Carnosine decreases IGFBP1 production in db/db mice through suppression of HIF-1

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Abstract

IGF binding protein 1 (IGFBP1) is a member of the binding proteins for the IGF with an important role in glucose homeostasis. Circulating IGFBP1 is derived essentially from the liver where it is mainly regulated negatively by insulin. Carnosine, a natural antioxidant, has been shown to improve metabolic control in different animal models of diabetes but its mechanisms of action are still not completely unraveled. We therefore investigate the effect of carnosine treatment on the IGFBP1 regulation in db/db mice. Db/db mice and heterozygous non-diabetic mice received for 4 weeks regular water or water supplemented with carnosine. Igfbp1 mRNA expression in the liver was evaluated using qPCR and the protein levels in plasma by western blot. Plasma IGF1 and insulin were analyzed using immunoassays. HepG2 cells were used to study the in vitro effect of carnosine on IGFBP1. The modulation of hypoxia inducible factor-1 alpha (HIF-1α) which is the central mediator of hypoxia-induction of IGFBP1 was analyzed using: WB, reporter gene assay and qPCR. Carnosine decreased the circulating IGFBP1 levels and the liver expression Igfbp1, through a complex mechanism acting both directly by suppressing the HIF-1α-mediated IGFBP1 induction and indirectly through increasing circulating insulin level followed by a decrease in the blood glucose levels and increased the plasma levels of IGF1. Reduction of IGFBP1 in diabetes through insulin-dependent and insulin-independent pathways is a novel mechanism by which carnosine contributes to the improvement of the metabolic control in diabetes.

Key Words
- carnosine
- IGFBP1
- liver
- HIF-1α

Introduction

Insulin-like growth factor binding protein 1 (IGFBP1) is one of the six proteins that binds and regulates the bioavailability of IGF1. IGFBP1 binds IGF1 with high affinity, and regulates IGF effects in a tissue-specific manner either by enhancing or damping IGF activity. IGFBP1 is expressed in the liver, kidney and decidua (Rajaram et al. 1997). The liver is the major source for the circulating IGFBP1 and its synthesis is centrally regulated by insulin that represses IGFBP1 at the transcriptional level (Brismar et al. 1994, Powell et al. 1995). Other factors, including hypoxia, pro-inflammatory cytokines, cAMP, glucocorticoids and oxidative stress stimulate the synthesis of IGFBP1 (Mesotten et al. 2002). Hypoxia inducible factor (HIF) is a transcription factor that binds
to the hypoxia responsive elements (HRE) in the promoter region of more than 100 genes including IGFBP1 and mediates the adaptive response to hypoxia (Semenza 2011). HIF is a heterodimeric factor composed of two subunits α and β, in which the α subunit is regulated by oxygen. In the presence of oxygen, HIF is hydroxylated by a specific Fe$^{2+}$, oxoglutarate dependent prolyl 4-hydroxylases (PHD) allowing HIF-1α to bind to the von Hippel-Lindau (VHL) tumor suppressor protein that acts as an E3 ubiquitin ligase and targets HIF-1α for proteasome degradation (Kaelin & Ratcliffe 2008).

Increased liver IGFBP1 synthesis is observed in states of insulin resistance, such as diabetes type 2 (Munoz et al. 1996, Clauson et al. 1998). Compelling evidence suggests that increased IGFBP1 is involved in the pathophysiology of diabetes complications (Crossey et al. 2000, Heald et al. 2001, Schrijvers et al. 2004, Ezzat et al. 2008) either through inhibition of IGF1 actions or by a direct effect of IGFBP1. High levels of IGFBP1 contribute directly to impaired metabolic control (Zachrisson et al. 2000) as illustrated in transgenic mice where overexpression of Igfbp1 results in fasting hyperglycemia (Murphy 2000) and long-term IGFBP1 infusion leads to increased blood glucose levels in rats (Lewitt et al. 1991). On the other side, in animal model of diabetes high levels of IGFBP1 have been associated with renal hypertrophy (Doublier et al. 2000, Van Buul-Offers et al. 2000). Furthermore, a polymorphism in the IGFBP1 gene, which affects the activity of IGF1, was associated with a decreased risk of developing diabetic nephropathy (DN) (Stephens et al. 2005).

