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ORIGINAL ARTICLE

The antineoplastic effect of carnosine is accompanied by induction of PDK4 and can be mimicked by L-histidine

Ulrike Letzien · Henry Oppermann · Jürgen Meixensberger · Frank Gaunitz

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Abstract Carnosine (β-alanyl-L-histidine) is a naturally occurring dipeptide that shows antineoplastic effects in cell culture as well as in animal experiments. Since its mode of action and the targets at the molecular level have not yet been elucidated, we performed qRT-PCR experiments with RNA isolated from glioblastoma cell lines treated with carnosine, β-alanine, L-alanine, L-histidine and the dipeptide L-alanine-L-histidine. The experiments identified a strong induction of expression of the gene encoding pyruvate dehydrogenase 4 (PDK4) under the influence of carnosine and L-histidine, but not by the other substances employed. In addition, inhibition of cell viability was only detected in cells treated with carnosine and L-histidine, with the latter showing a significantly stronger effect than carnosine. Since the tumor cells expressed the tissue form of carnosinase (CN2) but almost no serum carnosinase (CN1), we conclude that cleavage by CN2 is a prerequisite for the antineoplastic effect of carnosine. In addition, enhanced expression of PDK4 under the influence of carnosine/L-histidine opens a new perspective for the interpretation of the ergogenic potential of dietary β-alanine supplementation and adds a new contribution to a growing body of evidence that single amino acids can regulate key metabolic pathways important in health and disease.

Keywords Carnosine · L-Histidine · PDK4 · Glioblastoma · Cancer

Introduction

Carnosine (β-alanyl-L-histidine) was discovered more than a century ago as an abundant dipeptide in meat (Gulewitsch and Amiradzibi 1900). Since its discovery many different physiological functions have been attributed to carnosine, such as pH-buffering, metal-ion chelation and anti-oxidant capacity, and several in vitro and in vivo studies reported its function as an inhibitor of the formation of advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs) [for a recent review on the literature on carnosine's physiological functions see Boldyrev et al. (2013)]. Although, many physiological observations have been made and there is good experimental evidence for carnosine's ability to scavenge reactive oxygen species or its buffering capacities, there are many open questions on how carnosine exhibits its manifold functions at the molecular level. That is especially the case for its antineoplastic activity in mice that was already demonstrated in 1986 by Nagai and Suda (1986) and confirmed by Renner et al. (2010b) and Horii et al. (2012). The antineoplastic effect which has also been demonstrated in cell culture experiments (Holliday and McFarland 1996; Renner et al. 2008) has mainly been attributed to an influence

U. Letzien and H. Oppermann contributed equally to the work.

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U. Letzien · H. Oppermann · J. Meixensberger · F. Gaunitz (✉)
Klinik und Poliklinik für Neurochirurgie, Universitätsklinikum Leipzig AöR, Liebigstraße 20, 04103 Leipzig, Germany
e-mail: frank.gaunitz@medizin.uni-leipzig.de

U. Letzien
e-mail: Ulrike.Letzien@medizin.uni-leipzig.de

H. Oppermann
e-mail: Henry.Oppermann@medizin.uni-leipzig.de

J. Meixensberger
e-mail: Juergen.Meixensberger@medizin.uni-leipzig.de

on metabolic flux through glycolysis and reduced ATP and ROS concentration (Renner et al. 2010a; Iovine et al. 2011). However, neither the mechanisms responsible for the effect nor the molecular targets have been unequivocally identified. Therefore, we investigated whether carnosine influences the expression of genes involved in the metabolism of glucose with different qRT-PCR approaches using cells from three different cell lines derived from human glioblastoma. In addition, we analyzed whether the observed effects on gene expression and cell viability can be mimicked by the amino acids L-histidine, β-alanine and L-alanine or by the dipeptide L-alanine-L-histidine.

Materials and methods

Reagents

If not stated otherwise all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) or from Merck (Darmstadt, Germany). L-Histidine, β-alanine, L-alanine and carnosine were purchased from Sigma-Aldrich and L-ala-L-his from Bachem (Bubendorf, Switzerland).

Cell culture

All cells were cultivated in DMEM (4.5 g/l glucose, without pyruvate; Gibco, life technologies, Darmstadt, Germany) supplemented with 10 % fetal calf serum (FCS gold, PAA, Cölbe, Germany), 2 mM glutamax (Gibco), 50 µg/ml streptomycin and 30 µg/ml penicillin at 37 °C and 5 % CO_2 in humidified air in an incubator.

The human GBM cell lines T98G and U87 were originally obtained from the ATCC (Manassas, USA) and the line LN405 from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). All cells were genotyped (Genolytic GmbH, Leipzig, Germany) and their identity during the experiments confirmed.

qRT-PCR

For the isolation of RNA, 10^6 cells were seeded in 10-mm cell culture dishes (TPP, Trasadingen, Switzerland) with 10 ml of medium. Cells received fresh medium containing the individual supplements after 24 h and were cultivated for an additional period of time (for concentrations and incubation times see individual experiments). Then, total RNA was extracted using the RNeasy plus mini kit (Qiagen, Hilden; Germany) according to the manufacturer's recommendations and the RNA was stored at −80 °C until reverse transcription (RT), which was performed with 500 ng RNA and random primers, using the ImProm-II™ Reverse Transcription System (Promega, Mannheim, Germany) according to the manufacturer's recommendations. qRT-PCR was performed on a Rotor-Gene 3000 system (Qiagen) employing SYBR Green (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific, Germany). Data analysis was performed using the rotor-gene 6 software (Version 6.1/Build 93; Corbett Research). Sequences of forward and reverse primers used for expression analysis were analyzed using the NCBI/Primer-BLAST algorithms for potential mispriming sites, splice variants, melting temperatures and self-complementarity. Primers were finally purchased from Biomers (Ulm, Germany) (cartridge grade and MALDI analyzed). Further information on the primers employed and the amplicons produced is presented as Supplemental Material 1. Copy numbers of individual mRNAs were determined using linearized plasmid DNA containing the appropriate target sequences and normalized to the determined copy number of the reference gene TBP (TATA-box binding protein) which was found to be better suited for the experiments than β-actin (data not shown). All amplification reactions were controlled for the appropriate products by melting curve analysis and by 1.5 % agarose gel electrophoresis. In case of the quantification using the "RT^2 Profiler PCR Array for human glucose metabolism" (PAHS-006A, SABiosciences, Qiagen) expression data were analyzed by the $2^{-\Delta Ct}$ method. Therefore, relative fold changes were determined by subtracting the C_t of the housekeeping genes (contained within the assay by the manufacturer) from the C_t of the gene of interest, calculating the negative binary logarithm of the resulting ΔC_t followed by the comparison of the final values of treated samples with the untreated control.

