for the correction of the impaired glucose tolerance during the pre-diabetic state and prevention of final hyperglycemia in frank T2D through the engagement of novel signaling pathways has attracted much interest in pharmacological and clinical research. Remarkably, the search for natural products, produced by or extracted from lower eukaryotes, which mimic

the multiple insulin effects or potentiate the residual insulin action remaining left in the insulin-resistant tissues (Samad *et al.*, 2009; Malviya *et al.*, 2010; Smirin *et al.*, 2010) and thereby may be helpful for both patients suffering from type 1 diabetes (T1D) and T2D patients has been considerably intensified during recent years (Azimi-Nezhad *et al.*, 2008; Ceriello and Colagiuri, 2008; Esteghamati *et al.*, 2008). Moreover, the tremendous increase of the prevalence of T2D in the industrialized and developing countries (Swapan, 2006; Diaz-Apodaca *et al.*, 2010) considerably increased the burden to find novel efficient and safe treatment regimens.

#### DISCUSSION

##### Insulin-like molecules

Insulin is produced in large amounts in  $\beta$ -cells of the pancreas in mammals, but is also expressed in the most primitive vertebrates and in complex invertebrates, which are evolutionary about 500 million years old (Conlon *et al.*, 1988). Albeit traditionally linked to a specialized endocrine organ such as the pancreas, insulin-like molecules have been assigned a more general role throughout the phyla. Two major elements

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of glucose homeostasis are glycolysis and glycogen synthesis, two metabolic pathways that transcend more than a billion years of evolution. They agonize and antagonize one another to regulate and maintain the growth and viability of virtually all organisms. Therefore, boundaries of conventional wisdom that limit production and localization of hormones to endocrine glands and highly specialized target tissues may be too restrictive. Evidence for hormone synthesis without glandular localization comes from cells adapted to culture, diverse malignant cells derived from non-endocrine tissues, neurons from various organisms, and cells and tissues that emerged during very early stages of embryonic development.

Insulin is phylogenetically ancient, being found not only in mammals, but also in birds, reptiles, both teleost and elasmobranch fish, and the very primitive hagfish. In contrast, the assumption that lower species, including protozoa, express insulin-like molecules has remained controversial so far. Meanwhile insulin and insulin-like molecules from many different species have been sequenced. They exhibit many variations in their amino acid sequence but structurally important residues of the core region, notably the disulfide bridges and glycine residues at the bends, are generally conserved. This suggests that all insulins will obtain a tertiary structure similar to that of the porcine molecule. The insulins are members of a large superfamily of molecules which all have some degrees of homology in their sequence and probably in their tertiary structure. The insulin-like growth factors 1 and 2 (IGF-1/2), which are responsible for most of the mitogenic activity of serum, are single-chain proteins which contain sequences analogous to the A chain (extended at its carboxyterminus by a D-peptide chain), C peptide and B-chain moieties of proinsulin. They share close homology with insulin in the structurally important core regions, but are quite different throughout much of their surface (Blundell and Humbel, 1980). NMR spectroscopy has confirmed the similarity of the basic fold of IGF-1 to that of native insulin (Cooke *et al.*, 1991; Sato *et al.*, 1993). Their low, but unequivocal insulin-like activity correlates with possession of certain residues thought to be important for the activity of insulin itself. The presence of additional parts of the molecule (the C-peptide-like region and the D region) is believed to modify the receptor-binding region and/or the molecules's potential for conformational changes in course of interaction with the receptor (Belfiore *et al.*, 2017; Hakuno and Takahashi *et al.*, 2018).

### **Multicellular organisms**

Previously, an insulin-like polypeptide gene (ILP) was identified in the primitive cephalochordate *Amphioxus* (Chan *et al.*, 1990). The gene organization and sequence display similarities with both the insulin and the IGFs. *Amphioxus* occupies a key position in chordate development as a possible extant relative of the invertebrate progenitor from which the vertebrates emerged, and the ILP may represent a transitional form connecting insulin and IGF. On the other hand, ovarian relaxin, which has no insulin-like activity at all, has a sequence less closely related to that of insulin, although molecular modeling and more recently a detailed X-ray analysis have confirmed that relaxin does have an insulin-like fold (Bedarkar *et al.*, 1977; Isaacs *et al.*, 1978; Eigenbrot *et al.*, 1991). Interestingly, the major deviation of relaxin from the structure of insulin involves the last five residues at the truncated

