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Sugar- and lipid-derived aldehydes are reactive carbonyl species (RCS) frequently used as surrogate markers of oxidative stress in obesity. A pathogenic role for RCS in metabolic diseases of obesity remains controversial, however, due in part to their highly diffuse and broad reactivity, and to lack of specific RCS-scavenging therapies. Naturally occurring histidine dipeptides (e.g., anserine and carnosine) possess RCS reactivity, but their therapeutic potential in humans is limited by serum carnosinases. Here we present the rational design, characterization and pharmacological evaluation of ‘carnosinol’ (i.e. (2S)-2-(3-amino propanoylamino)-3-(1H-imidazol-5-yl)propanol) a derivative of carnosine with high oral bioavailability that is resistant to carnosinases. Carnosinol displayed a suitable ADMET profile and was determined to have the greatest potency and selectivity toward α,β-unsaturated aldehydes (e.g. 4-hydroxynonenal, HNE, acrolein) among all others so far reported. In rodent models of diet-induced obesity and metabolic syndrome, carnosinol dose-dependently attenuated HNE-adduct formation in liver and skeletal muscle while simultaneously mitigating inflammation, dyslipidemia, insulin resistance, and steatohepatitis. These improvements in metabolic parameters with carnosinol were not due to changes in energy expenditure, physical activity, adiposity or body weight. Collectively, our findings illustrate a pathogenic role for RCS in obesity-related metabolic disorders, and provide validation for a promising new class of carbonyl-scavenging therapeutic compounds rationally derived from carnosine.

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A Carnosine Analog Mitigates Metabolic Disorders of Obesity by Reducing Carbonyl Stress

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Abstract

Sugar- and lipid-derived aldehydes are reactive carbonyl species (RCS) frequently used as surrogate markers of oxidative stress in obesity. A pathogenic role for RCS in metabolic diseases of obesity remains controversial, however, due in part to their highly diffuse and broad reactivity, and to lack of specific RCS-scavenging therapies. Naturally occurring histidine dipeptides (e.g., anserine and carnosine) possess RCS reactivity, but their therapeutic potential in humans is limited by serum carnosinases. Here we present the rational design, characterization and pharmacological evaluation of ‘carnosinol’ (i.e. (2S)-2-(3-amino propanoylamino)-3-(1H-imidazol-5-yl)propanol) a derivative of carnosine with high oral bioavailability that is resistant to carnosinases. Carnosinol displayed a suitable ADMET profile and was determined to have the greatest potency and selectivity toward α,β-unsaturated aldehydes (e.g. 4-hydroxynonenal, HNE, acrolein) among all others so far reported. In rodent models of diet-induced obesity and metabolic syndrome, carnosinol dose-dependently attenuated HNE-adduct formation in liver and skeletal muscle while simultaneously mitigating inflammation, dyslipidemia, insulin resistance, and steatohepatitis. These improvements in metabolic parameters with carnosinol were not due to changes in energy expenditure, physical activity, adiposity or body weight. Collectively, our findings illustrate a pathogenic role for RCS in obesity-related metabolic disorders, and provide validation for a promising new class of carbonyl-scavenging therapeutic compounds rationally derived from carnosine.
Introduction

Overnutrition from fatty acids and complex carbohydrates is known to cause oxidative stress from multiple enzymatic and non-enzymatic sources due to the high caloric content and the prevalence of these macronutrients in the Western diet. A pathological role for oxidative stress in obesity has been clearly established through extensive clinical and experimental studies. Reactive sugar- and lipid-derived aldehydes (RCS, reactive carbonyl species) are spontaneously formed during preparation of high fat/sugar-containing foods under high heat (1), and are formed in vivo as a byproduct of oxidative stress. These RCS modify proteins via the covalent modifications of cysteine, arginine, lysine and histidine, and form adducts with phospholipids and DNA. Glucose-derived oxoaldehydes such as methylglyoxal (MGO) accumulate in oxidative tissues and react with protein functional groups, forming advanced glycation end-products (AGEs) (2,3). AGEs are a major cause of chronic inflammation, cardiovascular disease, diabetes, and even some cancers, all of which are associated with obesity (4-8). In addition to reducing sugars and sugar-derived break-down products, lipid-derived aldehydes are another significant source of carbonyl stress in vivo. Individuals consuming diets rich in vegetable and corn oil have a very high endogenous level of n-6 polyunsaturated fatty acids (PUFAs). The α,β-unsaturated carbonyls derived from n-6 PUFAs oxidation have particularly diverse biological effects. Of these, 4-hydroxynonenal (HNE) and acrolein (ACR) have been the most widely studied (9). These aldehydes rapidly form adducts with proteins and phospholipids (10), and chronic exposure to high levels of these aldehydes is toxic (11). Accumulation of HNE has been extensively documented in blood and tissue samples from obese/diabetic patients, typically as a marker of oxidative stress (12-16). However, emerging studies suggest that these reactive aldehydes are more than simply byproducts of oxidative stress. Rather, the carbonyl-modifying activity imposed by these reactive species may have a distinct pathogenic role in obesity-related disorders such as insulin resistance, chronic inflammation and fibrosis, dyslipidemia and liver disease (15,17-23). Thus, novel compounds that mitigate the production or enhance the removal of reactive carbonyl species (RCS) remain compelling therapies for cardiovascular and metabolic diseases associated with obesity.