Plasmids used as standards for qRT-PCR were either synthesized by Eurofins (Ebersberg, Germany) or cloned from amplification products by standard protocols.

cDNA from normal brain tissue was obtained from BioCat (Heidelberg, Germany).

Cell-based assays

For the determination of viability and toxicity the CellTiterGlo Assay, the CellTiterBlue Assay and the CytoTox-ONE Assay (all from Promega) were employed as described previously (Gaunitz and Heise 2003). The assays are based on the measurement of ATP (CellTiterGlo Assay), on dehydrogenase activity (CellTiterBlue Assay), and on lactate dehydrogenase activity in the supernatant as a result of necrotic loss of membrane integrity (CytoToxONE Assay). Luminescence and fluorescence were measured using either a Mithras LB 940 Multimode Microplate reader (Berthold Technologies, Bad Wildbad, Germany) or a Spectra Max M5 (Molecular Devices, Biberach, Germany).

Cells were initially cultivated in the wells of 96-well plates (μClear, Greiner Bio One, Frickenhausen, Germany) at a density of 2,500 cells/well (LN405) or 5,000 cells/well (T98G and U87) with 200 μl of medium. After overnight incubation, medium was withdrawn and replaced with 200 μl of fresh medium with the substances to be tested or vehicle. All assays were generally performed in sextuplicate at the time points indicated in the individual experiments.

Determination of intracellular L-histidine

Intracellular L-histidine was determined according to the method of Teufel et al. (2003). Briefly, 10^6 cells were seeded in 10-mm cell culture dishes (TPP, Trasadingen, Switzerland) with 10 ml of medium. After an overnight incubation, cells received fresh medium with or without carnosine or L-histidine. 72 h later cells were washed three times with ice-cold washing buffer (100 mM Tris–HCl, pH 8.0) and were finally collected in 1 ml washing buffer. After a short centrifugation (500*g*, 4 °C, 10 min) cells were resuspended in 300 μl of ice-cold lysis buffer (125 mM Tris–HCl, pH 9.5, containing 0.1 mM $MnCl_2$) and lysed by sonification using a Bioruptor® (Diagenode, Liège, Belgium) for 7.5 min with a 30 s on/30 s off protocol at maximum energy. After centrifugation (5 min at 17,000*g* at 4 °C) the supernatant was adjusted to a final protein concentration of 4 μg protein/100 μl and Tris–HCl to a final concentration of 50 mM (final $MnCl_2$ concentration 0.1 mM). 100 μl of this adjusted supernatant was dispensed into the wells of a black 96-well plate (Greiner Bio One, Frickenhausen, Germany). Then, 50 μl 1 % trichloroacetic acid and 50 μl *o*-phthalaldehyde solution (5 mg/ml in 2 mM NaOH) were added and after an incubation of 30 min at 30 °C the resulting fluorescence (λ_{ex} 340 nm and λ_{em} 440 nm) was quantified using a SpectraMax M5 Microplate Reader (Molecular Devices, Biberach, Germany).

Statistical analysis

Student's *t* test was performed using the algorithm implemented in Excel (Microsoft, Richmond, USA) (unpaired two-sample test with unequal variances). Calculation of ED50 was performed using Origin 8 (OriginLab Corporation, Northampton, USA) using the Boltzmann function to produce a sigmoidal curve from which the ED50 was derived. Relative data resulting from two experiments with a separate mean and standard deviation are presented using Gaussian error propagation according to the formula:

$$\frac{\Delta E}{E} = \sqrt{\left(\frac{\Delta a}{a}\right)^2 + \left(\frac{\Delta b}{b}\right)^2}$$