carboxy-terminus of the B-chain, which form an extension to the B-chain helix. Two even more distant members of the insulin family have been described. These were isolated from the neurons of the mollusc *Geodia cydonium*, the molluscan insulin-like peptide (MIP) (Smit *et al.*, 1988) with its gene (Schutze *et al.*, 1999) and from the silkworm *Bombyx morii* the prothoracicotropic hormone bombyxin (Nagasawa *et al.*, 1986; Adachi *et al.*, 1989). Both seem to have roles in growth and development. They both have the essential distribution of disulfide bonds and non-polar residues capable of packing a hydrophobic core of similar volume to that of insulin (Jhoti *et al.*, 1987). Thus, based on primary sequence, structural and physiological data of insulin-like molecules which have been identified in nerves, gut epithelium and other cells of invertebrates lacking typical pancreatic islets, such as of insects, mollusks, worms and sponges, the basic architecture of insulin seems to be of an ancient design, from which evolution has generated a family of molecules with a wide range of functions in multicellular lower eukaryotes.

### **Unicellular organisms**

The existence of material similar to mammalian insulin with regard to specific reactivity in the insulin radioimmunoassay and in the insulin bioassay has also been reported for a number of unicellular eukaryotes. Thus, accumulating evidence hints to a common origin of the insulin molecule about 1 billion years ago (LeRoith *et al.*, 1980). In detail, insulin-like material as detected in certain lower unicellular eukaryotes, such as ciliated protozoa (*Tetrahymena pyriformis*) as well as fungi (*Aspergillus fumigati*, *Neurospora crassa*) that were grown in simple synthetic media (LeRoith *et al.*, 1980) and even in prokaryotes (*E. coli*, halobacteria) (LeRoith *et al.*, 1981). Net production of insulin was demonstrated by an increase in those materials in the cells and the medium during the early logarithmic growth phase of the cells (LeRoith *et al.*, 1985). The characterization of these materials included specific radioimmunoassays, radiolabeled receptor-binding assays, chromatographic analyses and bioassays following purification of the extracts to near homogeneity using size exclusion, ion exchange and HPLC (de Pablo *et al.*, 1986). These findings point to a more fundamental and ancient biological role for insulin as well as to an evolutionary predecessor of insulin in unicellular eukaryotes (Lenard, 1992).

In the few cases where the primary structure of insulin-like materials from unicellular organisms has been elucidated, it became apparent of being highly divergent and displaying striking differences both concerning the length and the sequence of the polypeptide chain from that of the highly conserved (prepro)insulin from vertebrates (Sures *et al.*, 1980). Almost concomitant with the discovery of the bioeffects of vertebrate insulin on microbial metabolic processes, substances were discovered in a few species of unicellular eukaryotes that resembles vertebrate insulin.

During the beginning of the 1980's Roth and coworkers described and partially characterized substances resembling vertebrate insulins in *Tetrahymena pyriformis*, and in the filamentous fungi, *Aspergillus fumigatus* and *Neurospora crassa*. Materials extracted from cells and conditioned medium could be recovered as distinct peaks in the region characteristic of insulin (approximately 6 kDa) by Sephadex G-50 gel filtration. Standard radioimmunoassay (RIA) for porcine

insulin and a bioassay for lipid synthesis in rat adipocytes were used to detect activity eluting in the column fractions. The gel-filtered material from *Tetrahymena* had reactivity in RIA approximately equal to its activity in the bioassay, whereas the *Neurospora* material displayed an immunoreactivity: bioactivity of 1:3. The activity that stimulated lipogenesis could be neutralized, although not completely, by anti-insulin antisera. For extracts derived from *Tetrahymena* or *Neurospora*, either 75-95% of about 60%, respectively, of the active component was neutralized by anti-insulin antibodies. The substance is presumably more similar to insulin than any other known polypeptide and is nearly as well characterized as the circulating form of insulin in mammals. A pseudogene possessing an insulin-like protein sequence was cloned from *Neurospora crassa* (Muthukumar and Lenard, 1991). In contrast, the yeast genome sequence data bases do not contain open reading frames that are reminiscent of insulin peptides as yet. However, so far open reading frames coding for more than 100 amino acids have been considered as potential genes, only, unless they had been identified previously as genes by genetic means. Mammalian proinsulin, in general, consists of about 100 amino acids (Sures *et al.*, 1980). Thus, a gene potentially coding for an insulin-related peptide in unicellular eukaryotes has to await future identification. Unfortunately, no biological effects of the partially purified materials on lower eukaryotes have been published so far.