l-carnosine is a naturally occurring dipeptide, which is endogenously synthesized from β-alanine and l-histidine by an ATP-dependent carnosine synthase. Carnosine is present in high concentrations in the skeletal muscle, heart and nervous system and smaller quantities are synthesized in other tissues such as kidney, liver, stomach and lungs. Carnosine plays an important role in a number of biological functions, through its antioxidant, anti-inflammatory and anti-senesce properties (Lenz & Martell 1964, Gallant et al. 2000, Guiotto et al. 2005). Carnosine acts as a scavenger of reactive oxygen species including peroxyl radicals and superoxide (Boldyrev et al. 2013). The importance of carnosine in diabetes and its chronic complications was highlighted recently. A polymorphism in exon 2 of carnosinase (CN1), an enzyme that degrades carnosine, was associated with susceptibility for developing DN (Janssen et al. 2005). This polymorphism associated with resistance against DN was demonstrated in different populations (Freedman et al. 2007). Moreover, exogenous carnosine improved the glucose levels in different diabetic animal models (Yamano et al. 2001, Sauerhofer et al. 2007). Additionally, experimental treatment with carnosine in animal models has protective effects on the development of chronic complications in diabetes. Carnosine treatment decreases proteinuria and renal damage in diabetic mice (Peters et al. 2012), inhibits the production of fibronectin and TGF β in renal cells (Janssen et al. 2005) and improves wound healing in diabetes (Ansurudeen et al. 2012). It has been shown that anserine (methylated carnosine) also has a positive effect on blood glucose and plasma insulin concentration both in rodents and humans (Kubomura et al. 2010a,b).

Furthermore, Peters et al. (2012) have shown that in obese diabetic mice, renal CN1 activity is increased and histidine dipeptide concentrations are reduced. Carnosine supplementation mitigates DN, reduces renal vasculopathy, and normalizes vascular permeability in diabetic mice. In streptozotocin-induced, diabetic rats, carnosine treatment prevents apoptosis of glomerular cells and podocyte loss and vascular damage (Riedl et al. 2011).

Knowing IGFBP1 as a marker of hepatic insulin resistance and as a potential pathogenic factor for chronic complications of diabetes, we studied the influence of exogenous carnosine on IGFBP1 production and circulating levels in db/db mice.

### Materials and methods

#### Animals and experimental protocol

The db/db mouse was used as a model of type 2 diabetes and its lean heterozygote, littermate as the control (Charles River, Sulzfeld, Germany) (Stock 000662). Their phenotype consists of obesity, insulin resistance and diabetes, similar to type 2 diabetes in humans (Hummel et al. 1966). Only male mice were used for this study and housed in an animal facility that was maintained at 25 °C with a 12 h light:12 h darkness cycle. Animals had free access to water and standard rodent chow. The experimental protocol was approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals. Treatment was initiated at 6 weeks of age, before the db/db mice developed hyperglycemia. Mice were divided into four groups: i) diabetic mice with no treatment; ii) diabetic mice that received 5 g/l of l-carnosine (Sigma) in the drinking water for 4 weeks; iii) control mice with no treatment; and iv) control mice who received 5 g/l of l-carnosine in the drinking water for 4 weeks. Since l-carnosine was reported to be stable in the water bottles over a period of minimum 5 days at room
temperature, we chose to replace the water every 5 days (Sauerhofer et al. 2007). The water intake was estimated by weighing the water bottles every 5 days. Unless stated otherwise, each experimental group contained eight mice. At the end of the experiment, body weights and blood glucose levels were measured. Glucose levels were determined in blood collected from the tail tip using OneTouch Ultra Blood Glucose meter (LifeScan, Milpitas, CA, USA). The liver was harvested and snap frozen. At the end of the experiment, blood was collected in heparin tubes (BD Vacutainer, Plymouth, UK) and snap frozen.