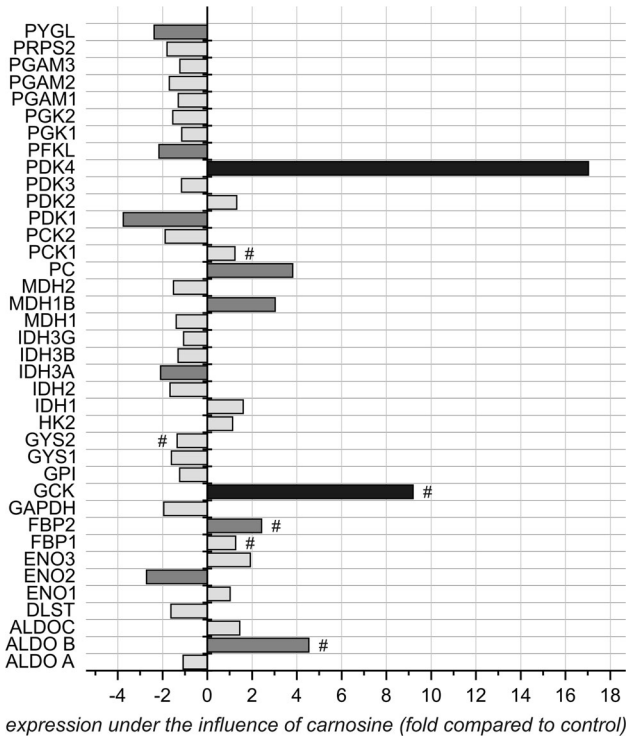


Fig. 1 Changes of mRNA expression in cells from the line U87 under the influence of 50 mM carnosine as revealed by human glucose metabolism "RT^2 Profiler PCR Array". Enhanced expression is indicated by *bars* towards right and reduced expression by *bars* towards left. *Black bars* indicate a more than sixfold change and *dark grey bars* a change >twofold. *ALDO A/B/C* aldolase A, B and C, *DLST* dihydrolipoamide *S*-succinyltransferase (E2 component of 2-*oxo*-glutarate complex), *ENO1* enolase 1, (alpha), *ENO2* enolase 2 (gamma, neuronal), *ENO3* enolase 3 (beta, muscle), *FBP1/2* fructose-1,6-bisphosphatase 1 and 2, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *GCK* glucokinase (hexokinase 4), *GPI* glucose-6-phosphate isomerase, *GYS1/2* glycogen synthase 1 (muscle) and 2 (liver), *HK2* hexokinase 2, *IDH1* isocitrate dehydrogenase 1 (NADP+), soluble, *IDH2* isocitrate dehydrogenase 2 (NADP+), mitochondrial, *IDH3A/B/G* isocitrate dehydrogenase 3 (NAD+) alpha, beta and gamma; *MDH1/1B* malate dehydrogenase 1 and 1B, NAD (soluble), *MDH2* malate dehydrogenase 2, NAD (mitochondrial), *PC* pyruvate carboxylase, *PCK1/2* phosphoenolpyruvate carboxykinase 1 (soluble) and 2 (mitochondrial), *PDK1/2/3/4* pyruvate dehydrogenase kinase, isozyme 1, 2, 3 and 4, *PFKL* phosphofructokinase, liver; *PGK1,2* phosphoglycerate kinase 1 and 2, *PGAM1/2/3* phosphogluco mutase 1, 2 and 3, *PRPS2* phosphoribosyl pyrophosphate synthetase 2, *PYGL* phosphorylase, glycogen, liver. Other genes from the array that were significantly expressed but unchanged under the influence of carnosine and that are not included in the figure: *TPI* triosephosphate isomerase, *TKT* transketolase, *TALDO1* transaldolase 1, *SUCLG1* succinate-CoA ligase, alpha subunit, *SDH A/B* succinate dehydrogenase complex, subunit A and B, *PGLS* 6-phosphogluconolactonase, *PDH A1/B* pyruvate dehydrogenase (lipoamide) alpha 1 and beta, *GSK3B* glycogen synthase kinase 3 beta, *GBE1* glucan (1,4-β-), branching enzyme 1, *FH* fumarate hydratase, *DLD* dihydrolipoamide dehydrogenase, *CS* citrate synthase, *ACO2* aconitase 2, mitochondrial, *ACLY* ATP citrate lyase. #: low expressed gene with $C_t \geq 30$

with ΔE is the propagated error; $E = a/b$; a, mean of data set 1; b, mean of data set 2; Δa, standard deviation of data set 1; Δb, standard deviation of data set 2.

Results

qRT-PCR analysis of genes influenced by carnosine

In order to analyze a possible influence of carnosine on enzymes involved in glucose metabolism, cells from the glioblastoma cell line U87 were incubated for 24 h in the absence and presence of 50 mM carnosine and analyzed by a "RT^2 Profiler PCR Array". The result of the experiment is presented in Fig. 1. The array identified two genes with a strongly enhanced expression under the influence of carnosine: glucokinase (GCK) was enhanced ninefold and pyruvate dehydrogenase kinase 4 (PDK4) 17-fold. In order to confirm this result and to identify whether the same effect can be observed in other cells of glioblastoma origin, we performed qRT-PCR analysis with the cell lines T98G and LN405 in addition to U87. We also included some targets that were not present on the "RT^2 Profiler PCR Array". A compilation of all data from all cell lines is presented in Fig. 2. As can be seen, the experiment confirmed a strong induction of PDK4 in U87 and T98G and a small induction in LN405. Although we also found the induction of GCK confirmed at least in U87 and T98G along with some other genes, we considered focussing on PDK4 because of its high induction and the reliable amount of mRNA found in non-stimulated U87 cells.