L-carnosine is a naturally occurring dipeptide (β-alanyl-histidine) which, along with its analogues, are potent endogenous scavengers of RCS and are highly concentrated (mM) in muscle and nervous tissues (24). The utility of L-carnosine as a pharmacological agent has been demonstrated in rodent models of metabolic syndrome and cardiovascular disease (25-27), and by its widespread use as eyedrop therapy in patients with ocular diseases (28). Major obstacles exist with respect to clinical applicability of L-carnosine as oral drug therapy, however. The largest of these is that
high serum and tissue carnosinase activity in humans abrogates bioavailability of circulating carnosine by rapid hydrolysis of the peptide bond (24).

Here, we present the rational design, characterization and pharmacological evaluation of ‘carnosinol,’ i.e. (2S)-2-(3-amino propanoylamino)-3-(1H-imidazol-5-yl)propanol, a reduced derivative of L-carnosine that is impervious to metabolism by carnosinase. Carnosinol displayed selectivity for reaction with RCS in vitro and in vivo, oral bioavailability and long duration in vivo, and negligible toxicity in human cell cultures and animal models. In rodent models of diet-induced obesity, carnosinol dose-dependently reduced systemic carbonyl stress, normalized glycemic control and many inflammatory parameters, and mitigated steatohepatitis. Collectively, these findings illustrate a distinct pathological role of RCS in metabolic diseases of obesity, and validate the use of a novel RCS-scavenging L-carnosine derivative to treat these diseases.
**Results**

**Rational design of Carnosinol**

In addition to the common properties which characterize a drug-like molecule (i.e. chemical and metabolic stability, bioavailability and safety), our *a priori* rationale was that a RCS-sequestering therapeutic compound should 1) be stable in plasma; 2) effectively scavenge (i.e., trap) circulating RCS; and 3) be highly reactive and selective towards damaging RCS (29). L-carnosine fulfils some of the above mentioned requirements (30). However, it lacks an important basic requirement to be a suitable RCS sequestering agent because it is unstable in the circulation due to the hydrolytic action of carnosinases.

Hence, the rational design of improved L-carnosine derivatives should be focused on molecules that, besides maintaining or even enhancing quenching activity and selectivity, are endowed with plasma stability and oral bioavailability. To this end, the ideal derivative should: (a) maintain or better optimize its quenching activity at least towards HNE, while preserving its selectivity, (b) maintain the active transport by human H+/peptide cotransporter-1 (hPepT1), and (c) eliminate the recognition by human serum carnosinase (30,31). As depicted in Figure 1A, this can be pursued by modifying the carboxyl group which (a) is not involved in the quenching mechanism, even though its complete deletion has a detrimental effect as seen in carcinine; (b) has a crucial role in carnosinase-1 (CN1) binding (Fig. 1B); and (c) has a marginal role in hPepT1 transport (Fig. 1C). Reduction of the carboxyl group has only a modest impact on the PepT1 interaction (Fig. 1C) since both the carboxyl and the hydroxyl functions elicit comparable and (weak) H-bonds with surrounding backbone atoms as also confirmed by very similar interaction energies (-19.056 vs. -17.934 kcal/mol). In contrast, the carnosine carboxyl group is engaged in a pivotal ion-pair with Arg350 in CN1 (Fig. 1B), and its reduction to the hydroxyl function has a dramatic impact on the stability of the complex CN1-carnosinol as confirmed by the reported drop in the interaction energy (-22.770 vs. -14.862 kcal/mol).

Based on these modelling results, the carboxyl reduction to yield carnosinol should meet all the above requirements and should even enhance the quenching activity since it optimizes some physicochemical parameters which influence the quenching mechanism (e.g. flexibility and lipophilicity). Besides a greater flexibility, physicochemical analysis reveals that carnosinol is also more lipophilic than carnosine as seen in experimental log P and log D7.4 values, a property that would benefit the molecular engagement by lipophilic RCS (Supplemental Table 1). Furthermore, carboxyl reduction did not significantly affect the ionization constant, nor the chelating activity towards transition metal ions such as Cu^{2+}, which are roughly comparable to those of carnosine (pK_{CuHL}= 12.73, pK_{CuL} = 5.49). Quantum mechanical simulations showed that carnosinol also possesses favourable stereo-electronic parameters (Supplemental Table 1).
Carnosinol is a selective and potent RCS-sequestering agent

The RCS-sequestering activity of carnosinol was first tested in vitro towards the most widely studied RCS involved in oxidative-based diseases and belonging to the chemical classes of α,β-unsaturated aldehydes, i.e. 4-hydroxynonenal (HNE), acrolein di-aldehydes, i.e. malondialdehyde (MDA) and glyoxal (GO), and keto-aldehydes i.e. methylglyoxal (MGO).