In contrast to these findings on the synthesis of insulin-like peptides by diverse unicellular organisms, the existence of insulin-like materials of proteinaceous nature in yeast with resemblance in structure and immunological crossreactivity to mammalian insulin has not been reported so far. However, in subcellular fractions of the yeast *Saccharomyces cerevisiae* some proteinaceous materials were identified that cross-reacted with anti-insulin antibodies raised against total porcine insulin in rabbits (E. Groß and W. Bandlow; personal communication).

#### **Insulin-mimetic Molecules**

Insulin may affect very primitive functions *via* ancient intracellular signaling systems. Literature citations spanning 40-45 years propose structural and functional homologs of vertebrate hormones in invertebrate and unicellular organisms. Indirect evidence is based on the existence of gene sequences for proteins and peptides, including their related controlling elements, and metabolic responses provoked by them. The apparent demonstration of those insulin-like materials in unicellular eukaryotes which somehow resembles mammalian insulin according to structural criteria and apparently manage to induce some insulin-like effects on the cells of their origin or typical insulin target cells, albeit to a limited degree, prompted a number of studies on the expression of molecules with insulin-mimetic action in those cells, i.e. materials which do not exhibit any resemblance to mammalian insulin with regard nature and structure, but have a physiological insulin-like role in the regulation of glucose and lipid metabolism in common, that in the literature has often been termed insulin-like activity. Due to the lack of adequate amounts of purified substance in most cases, insulin-like activity could be demonstrated so far only for the insulin-mimetic material prepared from *Neurospora crassa* which exerted moderate metabolic effects in mammalian adipocytes (Greenfield *et al.*, 1988; McKenzie *et al.*, 1988) and yeast.

#### **Phosphoinositolglycans from Yeast**

Phosphoinositolglycan-peptides (PIG-P) prepared *in vitro* from the glycosylphosphatidylinositol-anchored cell surface protein (GPI-AP) Gce1p of *Saccharomyces cerevisiae*, which binds and degrades cAMP in the immediate surface area (Müller *et al.*, 1992, 1997; Frick *et al.*, 1998a, b), and variant surface glycoprotein (VSG) of *Trypanosoma brucei*, which is part of the dense glycocalyx, as well as peptide-free PIG molecules derived thereof mimic the phosphorylation control elicited by insulin on a number of phosphoproteins upon incubation with isolated rat adipocytes (Alemany *et al.*, 1987; Stralfors and Alemany, 1990; Misek and Saltiel, 1994; Kessler *et al.*, 1998; Müller *et al.*, 1998) and exhibit potent insulin-mimetic effects in isolated rat adipocytes, cardiomyocytes and diaphragms (Misek and Saltiel, 1992; Müller *et al.*, 1997). Similar molecules prepared from isolated and cultured fat and muscle cells activate non-oxidative and oxidative glucose metabolism presumably by inducing dephosphorylation of GS, GP and pyruvate dehydrogenase as well as inhibition of PKA (Villar-Palasi and Zhang, 1990; Deeg *et al.*, 1992; Misek and Saltiel, 1992; Varela-Nieto *et al.*, 1996; Jones and Varela-Nieto, 1998). In contrast to former speculations that these compounds directly alter the activity of protein kinases and phosphatases relevant for the regulation of glucose and lipid metabolism in an allosteric fashion (Larner, 1987; Villar-Palasi and Zhang, 1990), strong experimental evidence was previously obtained for an efficient cross-talk of PIG molecules to the insulin signaling cascade downstream of the insulin receptor at the level of tyrosine phosphorylation of the IRS proteins (Frick *et al.*, 1998; Müller *et al.*, 1998d; Müller and Frick, 1999).