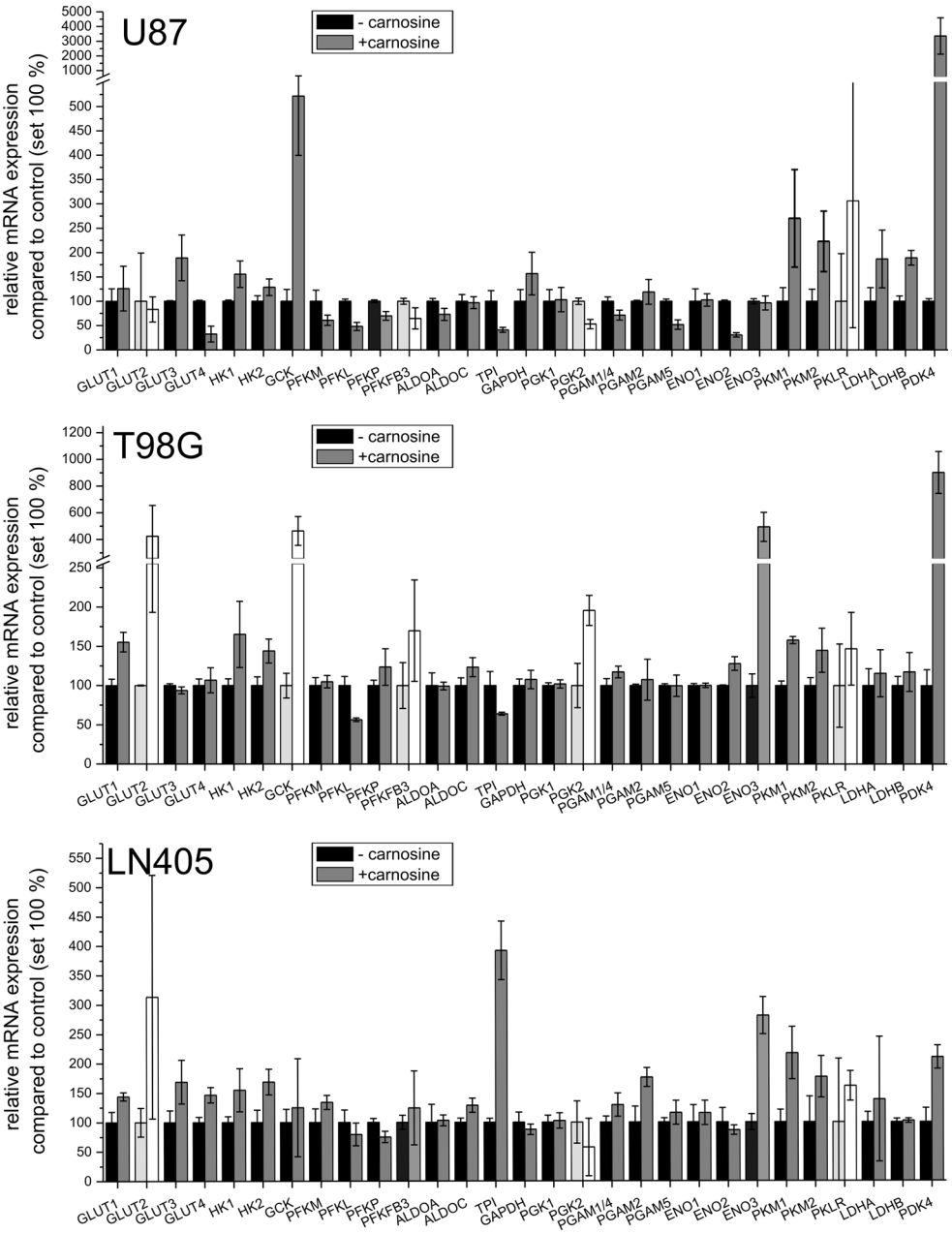


Fig. 2 Changes of mRNA expression in cells from the lines U87, T98G and LN405 under the influence of 50 mM carnosine as revealed by qRT-PCR. Total amount of mRNA of each gene tested was determined using standard curves and normalized to the expression of TBP. Abbreviations used are the same as in Fig. 1. Additional genes: *GLUT1/2/3/4* glucose transporter 1/2/3/4, *HK1* hexokinase 1, *PFKM* phosphofructokinase, muscle; *PFKP* phosphofructokinase, platelet; *PFKFB3* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme 3—splice variant UBI2K4, *PKM1/2* pyruvate kinase, muscle type 1/2, *PKLR* pyruvate kinase, liver and red blood cells, *LDH A/B* lactate dehydrogenase A/B. Genes with expression levels below the standard curve (<100 copies) are painted in *white* or *light gray*

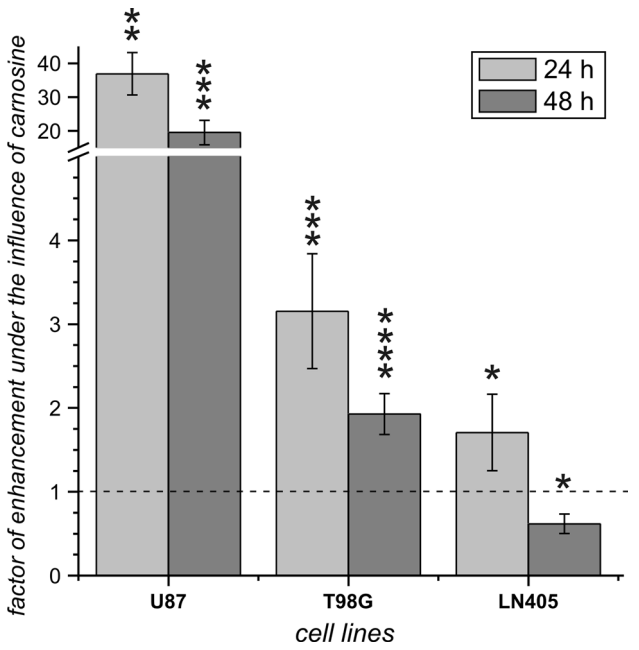


Fig. 3 Expression of PDK4 mRNA under the influence of carnosine after 24 and 48 h incubation. Cells from the cell lines LN405, U87 and T98G were incubated in the absence and presence of 50 mM carnosine. After 24 and 48 h cells were harvested, the mRNA isolated, reverse transcribed and subjected to qRT-PCR. The copy number of transcripts encoding PDK4 was normalized to the copy number of transcripts encoding TBP and finally the normalized relative copy numbers were compared between treated and untreated cells to determine the factor of enhancement under the influence of carnosine. The *dashed line* indicates the base line that would result without any effect of carnosine on the relative expression. Student's *t* test was performed using the results from determinations performed in quadruplicate to sextuplicate. $****p < 5 \times 10^{-6}$; $***p < 5 \times 10^{-5}$; $**p < 5 \times 10^{-4}$; $*p < 5 \times 10^{-3}$

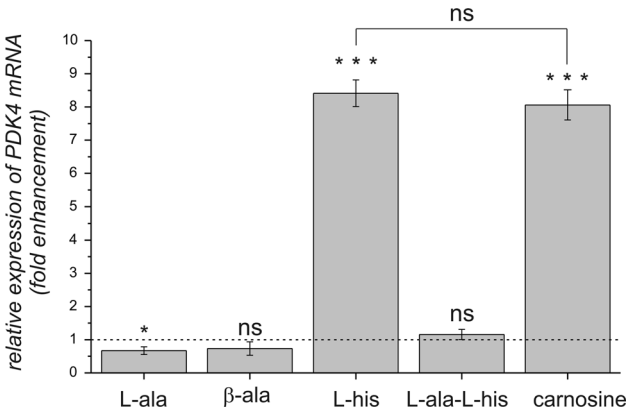


Fig. 4 PDK4 mRNA expression under the influence of different amino acids and dipeptides. Cells from the line U87 were incubated in the absence and presence of the test compounds indicated (each at 50 mM). After 24 h cells were harvested, mRNA isolated, reverse transcribed and subjected to qRT-PCR. The copy number of transcripts encoding PDK4 was normalized to the copy number of transcripts encoding TBP and finally the normalized relative copy numbers were compared between treated and untreated cells to determine the factor of enhancement under the influence of the test compounds. The *dashed line* indicates the base line that would result without any effect of carnosine on the relative expression. Student's *t* test was performed using the results from determinations performed in quadruplicate. $*p < 5 \times 10^{-2}$ and $***p < 5 \times 10^{-4}$; *ns* not significant

Since only a small induction was detected in cells from the line LN405, we addressed the question whether an effect can be observed after 48 h of incubation. Therefore, cells from the different lines were treated with 50 mM carnosine for 24 and 48 h. Relative levels of expression were compared between cells treated with carnosine and untreated cells and the corresponding factor of enhancement under the influence of carnosine is presented in Fig. 3. As can be seen, the enhancement is significant at 24 and 48 h in cells from the line U87 and T98G and significant in cells from the line LN405 at 24 h. Surprisingly, there is a small but significant drop in expression in the line LN405 at 48 h. At this point it should also be noted that the total effect observed differed quantitatively among independent experiments. This may be attributed to different influences from different charges of FCS (data not shown). However, the qualitative effect was confirmed in all experiments (24 h: U87—14 independent experiments; fold enhancement, 17.4 ± 10.6; range 7.1–36.9; T98G—3 independent experiments; fold enhancement 4.6 ± 3.5; range 2.1–8.6; LN405: 3 independent experiments; fold enhancement, 1.7 ± 0.5; range 1.3–2.2). Because of these differences all experiments presented in a single figure have been performed under the same conditions for all cells.

Effect of L-alanine, β-alanine, L-histidine and L-alanine-L-histidine on the expression of PDK4

In order to analyze the specificity of carnosine's effect on PDK4 expression, we asked whether one of its amino acid components, β-alanine or L-histidine, the isomer L-alanine or the dipeptide L-ala-L-his may exhibit the same effect. Therefore, cells from the line U87 were treated with the compounds for 24 h and subjected to qRT-PCR. The result of the experiment is presented in Fig. 4. All compounds were tested at a concentration of 50 mM. As can be seen in Fig. 4, L-histidine exhibits a strong effect on PDK4 expression that is not significantly different from that of carnosine. No significant effect was seen with β-alanine and L-ala-L-his, and a slight but significant reduction ($p < 0.05$) was determined with L-alanine.