Sequestering of HNE

The sequestering activity of carnosinol towards HNE and acrolein was first evaluated and compared with L-carnosine and for other small molecules known to possess similar properties (32). We directly compared the reactivity of carnosinol with the parent dipeptide, L-carnosine, with both acrolein (ACR) and HNE. Both compounds effectively reduced free aldehyde levels via conjugate formation over time, but interestingly, carnosinol showed a more rapid depletion of both ACR and HNE during the incubation as compared with L-carnosine (Supplemental Table 2). Selectivity was then determined by comparing carnosinol reactivity with HNE and the biogenic aldehyde pyridoxal (Table 1). The data are reported as consumption percentages (Q% ± SD) of HNE after 24 h and considering a quencher/RCS ratio of 1:1 for HNE and 10:1 for pyridoxal (30). Carnosinol was the most reactive compound in respect to L-carnosine and other well-known RCS-sequestering agents such as aminoguanidine, and pyridoxamine. Carnosinol was found to be less effective only by comparison with hydralazine, a commonly-prescribed anti-hypertensive medication with non-selective RCS reactivity as shown by its effects with pyridoxal. In contrast, carnosinol did not react with pyridoxal and hence it can be considered a selective RCS-sequestering agent, similar to the parent compound L-carnosine.

The reaction mechanism of Carnosinol towards HNE was fully elucidated on the basis of the reaction products identified and characterized by MS experiments (Supplementary Fig. 1). The proposed reaction mechanism (Fig. 2A) is similar to that already clarified for L-carnosine and based on the formation of an imine derivative (CI) that catalyses the Michael adduct (CII) (Fig. 2B).

Increased potency of carnosinol as compared with L-carnosine can be explained by the favourable stereo-electronic parameters as predicted by quantum mechanical simulations (Supplementary Table 1) and also by the ability of the hydroxyl group to form a hemiacetal intermediate with HNE which, in addition to the amino group of β-alanine, further catalyses the Michael adduct formation.

The quenching ability of carnosinol to inhibit HNE induced protein covalent adducts and its comparison with the known quenchers was then evaluated in a MS based assay, recently set-up in our laboratory (33). The assay consists of determining the ability of
the tested compounds to inhibit the formation of HNE induced adducts on a target protein, human ubiquitin, whose carbonylation specifically involves Lys6 and His68. Shown in Figure 3 is the MS spectrum of ubiquitin incubated in absence and presence of HNE, inducing the formation of an HNE Michael adduct characterized by a MW shifted by 156.11 Da with respect to native ubiquitin. Carnosinol dose-dependently inhibited ubiquitin carbonylation. The greater activity with respect to hydralazine when measured via HPLC as compared with MS can be easily explained by considering that hydralazine forms a reversible Schiff base with HNE as already reported (30), which cannot compete with the formation of Michael adducts on ubiquitin. By contrast, the more stable carnosinol–HNE adduct is not shifted in the presence of ubiquitin; thus hydralazine was less efficient when tested in the MS method. The % inhibition of HNE induced ubiquitin carbonylation for Carnosinol and some selected reference compounds are also reported in Table 1. Note again that hydralazine depletes pyridoxal levels completely, a feature of this drug that undoubtedly contributes to the adverse risk profiles related to vitamin B deficiency.

Carnosinol sequestering activity was then tested towards MGO and MDA by both HPLC and MS (Suppl Fig. 2 & 3, Table 2). Carnosinol was found more effective as a MGO-sequestering agent than L-carnosine and of the same order of potency of well-established scavengers such as aminoguanidine and hydralazine. An adduct characterized by a MOLD like structure was identified by MS as the major reaction product between carnosinol and MGO (Supplementary Fig. 2). The sequestering activity towards MDA was found superimposable in respect to carnosine and the N-propenal derivative of carnosinol was identified as the reaction product (Supplementary Fig. 3).

Taken together the data indicate that carnosinol is selective and very reactive towards biogenic sugar- and lipid-derived aldehydes, and the most potent RCS-sequestering agent among those commercially available.

**ADMET studies**

Carnosinol was evaluated in *in vitro* ADMET studies as well as in exploratory PK and toxicological studies (more information available in supplementary information). Carnosinol was found stable in human serum for at least 60 minutes, as well as in rat and human liver preparations, thus indicating that it is not hydrolyzed by carnosinases. *In vitro* experiments indicate a good safety profile of the compound (no inhibition of the most important isoenzymes of cytochrome P450, no interaction with hERG K+ channel, no cell toxicity up to 100 µM concentration in a human hepatoma cell line). The safety profile of carnosinol was then confirmed in rats in both single-dose and repeat-dose oral toxicity studies. PK studies in rats showed a moderately good oral bioavailability giving a significant exposure in plasma after oral administration at the dose of 45 mg/kg.
The data above reported demonstrate that carnosinol fulfils the optimization requirements we took into account in the rational design.