**Plasma assays**

Plasma samples for the determination of total IGF1 concentration were acid ethanol extracted prior to the RIA, and to further eliminate major interactions with IGFBPs, truncated IGFB1 was used as ligand (Bang et al. 1991). The intra- and inter-assay coefficients of variations (CV) were 4% and 8% respectively. The sensitivity of the RIA was 3 μg/l and the intra- and inter-assay CV were 3% and 10% respectively.

Insulin was determined by using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA). Leptin was determined by using a Mouse Leptin ELISA Kit (Crystal Chem).

**Carnosine concentration**

Carnosine concentrations were assayed in plasma and in liver homogenates by fluorometric determination after derivatization with carbazole-9-carbonyl chloride. Separation was performed by liquid chromatography according to the method previously described (Peters et al. 2010).

**RNA extraction and real-time RT-PCR**

Liver tissues were harvested and quickly submerged in RNA/later solution (Ambion, Austin, TX, USA). Total RNA from liver or HepG2 cells was extracted by using RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1 μg total RNA employing Superscript III reverse transcriptase with UDG transasecylase (Invitrogen) according to the manufacturer’s protocol. The cDNAs were stored at −20 °C until use in quantitative real-time PCR. The assay to semi-quantify specific mRNAs was carried out using the SuperScript III Platinum Two-step Quantitative RT–PCR system according to manufacturer’s instructions (Invitrogen). Real-time PCR was carried out using gene specific primer pairs for Igfbp1 (forward, 5'-ATCAGCCCATCTGGAC-3' and reverse 5'-TGCA-GCTAATCTCTAGCATT-3'), Vegf a (forward, 5'-TTAT-GCGGATCAAACCAGC-3' and reverse 5'-CTTTCATT-GGTCTGCCATTAC-3'), Pdk1 (forward, 5'-AGTGGG-TAGTCCTTATGACG-3' and reverse 5'-TTCCCAAGAC- CATCTTTCCC-3'), Bip3 (forward, 5'-ATTGTCAGTGCCG- CCAGAA-3' and reverse 5'-AGTCGCTGTACGCTTTG-GGT-3'), and Pgkd (forward, 5'-CTCTGTGTCACTTCCGT- CC-3' and reverse 5'-GTGGGACGGGTCTATGCTTCC-3'). Real-time PCRs were carried out in the ABI Prism 7300 Sequence Detection System (Applied Biosystems). PCR conditions were as follows: initial incubation for 2 min at 50 °C and 2 min at 95 °C, and a two-step cycling PCR protocol for 40 cycles at 94 °C for 15 s, at 60 °C for 30 s. The melting curve analysis was done using the program supplied by Applied Biosystems. The quality of the quantitative PCR run was determined by standard curves and melting curve analysis. Relative quantification was carried out by using the 2−ΔCT method.

**Western blot analysis for IGFBP1 and HIF-1α**

Liver tissues were homogenized in RIPA buffer (150 mmol/l NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/l Tris-HCl, pH 8.8, supplemented with freshly made protease inhibitor cocktail) and centrifuged at 4 °C with 20 000 g for 20 min. Proteins were quantified (Bio-Rad) and after equally loading (50 μg) were electrophoresed in 7.5 or 12% SDS–PAGE gel, and transferred to nitrocellulose membrane subsequently blocked with 5% nonfat milk. The primary antibody against HIF-1α (1:500 – Novus Biologicals, Littleton, Colorado, USA, NB 100-449) or IGFBP1 (1:2000 – Abcam, Cambridge, UK, ab 4242) was added and incubated overnight at 4 °C with gentle shaking. Membranes were washed three times in PBS containing 0.1% Tween 20. The secondary anti-rabbit antibody conjugated to HRP was added at a concentration of 1:3000 and incubated for 1 h at room temperature with gentle shaking, after which membranes were washed three times in PBS containing 0.1% Tween 20. Bound antibody was detected by ECL western blotting detection system (GE Healthcare, Piscataway, NJ, USA).