The generation of PIG(-P) structures by endogenous mechanisms in yeast has not been documented so far. However, yeast contains the processing machinery for lipolytic cleavage of GPI lipids and GPI-AP as well as for double lipolytic/proteolytic cleavage of GPI-AP which is prerequisite for the release of PIG (-P) molecules (Müller and Bandlow, 1993; Bandlow *et al.*, 1996; Müller *et al.*, 1996). The observation that in *Saccharomyces cerevisiae* human insulin accelerates and stimulates the GPI-specific phospholipase C (GPI-PLC) and increases the efficiency of double processing of GPI-AP at concentrations and under growth conditions of the cells and spheroblasts, which favor glycogen synthesis (Müller *et al.*, 1998c), is compatible with the assumption that the GPI-PLC is one of the key players regulating GS activity in yeast in concert with PP2A, PKA and cAMP-PDE (Müller *et al.*, 2000). The identical ranking in the efficiency of various insulin analogs in activating GS, PP2A, PKA, cAMP-PDE and GPI-PLC supports this view. It is tempting to speculate that the GPI-PLC may be epistatic to PP2A, PKA and cAMP-PDE in controlling their activity *via* a molecular mechanism involving soluble PIG (-P) molecules which induce phosphorylation of common key signaling components as has been demonstrated previously with the PIG-induced tyrosine phosphorylation of IRS proteins *via* the non-receptor tyrosine kinases pp60<sup>Lyn</sup> and pp125<sup>FAK</sup> in rat adipocytes (Müller *et al.*, 1999b, c).

#### **Glucose Tolerance Factor from Yeast**

60 years ago Schwarz and Mertz reported the extraction and partial purification of the so-called glucose tolerance factor (GTF) from Brewer's yeast powder (Schwarz and Mertz, 1957).

Their initial findings were subsequently confirmed and extended substantially with the demonstration that the partially purified extract manages the lowering of plasma glucose and lipids in animal models for diabetes and stimulation of glucose as well as lipid metabolism in insulin target cells *in vivo* and *in vitro*.

### Structure

Unfortunately, despite the potential relevance of the GTF for diabetes therapy, its nature, structure and (molecular) mode of action have not been elucidated so far. This seems to be mainly due to the inherent instability of the purified fractions. In fact, the efforts for the isolation of pure GTF from brewer's yeast and for its structural elucidation repeatedly failed (Mertz, 1975; Toepfer *et al.*, 1976; Tuman RW and Doisy, 1977; Tuman *et al.*, 1978; Mirsky *et al.*, 1980; Haylock *et al.*, 1983a; Davies *et al.*, 1985). On the basis of the partially purified, yet active and stable GTF preparations from brewer's yeast, GTF exhibits low molecular weight and resists proteolytic degradation (Mirsky *et al.*, 1980, 1981; Mirsky, 1993). Thus, the available partial characterization makes structural similarity with insulin already very unlikely (Votava *et al.*, 1973). Moreover, on basis of the proteolytic resistance, the GTF can be administered by the oral route.

Several decades ago GTF was defined by Mertz and coworkers as a Cr<sup>3+</sup>-containing complex consisting of nicotinic acid, the amino acids glycine, cysteine and glutamate and vitamin B3 (Toepfer *et al.*, 1976). Interestingly, chromium as an essential trace element has been hypothesized to be required for physiological insulin action (Samad *et al.*, 2009; Malviya *et al.*, 2010). The biologically active version of chromium is considered to be the so-called chromodulin, a complex with trivalent chromium. Chromodulin may support and regulate the cellular mechanisms which determine insulin sensitivity and responsiveness. Consequently, it was suggested that GTF facilitates the absorption of chromium with regard to kinetics and capacity (see below).