**Dose-dependent effects of carnosinol on systemic inflammation and metabolic derangements in fructose-fed rats**

Prior studies by our group and others have demonstrated that oral administration of L-carnosine and its derivatives effectively reduces atherogenesis (27,34,35), dyslipidemia and renal dysfunction (25) in rodent models of cardio-metabolic disease. To determine if carnosinol retained, or surpassed, the efficacy of L-carnosine, we tested the effect of low (10 mg/kg/day) and high dose carnosinol (45 mg/kg/day in d.w.) on cardio-metabolic and inflammatory parameters using a short-term intervention (3 weeks) in rats fed high fructose diet (60% in drinking water, Fig. 4A). An additional group of rats were treated for equivalent time with a clinically relevant dose of rosiglitazone, the peroxisome proliferator-activated receptor (PPAR) agonist widely prescribed for treatment of metabolic syndrome and type 2 diabetes, and which we recently determined to be a very effective therapy in this model (36). High Fructose-feeding led to significant gains in body weight (Fig. 4B and Supplemental Table 3) compared with control-fed rats, and drug treatment did not cause significant differences in body weight gain. Total food and water intake were similar between groups. Systolic blood pressure was substantially higher in fructose-fed rats, and this was dose-dependently reduced by carnosinol to levels comparable with rosiglitazone (Supplemental Table 3). This improvement in blood pressure with carnosinol mirrors the effect of L-carnosine supplementation on these parameters in similar models (25,26).

Next we sought to examine the effect of carnosinol on markers of systemic oxidative and inflammatory stress. Notably, carnosinol dose-dependently reduced serum AGEs (Fig. 4C), urinary 8-isoprostanes (Table 3), and pro-inflammatory cytokines (Fig. 4D-F) in fructose-fed rats. Importantly, the beneficial effects of carnosinol treatment on oxidative/inflammatory stress were paralleled by improvements in metabolic parameters. Carnosinol treatment led to dose-dependent reductions in serum triglycerides and cholesterol (Fig. 4G-H), improved glycemic control (Fig. 4I-J), and mitigated liver toxicity (Fig. 4K) and steatosis (Table 3) induced by fructose-feeding. All improvements in systemic metabolic and inflammatory parameters with carnosinol treatment were paralleled by significant reductions in plasma (Fig. 4L-M), kidney and liver HNE-adducts (Supplemental Figure 4). Liver and renal fibrosis were not significantly affected by fructose-feeding or drug treatment (not shown), although urinary creatinine clearance rate did decrease with fructose feeding (Table 3), and this effect was blunted with carnosinol and rosiglitazone treatment.
Carnosinol improves glycemic control and muscle insulin sensitivity in mouse models of severe carbonyl stress and diet-induced obesity

Glutathione peroxidase 4 (GPx4), also named phospholipid hydroperoxide glutathione peroxidase, is the only known seleno-enzyme that exclusively neutralizes lipid peroxides in membranes and lipoproteins (37). Homozygous-null GPx4 mice die at gestational day ~7, underscoring the critical role for this enzyme in development. GPx4 haploinsufficient (GPx4+/−) mice contain ~40% of wild-type GPx4 enzyme levels in oxidative tissues and are highly susceptible to environmental stressors due to enhanced lipid peroxidation and protein carbonylation (38,39). Recently, we observed that GPx4+/− mice display exacerbated metabolic derangements and cardiomyopathy when fed a n-6 PUFA enriched high fat, high sucrose (HFHS) diet (15). These cardio-metabolic disorders in GPx4+/− mice were accompanied by extensive carbonyl stress in liver and heart, and cardiac mitochondria in obese GPx4+/− mice had decreased fatty acid-supported respiration, and increased ROS production, compared with obese WT mice. These results are consistent with mitochondrial localization of GPx4 and its known role in protecting mitochondria from oxidative stress (40). Importantly, diabetic patients exhibit higher levels of HNE-adducts and lower GPx4 enzyme in their myocardial tissue compared with non-diabetic patients (15), a finding that corroborates previous observations concerning HNE-adducts in diabetic patients (12-14,16). Thus, GPx4+/− mice are ideal for pharmacological evaluation of carnosinol because much of the underlying stress-induced pathology of these mice is due to oxylipid-derived aldehydes.