Protein for HepG2 cells were extracted and separated by SDS–PAGE as described above, and transferred to PVDF membranes (GE Healthcare). After blocking, membranes were probed with related primary antibodies for HIF-1α (Novus Biologicals) or human IGFBP1 (Abcam) for 2 h at +22 °C. The membranes were then incubated with fluorescent conjugated secondary antibodyIRDye 800CW goat (polyclonal) anti-rabbit IgG (H+L) (Li-Cor, Lincoln,
NE, USA). Results were developed with the Li-Cor Odyssey system CLx (Li-Cor, Waltham, MA, USA). Band intensity of western immunoblots was measured with Image Studio Lite of the Li-Cor, Version 3.1.4 (Li-Cor). Protein concentration of HepG2 extract was measured with a BCA protein kit (Thermo Scientific, USA) to ensure equal loading.

Histological analysis

Frozen liver samples were placed in 4% paraformaldehyde and embedded in paraffin. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin.

Cell culture

A human hepatocellular carcinoma cell line, HepG2, obtained from the American Type Culture Collection known to produce IGFBP1 (Hilding et al. 2003), was used to investigate the effect of carnosine (Sigma–Aldrich) on IGFBP1 secretion. HepG2 cells were grown at 37 °C in 95% air–5% CO2 in 100-mm2 cell culture dishes and fed every 3–4 days with DMEM (Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Before the experiments the medium was replaced with serum-free DMEM containing 0.2% of albumin for 24 h. Hypoxia exposure was performed by incubating the cells at 1% oxygen using the Invivo 300 hypoxia chamber (Ruskinn, UK).

Carnosine dose used was 50 mmol/l carnosine, which is the lowest dose that decreased IGFBP1 levels in normoxia and this dose was further used for investigating the effect on hypoxia-induced IGFBP1 (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Luciferase experiments

HepG2 cells were transiently transfected with 300 ng HRE – luciferase reporter gene plasmid (pT81/HRE-luc) using Fugene reagent (Roche) according to the manufacturer’s instructions. Renilla luciferase vector which provide constitutive expression of Renilla luciferase was co-transfected with HRE – luciferase plasmid and used as internal control. The cells to be transfected were seeded in six well plates and transfected at 75–80% confluency, starved overnight and exposed then for 24 h to either normoxia (21% O2) or hypoxia (1% O2) in the presence of 50 mmol carnosine solution or PBS, used as control. The luminescence was measured in the cells extract using Dual-Luciferase Reporter Assay Kit (Promega). Relative light units were normalized to Renilla luciferase expression.

Statistical analyses

Data are expressed as mean±s.e.m. Comparison among groups was by ANOVA followed by Tukey’s multiple comparison post-test. P<0.05 was considered statistically significant.

Results

The data for water intake, baseline blood glucose and body weight of the animals are shown in Supplementary Table 1, see section on supplementary data given at the end of this article. As shown in Table 1, the treatment with carnosine for 4 weeks reduced the glucose levels in the db/db mice by 25%. Carnosine increases by 38% the plasma IGF1 levels in the db/db mice which have lower levels than control mice. Treatment with carnosine increased threefold plasma insulin concentration in the db/db mice that had, as expected, higher levels than control mice. Treatment with carnosine had no effect in the non-diabetic control animals on any of the variables previously mentioned.