Comparison of PDK4 expression under the influence of L-histidine and carnosine in U87, T98G and LN405 cells

Next, we compared the effect of L-histidine and carnosine on the expression of PDK4 in different cell lines. In order to do this, cells from the lines U87, T98G and LN405 were

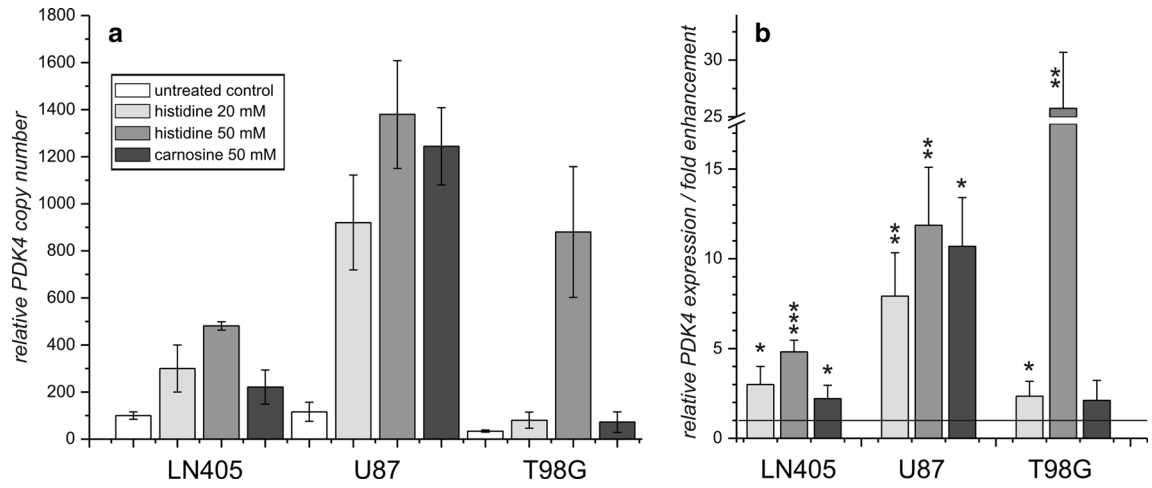


Fig. 5 Relative copy number of mRNA encoding PDK4 and expression under the influence of L-histidine and carnosine. **a** Cells from the cell lines LN405, U87 and T98G were incubated in the absence and presence of 20 and 50 mM L-histidine and in the presence of 50 mM carnosine. After 24 h cells were harvested, the mRNA isolated, reverse transcribed and subjected to qRT-PCR. The copy number of transcripts encoding PDK4 was normalized to the copy number of transcripts encoding TBP and is depicted as the relative copy number. In order to compare the relative copy number between the lines, the relative copy number in untreated cells from the line LN405 was set as 100 %. **b** The normalized relative copy numbers (panel **a**) were compared between treated and untreated cells for each cell line to determine the factor of enhancement under the influence of carnosine. The *dashed line* indicates the base line that would result without any effect of histidine or carnosine on the relative expression. Student's *t* test was performed using the results from determinations performed in quadruplicate. $***p < 5 \times 10^{-4}$; $**p < 5 \times 10^{-3}$; $*p < 5 \times 10^{-2}$

incubated in the absence and presence of 20 mM and 50 mM L-histidine and 50 mM carnosine. 24 h later a qRT-PCR experiment was performed. The result of the experiment is presented in Fig. 5. In the left panel (a) the relative copy number is shown. In order to compare differences between the lines the relative copy number of untreated cells from the line LN405 was set as 100 %. In the right panel (b) the enhancement of PDK4 expression under the influence of the substances is shown. As can be seen, the influence of L-histidine at a concentration of 20 mM is in all lines comparable to the enhancement obtained with 50 mM carnosine. At a concentration of 50 mM L-histidine, expression appears to be more strongly enhanced with an apparent very high effect in cells from the line T98G. Although the height of the enhancement has to be interpreted with care since the basal expression of PDK4 is low in this line (below the standards $\leq$100 copies), the effect of 50 mM L-histidine is significantly more pronounced than that of 50 mM carnosine ($p < 0.005$). With regard to LN405 the enhancement with 50 mM L-His compared to 50 mM carnosine is significantly higher ($p < 0.05$), whereas there is no significant difference in the line U87.

Cell viability under the influence of carnosine and L-histidine

The observation of a comparable induction of PDK4 expression under the influence of L-histidine and carnosine raised the question whether there is also an influence of L-histidine on cell viability in the tumor cell lines. To assess this, cells were incubated with different concentrations of carnosine and L-histidine for 24, 48 and 72 h and cell viability was determined by CellTiterGlo (Fig. 6a, b) and CellTiterBlue (Fig. 6c, d) assays. The result of the experiments is presented in Fig. 6. As expected, carnosine was clearly able to inhibit cell viability in all three lines (Fig. 6a, c). The same effect was observed with L-histidine (Fig. 6b, d), but at comparable concentrations the inhibitory effect of L-histidine was stronger than that of carnosine, for example, at 48 h with the CTB assay: 63.0 ± 16.9 % viability in 50 mM carnosine vs. 28.9 ± 5.1 % viability in 50 mM L-histidine (LN405); 58.0 ± 9.8 % viability in 50 mM carnosine vs. 43.9 ± 2.6 % viability in 50 mM L-histidine (U87); 64.1 ± 4.1 % viability in 50 mM carnosine vs. 3.5 ± 5.1 % viability in 50 mM L-histidine (T98G). Although it was difficult to calculate a reliable ED50 from different experiments, the general tendency was a strong response to L-histidine of T98G (ED50 7.7 mM; CTB at 48 h; sigmoidal fit with $R^2 = 0.997$) followed by LN405 (ED50 9.4 mM; CTB at 48 h; sigmoidal fit with $R^2 = 0.981$) and U87 (ED50 15.8 mM; CTB at 48 h; sigmoidal fit with $R^2 = 0.968$). A mathematically trustable calculation of the ED50 for carnosine was not possible, but it is obvious from Fig. 6 that all lines responded almost equally to the different concentrations of carnosine. A second observation was a microscopically evident cell death under the influence of L-histidine that was not

detected under the influence of carnosine (data not shown). In fact, we never detected any necrosis in cells treated with carnosine (≤50 mM). Therefore, we also employed the CytoToxONE assay to determine whether necrotic cell death occurs under the influence of L-histidine. A corresponding experiment is presented in Fig. 6e that demonstrates necrosis in cells from the line T98G after 48 h (starting at a concentration of 10 mM L-histidine) that was also significant at 72 h in the two other lines, but was less pronounced than in T98G. No cell death was detected under the influence of carnosine at concentrations ≤50 mM (data not shown).