To determine whether carnosinol is effective at mitigating obesity-related metabolic disorders, we administered the compound at the high dose (45 mg/kg/day in drinking water) in a cohort of HFHS diet-induced obese WT mice. Given our previous findings in GPx4+/− mice on HFHS diet, a cohort of GPx4+/− mice were used in parallel with WT mice to allow for assessment of carnosinol in a clinically relevant, translational model of severe carbonyl stress. Drug was administered starting after 8 weeks on the HFHS diet, and a control group was fed normal chow for duration of study (Supplemental Fig. 4A). As expected, HFHS diet increased body weight and adiposity in both WT and GPx4+/− mice (Supplemental Fig. 4B & C), and carnosinol had no effect on these parameters, although fasting serum triglycerides and cholesterol were decreased in carnosinol-treated mice (Supplemental Table 4). No effect of carnosinol was observed on whole body energy expenditure (Supplemental Figure 5), or food/water intake (data not shown) in mice compared with HFHS diet alone. Carnosinol treatment led to enhanced glucose disposal following oral glucose challenge in obese WT but not GPx4+/− mice (Fig. 5A-C). This improved glucose disposal may be attributed in part to an increased skeletal muscle insulin sensitivity, as carnosinol normalized insulin-stimulated 2-deoxyglucose (2-DOG) uptake in extensor digitorum longus (EDL) to levels similar to control-fed lean mice (Fig. 5D). No improvement in insulin sensitivity was observed with
carnosinol in soleus from obese WT mice however, although a modest improvement was observed in obese GPx4+/− soleus (Fig. 5F).

Reactive aldehyde derivatives of lipid peroxidation have recently come into focus as novel redox signaling agents which paradoxically have beneficial and pathological roles, depending on concentration and tissues/cells affected (11,41). Protein-HNE adducts are known to be increased in skeletal muscle of type 2 diabetic and obese/insulin-resistant patients, and these adducts are associated with the severity of insulin resistance (42). Experimental models have reported that lipid peroxidation in skeletal muscle blunts insulin signaling and glucose uptake in skeletal muscle via HNE-adduct formation (43,44). Lipid peroxidation and HNE-adduct formation has also emerged as a potential causal factor in hyperinsulinemia and eventual loss of pancreatic β-cell function in models of obesity/overnutrition (45-47), which is further evidence of a system-wide pathological role for reactive aldehydes in metabolic syndrome. Here, HNE-adduct formation in mixed gastrocnemius skeletal muscle (Fig. 5F, G) and pancreas (Fig. 5H, I) increased with HFHS diet, particularly in GPx4+/− mice. Carnosinol effectively mitigated the accumulation of these adducts in both WT and GPx4+/− mice on HFHS diet, suggesting that this may be one aspect of the mechanism by which carnosinol improves systemic glycemic control in the obese mice.

Liver inflammation and fibrosis are mitigated by carnosinol in mouse models of severe carbonyl stress and diet-induced obesity

Non-alcoholic steatohepatitis (NASH) is among a cluster of obesity-related pathologies and closely linked with insulin resistance. NASH is distinct from fatty liver disease in that intralobular inflammation and fibrosis is present, in addition to the steatosis (48). The fibrosis component of this disease has been specifically identified by numerous studies to be the most likely to predict adverse outcomes in patients (49). Thus, therapeutic strategies that specifically target liver inflammation and fibrosis in obese patients will be highly valued by clinicians (50). A number of studies have implicated RCS as having a causal role in NASH, due in large part to the known effect of RCS on activation of the pro-inflammatory receptor for AGEs (RAGE) pathway (22,51). In the recent study from our group, obese GPx4+/− mice were found to have elevated RAGE expression in their heart, and this corresponded to greater cardiac inflammatory cytokine expression and fibrosis (15). In the present study, RAGE expression in liver was unchanged in WT and GPx4+/− mice with HFHS diet, but carnosinol significantly decreased RAGE expression in both groups (Fig. 6A). Expression of pro-inflammatory cytokines TNFα and IL-6 was significantly higher in liver of the HFHS-fed mice, and carnosinol mitigated expression of TNFα, but not IL-6, in this tissue (Fig. 6B & C).
To further examine the effect of carnosinol on liver pathology in obesity, sections of liver tissue were fixed and stained with oil red O and picrosirius red to label triglycerides and collagen, respectively. As in the previous study, HFHS diet increased liver triglyceride content in WT and GPx4^{+/−} mice, with substantially greater lipid deposition occurring in the obese GPx4^{+/−} mice (Fig. 6D & E). Interestingly, the increase in liver triglyceride deposition with HFHS diet was not accompanied by changes in serum triglycerides (Supplemental Table 4) – although this could be attributable to necropsy being performed while mice were in fasted state. While total triglyceride content in liver did not significantly change with carnosinol (data not shown), a shift from predominantly macro- to micro-vesicular steatosis was observed with carnosinol in obese WT and GPx4^{+/−} mice (Fig. 6E). Carnosinol also blunted fibrosis in the liver as indicated by picrosirius red staining (Fig. 6F). In order to determine if HFHS diet and/or carnosinol treatment altered collagen cross-linking in the tissue, the ratio of insoluble to soluble hydroxyproline quantity in liver extracts was measured. HFHS diet led to an increase in insoluble hydroxyproline fraction in WT and GPx4^{+/−} livers (Fig. 6G), and carnosinol normalized these levels. Indeed, carnosinol treatment was associated with significantly lower levels of collagen overall, including soluble hydroxyproline content (Fig. 6H), regardless of diet or genotype. This decrease in collagen content caused by carnosinol treatment in obese WT and GPx4^{+/−} mice does not appear to due to decreased collagen gene expression, as no effect of carnosinol on collagen-1 (Fig. 6I) or collagen-4 (not shown) mRNA was observed.