### Signaling and Activity

The glucose-lowering activity of the GTF, as manifested in lowering of non-fasting plasma glucose levels in normal and genetically diabetic db/db mice (Tuman *et al.*, 1977, 1978) and in other hyperglycemic diabetes animal models and diabetic patients (Mertz and Schwarz, 1959; Mertz, 1975; Tuman and Doisy, 1977; Tuman *et al.*, 1978; Mirsky, 1993; Grant and McMullen, 1982a, b), was attributed in the past to (i) an improvement of pancreatic islet tissue function and viability leading to a more robust insulin secretory response, in particular in the diabetic state (Liu *et al.*, 2010), (ii) a direct insulin-mimetic activity, which is independent of the simultaneous presence of insulin (Hwang *et al.*, 1987), and (iii) a potentiation of the endogenous insulin action, i.e. sensitization of the peripheral target tissues towards insulin (Evans *et al.*, 1973; Tuman *et al.*, 1978; Grant and McMullen, 1982a, b), compatible with the findings that the GTF-induced stimulation of glucose metabolism in primary and cultured cells was critically dependent on the simultaneous presence of insulin (Toepfer *et al.*, 1976; Tuman *et al.*, 1978; Grant and McMullen, 1982a; Davies *et al.*, 1985; Holdsworth and Neville, 1990) and even much higher in the combination with insulin compared to the sum of the effects elicited by GTF and

insulin separately (Weksler-Zangen *et al.*, 2012). This apparent correlation or synergism between GTF activity and insulin has been explained previously by a direct interaction between GTF and insulin (Evans *et al.*, 1973) or by GTF acting as cofactor for insulin for the enhancement of its binding to the receptor (Schwarz and Mertz, 1957; Haylock *et al.*, 1983a, b; Davies *et al.*, 1985; Holdsworth and Neville, 1990). At variance, other investigations have demonstrated GTF action on glucose transport in isolated rat adipocytes, 3T3 adipocytes, primary cardiomyocytes and L6 myoblasts in the absence of insulin (Tokuda *et al.*, 1987; Fischer *et al.*, 1992; Weksler-Zangen *et al.*, 2012). This argues for operation of an insulin-independent insulin-mimetic activity of GTF.

In one of the most recent study the effects of oral bolus administration of GTF and intraperitoneal injection of insulin and of their combination were compared using streptozotocin-diabetic (STZ) (Weksler-Zangen *et al.*, 2001) and hyperglycemic Cohen diabetic-sensitive (hyp-CD) rats (Weksler-Zangen *et al.*, 2008, 2012). Interestingly, GTF and insulin (low dose) caused reductions by 17/33% and 12.5/34%, respectively, in the postprandial blood glucose levels of hyp-CD/STZ rats. Moreover, the combination yielded 42/58% blood glucose lowering, compatible with a potentiating synergistic mode of interaction between insulin and GTF in STZ rats and a subadditive one in hyp-CD rats. Importantly, insulin release was not affected by GTF in hyp-CD rats. However, other research groups failed to demonstrate amelioration of spontaneously occurring diabetes in db/db mice in course of administration of GTF-containing brewer' yeast (Flatt *et al.*, 1989).

Importantly, GTF induced the concentration-dependent upregulation of deoxy-glucose transport into cultured L6 myoblasts and 3T3-L1 adipocytes to an extent comparable with insulin. Furthermore, GTF provoked the concentration- and time-dependent stimulation of IRS-1 tyrosine phosphorylation as well as serine/threonine phosphorylation of PKB and MAPK in differentiated 3T3-L1 adipocytes, which did not depend on insulin receptor autophosphorylation. So far the available data demonstrate the induction of tyrosine phosphorylation of IRS-1 and serine/threonine phosphorylation of PKB, i.e. the activation of several critical components in the insulin signaling pathway, in adipose and muscle cells *in vitro* by GTF in insulin-like fashion, and even in the absence of insulin. A number of investigations failed to show tyrosine phosphorylation of the insulin receptor  $\beta$ -subunit, as the initial step of insulin signaling, in response to GTF at a broad range of concentrations and time points studied. Only a single study succeeded in the demonstration of a 8-fold elevated tyrosine kinase activity of the insulin receptor in course of challenge with GTF. It was concluded that GTF, which harbors a chromodulin molecule, together with insulin and the insulin receptor of insulin target cells forms a triple complex which makes the receptor more susceptible for activation by the bound insulin (Hosseinzadeh *et al.*, 2013). Furthermore, GTF is known to exert elevated phosphorylation of p42/44 MAPK in 3T3-L1 adipocytes, as is true for insulin, and therefore is believed to be involved in processes of gene expression and mitogenesis. This assumption is in concert with previous findings that yeast extract or partially purified GTF present in the culture medium foster the growth of yeast cells (Berdicevsky and Mirsky, 1994). Taken together, GTF seems