Expression of carnosinase

Because of the strong effect of L-histidine on cell viability and PDK4 expression, we wondered whether the cleavage of carnosine is a prerequisite for the observed effects under the influence of carnosine. Therefore, we analyzed whether the two described carnosinase genes (CNDP1 and CNDP2) are transcribed in the cells from the three lines and compared the level of expression to the expression of carnosinases in normal brain tissue by qRT-PCR. In these experiments, mRNA encoding serum carnosinase (CN1) was almost not present in cells from the lines LN405 and T98G and its expression in U87 cells was detectable but still ~2,000 to 3,000 times less than in the four normal brain tissue mRNAs employed (Fig. 7). The expression of CNDP2 was comparably high in the three lines with an increase in the level of mRNA encoding tissue carnosinase (CN2, also designated cytosolic nonspecific dipeptidase) from LN405 to U87 (two times more than LN405) and to T98G (three times higher expression than in LN405). A comparison with normal brain tissue revealed an equal expression in T98G cells as compared to the temporal lobe and a ~3 times higher expression in tissue from the occipital lobe, the parietal lobe and the frontal lobe.

Intracellular L-histidine in cells cultivated in the absence and presence of carnosine and L-histidine

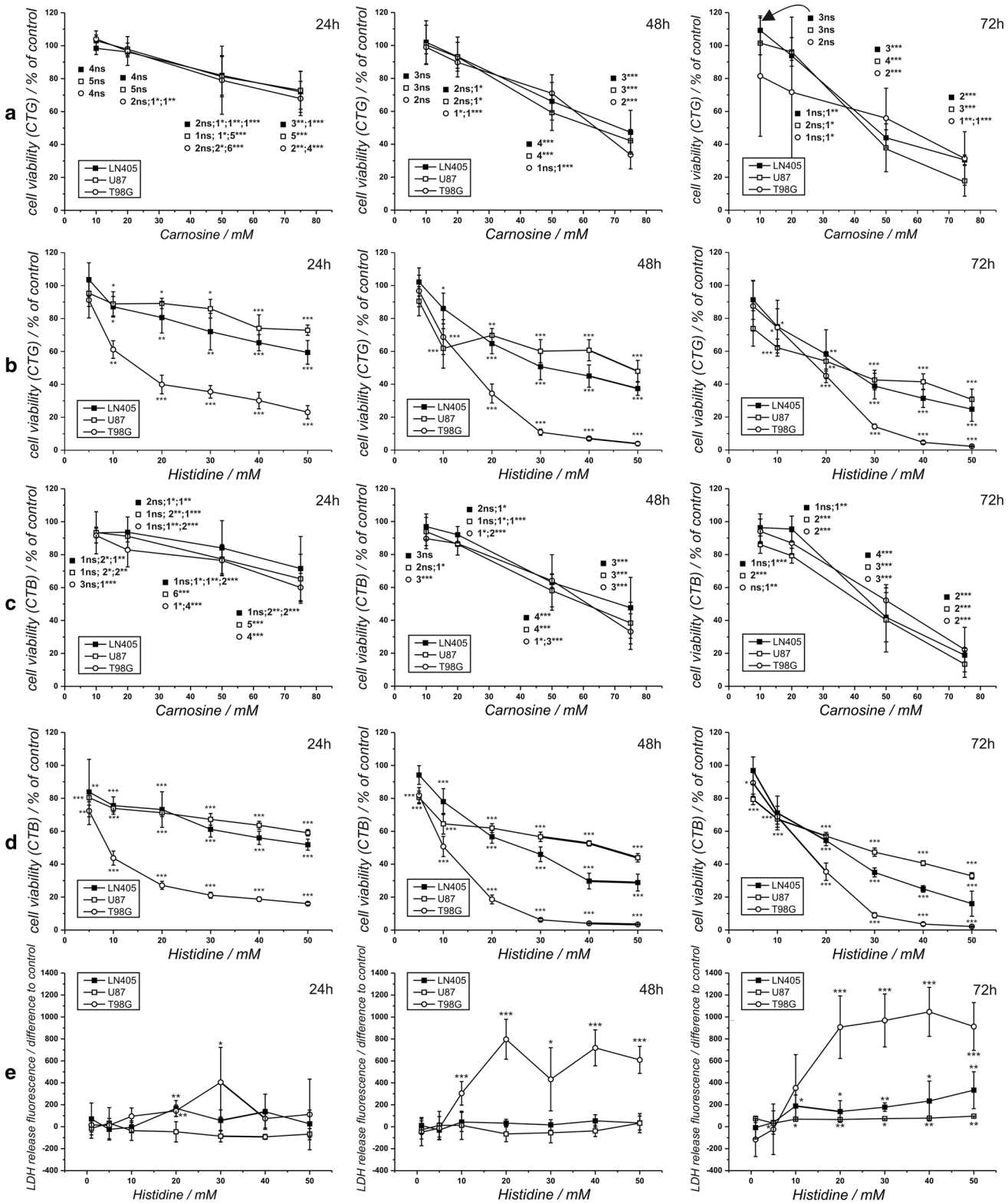
In order to determine whether the exposure of cells to medium containing carnosine or L-histidine does result in an increased intracellular concentration of L-histidine, cells from the three different lines were incubated for 72 h in medium containing 50 mM carnosine or 20 mM L-histidine, and the relative intracellular concentration of L-histidine was compared to cells cultivated in standard medium (containing 0.2 mM L-histidine). The result of the experiment is presented in Fig. 8. As can be seen, intracellular L-histidine concentrations are significantly elevated in cells from the lines U87 and T98G but not in cells from the line LN405 which has the lowest CN2 expression (Fig. 7). The presence of L-histidine in the medium resulted in a significantly increased concentration of L-histidine in all lines.