**Pharmacodynamics and reactivity of carnosinol in mouse models of obesity and in human serum**

In order to ascribe the beneficial effects of carnosinol in our obese mouse models to its aldehyde-scavenging capacity, *in vivo*, we developed a high resolution LC-MS based approach to measure free carnosinol and carnosinol-aldehyde adducts in biological material. Pooled samples of tissue from skeletal muscles, pancreas, liver, kidney and adipose tissue were analyzed using this method, and free carnosinol was found to be detectable only in liver, kidney and adipose tissue (Supplemental Table 5). Carnosinol levels in liver and kidney were lower in the HFHS fed GPx4^{+/−} mice compared with WT.

Importantly, the lower concentration of free carnosinol in these mice corresponded with a higher concentration of carnosinol-acrolein adducts in the liver, as compared with HFHS-fed WT mice treated with carnosinol (Fig. 7A). Specifically, a signal at 269.16082 (i.e. the expected m/z value for carnosinol-acrolein Michael adduct) was detectable at the same retention time observed in a reference sample prepared by spiking an aliquot of carnosinol-acrolein adduct in rat liver homogenate at a final concentration of 0.5 µM (bottom panel, Fig. 7A). The narrow mass range considered for extracting the chromatograms in Fig. 7A (i.e. 1 ppm mass tolerance) and the reproducible retention time if compared with a spiked sample is a conclusive evidence of the formation of a
carnosinol metabolite after supplementation. Tandem mass spectra of the signal at 269.16 from traces in Fig. 7A were able to confirm the structure of the putative adduct (Supplemental Figure 7). No signal of adducts with other reactive carbonyl species (e.g. HNE, HHE, malondialdehyde) were detectable in control or carnosine supplemented animals. This may be due to the instability of carnosinol-HNE adducts in liver homogenate, which we determined in separate experiments. Only 63.24±3.47% of an initial amount of 5 µM carnosinol-HNE conjugate was detectable after 2-hour incubation in liver homogenate, with an estimated half-life of 3 h, according to a first order decay model. This instability was not seen with carnosinol-acrolein adduct following incubation in liver homogenate.

To further characterize carnosinol reactivity and stability of aldehyde adducts, we tested the HNE-scavenging capacity of carnosinol in human serum by spiking the serum with a known concentration of pre-formed carnosinol-HNE, and also carnosinol alone. Using our high resolution LC-MS approach we found that not only is carnosinol-HNE detectable, but unlike in tissues, the adduct is highly stable in human serum, with >85% of a carnosinol-HNE adduct remaining after 2 hours incubation time (Fig. 7B and Supplemental Figure 8). Moreover, carnosinol can form adducts with trace amounts of HNE that are already present in human serum.
Discussion

An association between sugar- and lipid-derived RCS and obesity has been known for many years. The extent to which carbonyl stress plays a causal role in the metabolic disorders of obesity has remained unclear, however. The present study sought to investigate the pathological role of RCS in obesity with the rational design and pharmacological evaluation of carnosinol, a chemical analog of L-carnosine with high oral bioavailability and RCS-scavenging capacity. Following the design and characterization of carnosinol, we performed a comprehensive set of in vitro and in vivo studies profiling its effects across a range of experimental models, including rodent models of diet-induced obesity and metabolic syndrome. Our findings support the hypothesis that sugar- and lipid-derived RCS have a causal role in metabolic disorders associated with obesity. Moreover, we show that carnosinol represents a promising lead compound in a new class of RCS-scavenging agents derived from the histidyl dipeptide L-carnosine.

Recently, several important studies in experimental and clinical models have illustrated the unique pathological role of lipid-derived aldehydes, particularly HNE, to development of cardio-metabolic disease. A study in healthy lean men determined that one week of a high fat, high carbohydrate (i.e. hypercaloric) diet induced systemic insulin resistance and glucose intolerance. Importantly, the authors showed that HNE-modification of glucose transporter-4 (GLUT4) in adipocytes, on a residue near the glucose transport channel, played a role in diminished glucose uptake following the hypercaloric diet (20). Other studies have documented lipid peroxidation and HNE to be mediators of chronic inflammation and insulin resistance in adipose tissue (12,19,52) and skeletal muscle (42,43) with obesity. From a mechanistic standpoint, the most important outstanding question involve the temporality and tissue-dependency of the RCS. Specifically, it is still unclear how long the oxidative stress must persist after onset of caloric overload, in order for RCS pathogenicity to emerge. Moreover, it is still not clear whether RCS are equally toxic and pathogenic in every tissue. Considering the critical role of the liver, skeletal muscle, and adipose tissue in glucose disposal and intermediary metabolism, these organs represent the most obvious target for examination. For example, a likely explanation for the decrease in serum and liver triglycerides with carnosinol treatment in the rodent models is that this effect is secondary to the improvement in insulin sensitivity and glycemic control. It also is noteworthy that in the carnosinol-treated mice, the adipose tissue retains the highest concentration of the drug, behind liver and kidney. Although the adipose tissue was not a major focus of this study, much work over the past decade has documented the presence and pathogenicity of RCS in adipose tissue as it pertains to metabolic syndrome (12,19,52,53). Thus, the effect of carnosinol on RCS in the adipose tissue,
and subsequently the impact on metabolic parameters as a result, may be playing a significant role in the outcomes of the present study.