to trigger its insulin-mimetic and potentiating effects by either the direct interference with the insulin signaling pathway or the engagement of a different cellular signaling pathway that cross-talks to the insulin signaling pathway at sites downstream of the insulin receptor (which remain to be identified) and ultimately leads to regulation of glucose transport, glucose and lipid metabolism and gene expression in an insulin-like fashion. However, so far, it can not be excluded that GTF exerts (part of) its insulin-mimetic and potentiating effects through blockade of the dephosphorylation of key signaling components downstream of the insulin receptor, as has been demonstrated for vanadium-complexes and their capability to support insulin-like phosphorylation (Smith *et al.*, 2008; Thompson *et al.*, 2009; Clark *et al.*, 2014; Domingo and Gomez, 2016). Of huge relevance for a putative application in diabetes therapy is the preliminary finding that GTF fails to elicit (auto)phosphorylation of the IGF-1 receptor in immortalised human prostate epithelial p69 cells, which express functional IGF-1 receptor (Mirsky N, Mizrahi T and Shitrit N, personal communication). Taken together, these results strongly argue for potent insulin-mimetic and insulin-potentiating activities of GTF both *in vivo* and *in vitro*, which are mediated by cellular signaling components and cascades located downstream of the insulin receptor and causally involved in the insulin-like stimulatory effect on glucose clearance by peripheral tissues rather than on stimulation of insulin release.

### Role of chromium

Strikingly, the symptoms caused by severe chromium deficiency closely resemble T2D (Anderson, 2000). Conversely, the administration of chromium yeast as nutritional supplement was reported to lower fasting blood glucose and lipids as well as plasma insulin levels in T2D patients (Anderson, 1997; Ravanshad *et al.*, 2005), normal probands (Vinson and Bose, 1984) and streptozotocin-induced diabetic rats (Lai *et al.*, 2006), albeit with quite considerable differences in efficacy between the studies. Consequently, various pharmaceutical companies promote the potential advantages of chromium supplementation for the prevention and therapy of T2D, albeit its clinical results and benefits for the patients are far from being unequivocal and clear-cut (Moyad, 2008). The inconsistencies may be due to the wide variation of the chromium status of the patients, the doses required and administered, as well as the type of supplementation (Balk *et al.*, 2007). Organic chromium yeast, commercially available as "Bioactive Chromium DIA" (ChromoPrecise®Pharma Nord, Vejle, Denmark), has been commonly accepted as a safe and efficient form for chromium supplementation. Furthermore, in course of comparative studies between brewer's yeast and CrCl<sub>3</sub> it was unambiguously shown that in diabetic patients brewer's yeast is more potent in blood glucose-lowering than CrCl<sub>3</sub> but ineffective in healthy animals and probands (Offenbacher and Pi-Sunyer, 1980; Rabinowitz *et al.*, 1983a, b; Muzik *et al.*, 2011). Those results prompted the assumption that unfractionated brewer's yeast, which is prepared as dried and inactive cells lacking any fermenting activity (Holdsworth and Neville, 1990), provides the best source for the GTF (Grant and McMullen, 1982a, b) and may be useful for the cure of T2D and the prevention of diabetic late complications (Vincent, 2004; Malviya *et al.*, 2010).

In a previous study with T2D patients (100 µg chromium yeast for the first two weeks followed by 200 µg for the next six weeks followed by wash-out for six weeks) it was found that chromium supplementation during the first and second period leads to a significant and additional non-significant reduction, respectively, of the fasting plasma glucose (Racek *et al.*, 2013). In agreement, the levels of HbA<sub>1c</sub> were reduced significantly during both periods. These beneficial effects were reversible since after withdrawal of chromium supplementation, the fasting plasma glucose and HbA<sub>1c</sub> values were found to be identical with their pre-intervention values. Conversely, chromium supplementation did not alter significantly the levels of serum lipids. The authors concluded that T2D patients could benefit from chromium supplementation on the basis of its putative capability to improve their insulin sensitivity and glucose tolerance, i.e. lower their insulin resistance, rather than to provoke or facilitate their insulin secretory potency. However, the authors stated that despite these rather promising data, the study has to be considered as preliminary on basis of the low sample size and limited degree of control (Racek *et al.*, 2013).