Discussion

In recent years, the antineoplastic effect of carnosine that was first described by Nagai and Suda (1986) has been confirmed by different groups in cell culture (Holliday and McFarland 1996; Renner et al. 2008; Iovine et al. 2011) and in vivo (Renner et al. 2010b; Horii et al. 2012). Therefore, a potential use of carnosine as a therapeutic drug for the treatment of cancer patients has already been discussed (Gaunitz and Hipkiss 2012; Hipkiss and Gaunitz 2013). Unfortunately, the molecular targets and the mode of action responsible for the observed effects have not been elucidated, although there have been indications that glycolytic flux and ATP production may be cellular targets (Renner et al. 2010a; Iovine et al. 2011). This hypothesis was also supported by experiments with yeast that demonstrated that carnosine slows growth rate and increases cell death only in cells that depend on glycolysis for ATP production and not in cells that can produce their ATP by oxidative phosphorylation (Cartwright et al. 2012).

In the present work, we demonstrate that carnosine induces expression of PDK4 in glioblastoma cells. To our knowledge, this is the first clear evidence that the dipeptide is able to influence gene expression. We could also demonstrate that the same effect on PDK4 expression is observed under the influence of L-histidine but not in the presence of β-alanine, L-alanine or L-alanine-L-histidine. In addition, the inhibitory effect of carnosine on tumor cell viability was also visible in the presence of L-histidine, strongly pointing towards the interpretation that cleavage of carnosine by the presence of cytosolic carnosinase 2 leads to the formation of L-histidine that is in turn responsible for enhanced expression of PDK4 and loss of cell viability. Transcriptional effects of L-histidine have also been shown by Son et al. (2005) who demonstrated an inhibition of TNF-α-induced IL-8 expression in Caco-2 and HT-29 cells which is not the case in cells treated with carnosine (Son et al. 2008). Interestingly, the same authors also confirmed that carnosine is not cleaved in Caco-2 cells (Son et al. 2008).

One question that needs to be answered is the missing effect of L-alanine-L-histidine on the expression of PDK4 and its only weak but significant effect on cell viability (CTG assay) after 72 h in U87 cells incubating them in 75 mM L-alanine-L-histidine (79.5 ± 5.2 % viability compared to 17.8 ± 9.3 % in 75 mM carnosine; $p < 0.005$; data not shown). At this point, it should also be noted that this observation also excludes any unspecific effect of carnosine, e.g., by changing osmotic pressure or pH. One possibility is that L-alanine-L-histidine may not



enter the cells as good as carnosine. Carnosine can be taken up at least by one of four transporters. These are PEPT1, PEPT2 (oligopeptide transporter 1 and 2; also designated SLC15A1 and 15A2) and PHT1and PHT2 (peptide/histidine transporter 1 and 2; also designated SLC15A4; SLC15A3) (see Smith et al. 2013; Daniel and Kottra 2004

◀**Fig. 6** Viability of cells at different concentrations of carnosine and L-histidine after 24, 48 and 72 h of incubation. Cells from the lines T98G, LN405 and U87 were cultivated at an initial culture density of 2,500 cells/well (LN405) or 5,000 cells/well (T98G and U87) in 96 well plates. 24 h later they received fresh medium with different concentrations of carnosine and L-histidine and cell-based assays were performed 24, 48 and 72 h after addition of the compounds. CellTiterGlo Assay (**a**) and CellTiterBlue assay (**c**) from cells incubated with carnosine. Mean and standard deviations were calculated from data of different independent experiments each performed in sextuplicate. *Numbers* indicate in how many experiments the reduction of viability was significant and *asterisks* indicate the level of significance: *ns* not significant, $***p < 5 \times 10^{-4}$; $**p < 5 \times 10^{-3}$; $*p < 5 \times 10^{-2}$. CellTiterGlo Assay (**b**) and CellTiterBlue Assay (**d**) from cells incubated with L-histidine. Experiments were performed in sextuplicate. *Asterisks* indicate level of significance (see legend to **a**). **e** CytoToxONE Assay with cells cultivated in L-histidine. Experiments were performed in sextuplicate. *Asterisks* indicate level of significance (see legend to **a**)

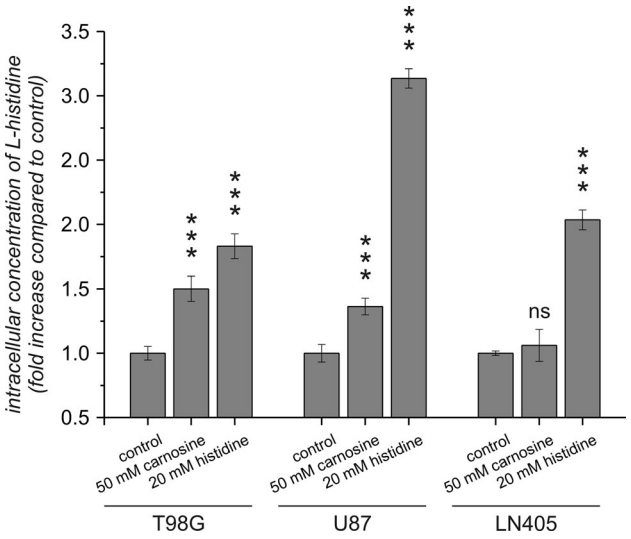


Fig. 8 Increase of intracellular L-histidine in the absence and presence of L-histidine and carnosine in the culture medium. Cells from three different lines were incubated for 72 h in the presence of 50 mM carnosine and 20 mM L-histidine and the relative intracellular concentration of L-histidine was determined and compared to cells cultivated in standard cultivation medium (0.2 mM L-histidine). All experiments have been performed in sextuplicate. $***p < 5 \times 10^{-4}$; *ns* not significant

for review). PEPT1 and 2 possess the capability to transport all 400 different dipeptides (Daniel and Kottra 2004) and should therefore also transport L-alanine-L-histidine. With regard to the PHTs we did not find experimental evidence in the literature whether they are able to transport L-alanine-L-histidine as it has been described for the transport of carnosine by PHT1 (Yamashita et al. 1997) and PHT2 (Sakata et al. 2001). At this point it should be noted that the PHTs in comparison to the PEPTs also transport L-histidine. The most likely interpretation of the missing effect of L-alanine-L-histidine may simply be that the effects observed are in fact induced by L-histidine which is released from carnosine, but cannot be cleaved from L-alanine-L-histidine under the influence of CN2. As Lenney et al. (1985) have demonstrated, L-alanine-L-histidine is not just a substrate of CN2 but instead inhibits the enzyme already at a concentration of 0.1 mM by 84 %. We are also aware that Teufel et al. (2003) pointed out that the optimal pH for degradation of carnosine by carnosinase 2 is around 9.5 which is well above the physiological pH. However, one may consider that the physiological conditions may be different from the experimental ones, and it also has to be considered that given the high effect of L-histidine even a small continuous release of L-histidine from carnosine may be enough to result in the observed physiological effects. Moreover, the results presented in Fig. 8 clearly demonstrate that the intracellular concentration of L-histidine is elevated at least in cells from the lines T98G and U87 when carnosine is present in the medium. One spillover that is also interesting to note is the absence of CN1 expression in