Though it is clear that lipid peroxidation and RCS do indeed have deleterious effects in the context of obesity, there are also complex time-, concentration- and tissue-dependent factors to consider. Studies in pancreatic β-cells have shown that short-term exposure to low levels of lipid peroxidation and subsequent 4-hydroxyalkenal formation stimulates an adaptive response mediated by peroxisome proliferator activated receptor δ (PPARδ), which causes increased glucose-stimulated insulin secretion (46,47). In a previous study, we found that numerous enzymes involved in redox buffering and fatty acid metabolism are enhanced in rat myocardium in parallel with lipid peroxidation following 12 weeks of HFHS diet (54). Such a ‘hormetic’ effect of oxidative stress has been documented by other groups using similar obese models (55). Certainly, much remains to be determined about the precise mechanisms and factors involved in lipid peroxidation and subsequent RCS with obesity.

A major determinant of lipid peroxidation now known to be intimately involved in regulating disease pathology is the expression and activity of the seleno-enzyme GPx4. As one of only three antioxidant enzymes essential for development (56), GPx4 has recently been the target of intense scrutiny by investigators. In particular, a critical role for GPx4 in regulating ferroptosis and subsequent organ failure has been reported (57-61). Furthermore, genetic variants of gpx4 that result in diminished activity and/or enzyme content are associated with obesity and cardiovascular disease in humans (62-64). We previously reported that GPx4-deficient (GPx4+/−) male mice acquire severe insulin resistance, steatohepatitis, and cardiomyopathy on HFHS diet, and that diabetic patients have diminished GPx4 content and elevated HNE-adduct levels in their hearts as compared with age-matched nondiabetic patients (15). In the present study, we used male GPx4+/− mice as a model to pharmacologically assess carnosinol in a context of enhanced lipid peroxidation induced by obesity. As shown in Figure 5, GPx4+/− mice have greater levels of HNE-adducts in skeletal muscle and pancreas following HFHS diet than WT. Carnosinol was effective at mitigating HNE-adducts in these organs from both WT and GPx4+/− mice, but only fully effective at mitigating insulin sensitivity and glucose tolerance in WT. A reason for this may be that the dose of carnosinol used in this study was insufficient for complete therapeutic effect in the GPx4+/− mice. Evidence supporting this conclusion is that there was a greater concentration of carnosinol-ACR adduct in HFHS-fed GPx4+/− livers (Fig. 7A), which corresponded with lower concentration of free carnosinol in liver and kidney of the HFHS-fed GPx4+/− mice (Supplemental Table 5). These findings are consistent with a greater ‘aldehyde load’ in the GPx4+/− mice on HFHS diet, and a correspondingly greater demand for carnosinol in these mice as compared with WT mice on a HFHS diet. Taken together, this is
compelling evidence that the therapeutic effect of carnosinol is mediated, at least in part, by its RCS-scavenging capacity.

Other compounds that scavenge RCS have shown significant therapeutic potential in pre-clinical studies using cardiovascular, renal and metabolic disease models. Of all those tested to date, aminoguanidine and members of the B vitamin family (pyridoxamine, thiamine) have shown the most promising therapeutic effects (29,65). However, in randomized controlled trials (RCTs) these compounds have yielded largely disappointing results (with exception of pyridoxamine), likely due to their promiscuous reactivity to all aldehydes (e.g., pyridoxal). Such off-target effects, particularly in the case of aminoguanidine (66), have made existing RCS-scavenging therapies unsuitable for mainstream clinical practice (67). These findings underscore the complexity of carbonyl biochemistry in physiological systems, and illustrate that in principle, agents that scavenge or block RCS do have clinical potential. Thus, development of novel RCS-scavenging therapies are warranted.

Recently, intervention studies based on a daily dose of L-carnosine (2 g/day for 12 weeks) in overweight and obese subjects resulted in preservation of insulin sensitivity and insulin secretion, and normalized glucose tolerance compared with placebo group. Furthermore, L-carnosine supplementation reduced 2-h insulin levels after OGTT in a subgroup of individuals with impaired glucose tolerance (68). Although L-carnosine was not detected in serum, it was easily detectable in the urine as a conjugate with acrolein (69). The in vivo RCS-scavenging capacity of L-carnosine was further confirmed by two independent studies reporting the presence of covalent adducts between carnosine and lipid peroxidation by-products (e.g. acrolein, HNE and HHE) in the urine of human volunteers (70,71).