The outcome of a recent study revealed that consumption of six tablets per day of brewer's yeast for 3 months reduces the levels of fasting blood glucose and HbA<sub>1c</sub> as well as increases insulin sensitivity (Hosseinzadeh *et al.*, 2013), compatible with some previously reported findings (Bahjiri *et al.*, 2000; Ravanshad *et al.*, 2005; Racek *et al.*, 2006; Muzik *et al.*, 2011), but in disagreement with others (Grant and McMullen, 1982a, b; Haylock *et al.*, 1983a). These discrepancies may be due differences in the sample number and treatment period. Furthermore, it is conceivable to assume that the efficacy of the absorption of brewer's yeast critically depends on the chromium status and is higher in individuals with poor chromium status. As a consequence, in case of chromium deficiency in diabetic patients their chromium pool would be saturated faster with excretion of the potential surplus through the urine (Haylock *et al.*, 1983b). This view is in agreement with the hypothesis, that T2D patients in general suffer from chromium deficiency to a certain degree (Moyad, 2008; Azimi-Nezhad *et al.*, 2008; Esteghamati *et al.*, 2009) and benefit from chromium supplementation provoked by an effective source, such as brewer's yeast, leading to improved glucose tolerance (Ceriello and Colagiuri, 2008). This would necessitate careful patient selection for relevant clinical studies, since the positive clinical response, as manifested in lowered blood glucose and increased insulin sensitivity, should be of higher likelihood in insulin-resistant T2D patients (Simonoff *et al.*, 1992; Wang and Cefalu, 2010). Interestingly, the administration of neither brewer's yeast nor torula yeast to healthy probands for 12 weeks led to significant upregulation of insulin sensitivity (Wang, 1989), in contrast to a more recent study (Hosseinzadeh *et al.*, 2013). This discrepancy may be explained as follows: (i) Different methods for the determination of insulin sensitivity, in particular for the measurement of fasting blood glucose and the 2-h plasma insulin concentration were used, based on the calculation of the insulin to glucose ratio and assessment of the homeostasis model, correspondingly (Berdicevsky and Mirsky, 1994; Kleefstra *et al.*, 2007). (ii) In the former study healthy probands had been recruited, exclusively, which could have negative impact on the results. (iii) The phenotype of the probands is crucial for the investigation of the clinical response

towards brewer's yeast, since about 40% of its variance could be attributed to baseline insulin sensitivity (Wang *et al.*, 2007). Another portion of this variance may be caused by the inability of some persons to convert non-biological into biologically active forms of chromium (Wang, 1989). It remains to be clarified whether the upregulated utilization of chromium present in brewer's yeast is caused by a naturally operating physiological feedback mechanism of the body and responsible for the positive effect of brewer's yeast in patients suffering from chromium deficiency. It seems to be curious that brewer's yeast exerts effects comparable to other forms of chromium albeit it harbors considerably lower concentrations of chromium. However, this can be regarded as compatible with the view that chromium bound to an organic complex in yeast is of advantage with regard to glucose uptake and metabolism, body mass, blood carbohydrates and lipids as well as lipids (Cefalu *et al.*, 2010; Krol *et al.*, 2011,) and that a distinct yeast component mediates or potentiates the insulin-sensitizing effect of chromium. Two studies confirm the improvement of insulin resistance upon coadministration of brewer's yeast and chromium, the one in course of treatment of two groups of aged healthy probands with 5-g pills of brewer's yeast and 200- $\mu$ g capsules of chromium chloride (Offenbacher and Pi-Sunyer, 1980), the other one performed with 61 years old T2D patients who received four tablets of brewer's yeast and 100  $\mu$ g chromium, in addition, per day (Racek *et al.*, 2006). The finding of a considerable decline of plasma insulin in Chinese adult probands of mean age of 51 years with various degree of insulin resistance ranging from the pre-diabetic to the frank diabetic state who were treated with 10-g capsules of brewer's yeast daily during a clinical trial (Li, 1994) can also be explained by a gain in insulin sensitivity. This is also true for a study on the effects of inorganic chromium and brewer's yeast supplementation on glucose tolerance and serum lipids in T2D patients (Bahijiri and Mufti, 2002), which unraveled a direct correlation between the amount of extract and chromium ingested and the improvement of the metabolic parameters (Bahijiri *et al.*, 2000).