Carnosine normalizes high levels of IGFBP1 in diabetes

The liver Igfbp1 mRNA levels were increased in the db/db mice compared with non-diabetic control animals (Fig. 1). Treatments of both control and db/db mice with carnosine
caused a decrease in Igfbp1 mRNA expression (by 50%). Carnosine increased the repressed levels of Igf1 mRNA expression (Fig. 2) in concordance with the previously mentioned effect on the circulating levels of IGF1 in diabetic animals.

The effects of carnosine on IGFBP1 were restricted to the diabetic mice since neither Igf1 expression or plasma protein levels were modulated by carnosine treatment in the non-diabetic mice.

The exogenous supplementation of carnosine increased by almost twofold its accumulation in the liver of the db/db mouse that exhibited lower levels than the non-diabetic mice (Fig. 3). The carnosine treatment had no effect on the liver accumulation of carnosine in control non-diabetic mice despite similar increase in plasma carnosine levels in both the diabetic and the non-diabetic animals. This was followed by a decrease in the liver steatosis just in diabetic animals but not in non-diabetic control mice (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Carnosine modulates IGFBP1 levels at multiple levels

The next step of our investigation was to study the mechanisms by which carnosine modulated IGFBP1. Having in mind the central role of insulin on IGFBP1 regulation, the noted increased in the circulating insulin in the db/db mice after treatment with carnosine provides an important explanatory mechanism for the repression of IGFBP1 after treatment with carnosine.

However the impressive specific accumulation of carnosine in the liver prompted us to continue our investigation by studying additional mechanisms that could be activated in the liver independent of insulin levels. Having in mind the role of hypoxia in diabetes and for IGFBP1 regulation we studied the effect of carnosine on hypoxia-induced IGFBP1.

For this end, we have used HepG2 which are cells extensively used for studies concerning both HIF and IGFBP1 regulation. Exposure of HepG2 cells to hypoxia was followed by an increase in Igfbp1 both at mRNA levels (Fig. 4a) and protein levels (Fig. 4b and c). Carnosine diminished this effect by normalizing the hypoxia-induced levels of both Igfbp1 mRNA (Fig. 4a) and protein (Fig. 4b and c).

Carnosine destabilizes HIF-1α and decreases its activity

Keeping in mind that the main adaptor of the cells to hypoxia is HIF and that IGFBP1 is induced by HIF-1α (Tazuke et al. 1998), we next investigated the effects of carnosine on HIF stability and function. Carnosine destabilized HIF-1α in hypoxia in the HepG2 cells as shown in Fig. 5a. The functional inhibition of carnosine treatment on HIF-1α activity was further proved using a transient transfection with a HRE-reporter plasmid. Carnosine decreased the hypoxia induced HRE-activity (Fig. 5b) in concordance with the effects observed on target genes (Fig. 6a, b and c).
Discussion

In this study, we report that carnosine, significantly decreased the high levels of IGFBP1 seen in diabetes. We therefore provide a new mechanism by which carnosine improves the metabolic control in diabetes and protect against the development of complications in diabetes.

Even though carnosine shown protective effect in several animal models for complications of diabetes (Pfister et al. 2011, Riedl et al. 2011, Ansurudeen et al. 2012, Yapislar & Aydogan 2012, Brown et al. 2014, Peters et al. 2014, Menini et al. 2015) and strongly suggested to be relevant for diabetes complications in humans (Ahluwalia et al. 2011, Kurashige et al. 2013) the exact mechanism of action is still unraveled. Here we suggest a potential mechanism by showing that carnosine complexly modulates IGFBP1 by different mechanisms, i.e. indirectly by increasing the insulin levels and directly by interfering with the HIF dependent IGFBP1 induction.

Increased IGFBP1 has been associated with risk of development of cardiovascular diseases (Wallander et al. 2007), atherosclerosis (Wang et al. 2012) and kidney disease (Lindgren et al. 1996). It is therefore tentative to propose that at least part of the protective effect of carnosine in diabetes is mediated through its inhibiting effect on IGFBP1.