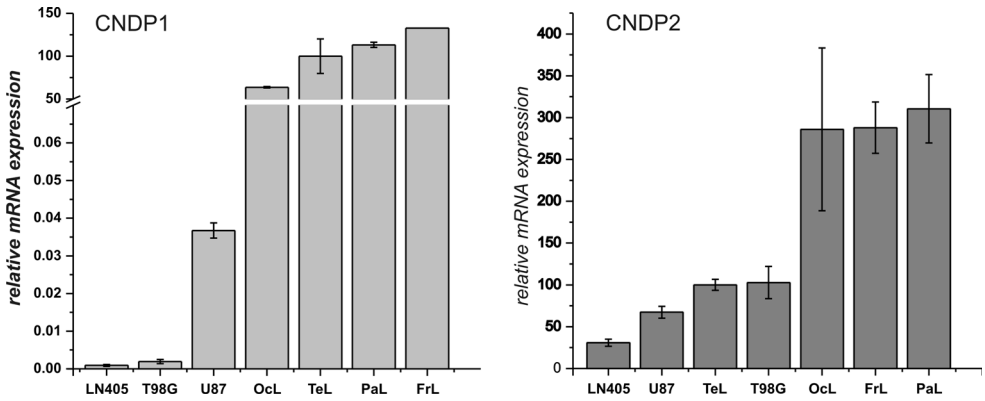


Fig. 7 Expression of CNDP1 and CNDP2 in glioblastoma cells and normal brain tissue. For the experiment RNA from the lines T98G, LN405 and U87 was reverse transcribed and the expression of CNDP1 and CNDP2 was analyzed by qRT-PCR and compared to the expression in normal brain tissue. *OcL* occipital lobe, *TeL* temporal lobe, *PaL* parietal lobe, *FrL* frontal lobe. Relative mRNA expression was calculated by normalization to expression of TBP

the glioma cells since Gautam et al. (2012) just recently demonstrated that CN1 protein levels are lowered in patients with glioblastoma.

Another question that needs to be addressed by further experiments is the connection between the antineoplastic effect of L-histidine and carnosine and the enhanced expression of PDK4. PDK4 is one of four human kinases that inhibit the pyruvate dehydrogenase complex via phosphorylation of its E1 component. Therefore, its overexpression will result in a reduced conversion of pyruvate to acetyl-CoA which is the only entry point for carbohydrates derived from circulating glucose (or intramuscular glycogen) into the mitochondria for complete oxidation (Pilegaard and Neufer 2004). Although our experiments do not reveal whether the enhanced expression of PDK4-mRNA is accompanied by enhanced enzymatic activity of PDK4, the observation that pyruvate can prevent the growth inhibition induced by carnosine (Holliday and McFarland 1996) is a strong hint pointing towards a direct influence of pyruvate dehydrogenase kinases on tumor cell growth since PDK4 (and also PDK1 and PDK2) are allosterically inhibited by pyruvate (Roche and Hiromasa 2007). Since the glioblastoma cells do not produce ATP by oxidative phosphorylation (Renner et al. 2010a) one may speculate that production of acetyl-CoA or its entry into the citric acid cycle may play a distinct role in tumor cells aside from the production of ATP. In fact, although inhibitors of pyruvate dehydrogenase kinases are discussed as therapeutic tools for the treatment of tumors (Sutendra and Michelakis 2013), it was demonstrated that PDK4 mRNA is commonly downregulated in tumors compared to their tissues of origin and that overexpression decreases cell proliferation (Grassian et al. 2011). Since a detailed discussion on the role of the citric acid cycle and acetyl-CoA as well as on thiamin diphosphate-dependent enzymes in tumor metabolism is beyond the scope of this manuscript, readers should refer to recent publications by Bunik et al. (2013) and Icard et al. (2012).

Aside from regulation of PDK4 expression, we cannot rule out the possibility that carnosine also has other influences on (glycolytic) ATP production that contribute to its antineoplastic effect and may not be related to free L-histidine. This consideration comes from experiments with yeast. As Cartwright et al. (2012) have demonstrated, no loss of viability was observed when yeast cells were grown with 2 % glucose in the presence of 10 mM L-histidine but exhibited only 90 % viability when cultivated under the same conditions in the presence of 10 mM carnosine. At this point it should also be noted that carnosine exhibited a strong effect on cell viability in cells from the line LN405, although expression of CN2 is low in this line and only a small activation of PDK4 expression was observed. Whether the decrease in overall ATP production determined by the CellTiterGlo viability assay in the cultures of our experiments results from a reduced number of cells or may also reflect an inhibition of glycolytic ATP production remains speculative but cannot be ruled out. In conclusion, for the first time it is demonstrated that in the presence of carnosine, transcriptional regulation can be altered and that the effect is mimicked by L-histidine. Aside from many other functions attributed to carnosine it can therefore also be considered as a vehicle for L-histidine that protects this amino acid from becoming part of the pool of amino acids entering protein biosynthesis and which releases it in cells with cytosolic carnosinase 2. In addition, a strong influence of a proteinogenic amino acid on tumor growth is demonstrated that adds to the growing body of evidence that amino acids participate in and regulate key metabolic pathways (Wu 2013).

In view of a potential use of carnosine as a cancer therapeutic, it would be highly interesting whether there are L-histidine containing peptides or derivatives that under physiological conditions in humans are a substrate for carnosinase 2 but not for carnosinase 1 to be used to target glioma cells without being degraded by serum carnosinase. As already demonstrated at least purified recombinant CN1 and CN2 exhibit different substrate specificities under experimental conditions (Teufel et al. 2003). A different approach would be to inhibit CN1 which might be possible as suggested by Vistoli et al. (2006).

We hope that our work will encourage further investigation of carnosine's effects in cells derived from other tumors and normal tissue. At least non-tumorous human embryonic kidney 293 (HEK293) cells respond to the presence of carnosine with reduced growth and a small but significant enhancement of PDK4 expression (Supplemental Material 2). Although HEK293 cells are immortalized and are therefore not really normal parenchymal cells, it will be necessary to analyze carnosine's effects also in normal tissue in more detail when considering a possible application as an anti-cancer drug.

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