A systematic review of randomized clinical trials dealing with the benefits of dietary chromium supplementation on the metabolic state in normal, pre-diabetic and type 2 diabetic probands revealed the obvious differences between their outcomes may be due to inadequate standardization and control with regard to (i) the formulation and dose of the brewer's yeast used, (ii) the at-risk population selected, (iii) the level of exercise performed by the subjects, (iv) the degree and mode of the control of the T2D (by diet and/or anti-diabetic drugs) and (v) the BMI of the subjects, which indicates their nutritional and, in consequence, their chromium status at baseline (Althuis *et al.*, 2002). These drawbacks hamper the interpretation of the currently available clinical studies with regard to improvement of insulin sensitivity and/or insulin resistance in response to brewer's yeast and/or chromium as well as the putative impact of the chromium status. In a meta-analysis of randomized controlled trials for studying the effect of chromium supplementation *via* brewer's yeast on glucose and lipid metabolism it became apparent despite the statistical heterogeneity that administration of elevated amounts of brewer's yeast, such as corresponding to 10  $\mu$ g chromium per day, provoked more pronounced reductions in fasting plasma glucose compared to lower amounts (Anderson *et al.*, 1997;

Kleefstra *et al.*, 2007) as realized for instance with 6 and 8  $\mu$ g per day (which actually did not lead to significant differences) (Rabinowitz *et al.*, 1983). It remains to be elucidated whether the apparent dose-dependent effects on glucose and lipid metabolism provoked by brewer's yeast (Mertz, 1976), at least within a certain range and to a moderate degree, are related to variation of the resulting concentration of chromium or of the expression of a relevant yeast component or both.

Interestingly, the adequate intake of chromium was set by the National Academy of Science (USA) as 30  $\mu$ g per day for adults (Racek *et al.*, 2006). In studies, which present calculations of the daily chromium intake of their subjects based on their diet records, inadequate chromium supplementation as low as about 1 to 2  $\mu$ g per day were deduced. They also revealed the pronounced dependence of the daily chromium intake on the socioeconomic origin, the grade of education and the degree of wealth and health with the simple conclusion that well-nourished healthy people have sufficient daily chromium intake. Those dietary divergences could be responsible for the apparent differences in the hypoglycemic and insulin-sensitizing effects of the yeast tablets between different studies and study populations.

However, several efforts for the synthesis of complexes between chromium and these three amino acids led to rather unstable structures which displayed a considerably lower activity in comparison to natural GTF (Toepfer *et al.*, 1976; Mertz, 1993). Most important, GTF prepared from chromium-rich and chromium-deficient yeast displayed very similar insulin-mimetic and potentiating activity (Haylock *et al.*, 1983b; Davies *et al.*, 1985; Simonoff *et al.*, 1992), making a direct relationship between the activity of GTF and its chromium content less likely. This interpretation is supported by additional efforts for the generation of pure and homogenous GTF from yeast extracts which did not reveal an unambiguous correlation between chromium content and GTF activity (Davies *et al.*, 1985; Haylock *et al.*, 1983a, b). Recent unpublished findings from the Mirsky-group using elaborate state-of-the-art equipment for the detection of trace elements did not provide experimental evidence for the presence of chromium in the analyzed fractions of yeast extract with GTF activity.

## CONCLUSION

The most comprehensive meta-analysis covering the clinical trials for the elucidation of the beneficial effects of brewer's yeast for the therapy of T2D from 1996 to 2008 and conducted in 2009 (Nahas and Moher, 2009) detected major limitations in the majority of those studies which encompass sample size (too small), duration of intervention (too short), design (non-randomized) and type as well as dose of the brewer's yeast (huge variation). These obstacles may contribute to the heterogenous study results and inconsistent conclusions drawn thereof. Thus, additional studies with a higher number of T2D patients of different, but defined stages of insulin resistance and disease compensation and performed using state-of-the-art design with optimal doses are urgently required to confirm the moderate beneficial effect of brewer's yeast supplementation on the glycemic and lipidemic indices in T2D patients. Furthermore, future studies should be designed to cover

putative additional actions of brewer's yeast on other diabetes parameters such as oxidative stress markers.

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