Carnosine has a combined effect by both decreasing IGFBP1 and increasing circulating IGF1 levels with potential beneficial effect on glucose metabolism since it reverses the characteristic pathologic changes of both IGFBP1 and IGF1 in diabetes (Clauson et al. 1998). The mechanism by which carnosine increases the liver expression of IGF1 is not clearly known. Insulin has a known direct effect on IGF1 production (Brismar et al. 1994) but a direct effect of the dipeptide or of the IGFBP1 on IGF1 expression cannot be excluded.

Moreover, carnosine increases threefold plasma insulin levels in db/db animals that contribute directly to the improvement of glucose levels. The increase of insulin levels secondary to carnosine treatment is in agreement with previous observations in other models of diabetes (Nagai et al. 2003). It definitely contributes to the repression of the carnosine in IGFBP1 in vivo since insulin is a major regulator of the production of IGFBP1 from the liver.

**Figure 3**
Carnosine concentration in the liver was significantly reduced in diabetic mice (D) compared to controls (C). Carnosine treatment did improve the decreased levels of carnosine in the liver of diabetic animals but had no effect on liver of control, non-diabetic animals. ***P<0.001 vs C and 1P<0.05 vs D.

**Figure 4**
Effect of hypoxia on Igfbp1 expression in HepG2 cells. HepG2 cells were starved overnight then exposed to hypoxia for 24 h. Carnosine was added just prior to placing the cells in hypoxia. (a) Total RNA was extracted, and the Igfbp1 expression determined by qPCR. Hypoxia increase by eightfold the mRNA expression of Igfbp1 and carnosine significantly blunted that response. (b) The IGFBP1 secreted in the medium and (c) protein expression in HepG2 cells were increased under hypoxia and carnosine treatment significantly decreased IGFBP1. The values represent the mean ± S.E.M. of three independent experiments. *P<0.05 vs. Hx, ***P<0.001 vs. Nx, 1***P<0.001 vs. Hx and 3***P<0.001 vs. Nx.
Furthermore, hypoxia was recently identified as an additional pathogenic factor in diabetic complications beside hyperglycemia (Catrina 2014). IGFBP1 is highly stimulated by hypoxia (Tazuke et al. 1998) and is a direct target of HIF, which is the main adaptor of the cells to hypoxia. We demonstrate in this study that carnosine reduces IGFBP1 in diabetes by a complex mechanism involving also a direct decrease of HIF stability and function.

This is in agreement with a recent study that found a destabilizing effect of carnosine on HIF-1α in a cancer cell line (HCT-116) (Iovine et al. 2014). Furthermore, in a mouse model of retina ischemia it has been shown that carnosine treatment decrease HIF-1α at the protein level after 6 h ischemia (Ji et al. 2014).

In conclusion, we were able to show that carnosine decreased the pathological levels of IGFBP1 in db/db mice. This effect was both by modulating the hypoxia-driven IGFBP1 increase and by increasing the insulin levels and was followed by improvement of blood glucose levels.

**Figure 5**
Carnosine decreases the functional activity of HIF-1α. (a) Carnosine downregulates HIF-1α in hypoxia in the HepG2 cells, *P<0.05 vs Hx. (b) HepG2 cells were transiently transfected with HRE-luciferase and CMV-Renilla reporter vectors and relative luciferase activity was measured after 24 h incubation in the presence or absence of 50 mmol carnosine solution and of hypoxia. Bars represent mean of three experiments ± S.E.M. of relative luciferase activity (firefly luciferase activity/Renilla luciferase activity) normalized to the relative luciferase activity in cells exposed only to normoxia. *P<0.05 (significantly different from corresponding HepG2 cells non-exposed to carnosine). The values represent the mean ± S.E.M. of four independent experiments.

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In conclusion, we were able to show that carnosine decreased the pathological levels of IGFBP1 in db/db mice. This effect was both by modulating the hypoxia-driven IGFBP1 increase and by increasing the insulin levels and was followed by improvement of blood glucose levels.
Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0571.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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