Based on these translational studies it would seem that orally supplemented L-carnosine, despite its rapid serum degradation by carnosinases, provides a promising therapeutic effect in obese subjects which may be linked to a RCS-scavenging mechanism. However, several studies indicate that the beneficial effect of L-carnosine is strictly associated with its serum stability and bioavailability. An allelic variant of CN1 which results in increased enzyme activity (and consequently lower serum L-carnosine levels) is linked to incidence of diabetic nephropathy (72). Transgenic db/db mice overexpressing human CN1 and with reduced serum carnosine levels exhibit higher fasting plasma glucose and HbA1c levels, to the extent that the glucosuria in these mice causes significant reduction of body weight (73). It can be inferred from these studies that serum L-carnosine levels are directly linked to glycemic control, and human CN1 presents an attractive drug target for this patient population.

Two approaches were envisioned by our group which could circumvent the challenge posed by endogenous carnosinases: i) to inhibit these enzymes during
simultaneous oral L-carnosine therapy; and ii) to design stable carnosine peptide-mimetics resistant to carnosinases. The former approach was not pursued due to high potential for toxicity, as CN1 is critical for production of neurotransmitters, histidine metabolism, and other vital functions. To address the latter approach, we previously synthesized and characterized the carnosinase-resistant enantiomer D-carnosine, and found that this compound exhibited significant RCS-scavenging effect in vitro and in vivo, but had poor intestinal absorption due to low affinity and transport by PepT1 (74). Other groups have successfully made carnosine derivatives that are resistant to carnosinase which initially showed therapeutic potential, but failed in pre-clinical testing due largely to decreased absorption (75,76). An octylester derivative of D-carnosine showed enhanced intestinal absorption and therapeutic effects in a mouse model of cardio-metabolic disease (34), but translational applicability of this compound is low due to potency issues likely stemming from dosing limitations resulting from diminished absorption. Carnosinol is the most promising L-carnosine derivative that has been synthesized to this point. It is easily transportable by PepT1 and not metabolized by CN1 (Fig. 1), it maintains the outstanding safety profile of L-carnosine, and it was determined to be more reactive towards HNE, MGO and ACR as compared with L-carnosine (Tables 1, 2 and Supplemental Table 2). As such, it represents a promising lead compound for counteracting RCS, particularly lipid peroxidation-derived α,β-unsaturated aldehydes, which have recently come to the forefront as primary driving forces in chronic disease.

Conclusions/Translational Perspective

The consensus finding of these studies support the hypothesis that sugar- and lipid-derived RCS have a causal role in metabolic disorders associated with obesity, and further demonstrate that carnosinol represents a very promising lead compound in a new class of RCS-scavenging agents derived from the histidyl dipeptide L-carnosine.
Methods

A more detailed description of materials and methods used in this study can be found in the online Supplemental Materials.

Materials and Reagents

Solvents for HPLC and LC-MS and all the chemicals of analytical grade were purchased from Sigma–Aldrich (Milan, Italy). L-Carnosine and carnosinol were kind gifts provided by Renato Canevotti and Stefania Gagliardia of Flamma S.p.A. (Chignolo d’Isola, Italy). For molecular and biochemical endpoints, all reagents were from Sigma-Aldrich unless otherwise noted above.

ADMET studies

ADMET studies were performed by Nikem research (Milan, Italy) following standardized experimental protocols. In vivo studies were carried out in agreement with the Italian Law (D. L.vo 116/92). PK studies were performed by using fasted CD male rats (Charles River Laboratories, Calco, Italy). More details and descriptions of the ADMET studies are provided in the Supplemental Materials.

Rodent models of obesity/metabolic syndrome

Both rat and mouse models of diet-induced obesity were used in this study. Male Sprague-Dawley rats (Harlan Laboratories Inc., Udine, Italy) of 8 weeks of age and weighing 200 ± 20 g were used for the study. Rats were housed under constant environmental conditions, with standard laboratory rat chow or 60% high fructose diet obtained from Mucedola s.r.l., Settimo Milanese, Milan, Italy and tap water ad libitum. In particular, the control diet contained 60% corn starch (carbohydrates), 20% casein (protein), 0.3% methionine, 5% lard (fat), 8% cellulose, 5% mineral mixture and 1% vitamin mixture, and zinc carbonate 0.004%. The fructose diet contained all the ingredients except corn starch, which was replaced by an equal quantity of fructose. Animals were acclimatized for a period of at least seven days before the use. The study was approved by the Animal Ethics Committee of University of Milan, Italy and communicated to the Italian Ministry of Health, corresponding to article seven of the D.L. 116/92. Subsequently, rats were randomly divided into five groups. Control group (CTR group) received a standard rat chow diet for six weeks, whereas the other four groups were given fructose-enriched diet for six weeks. Three weeks after starting the fructose diet, two groups were treated with carnosinol at two doses: 10 and 45 mg/kg/day. An additional group was treated with 10 mg/Kg/day of rosiglitazone (Glaxo-Smith-Klein, Middlesex, UK), and the fifth group continued with fructose-enriched diet alone (Hi Fructose group, HF). RGZ was orally administered by gastric gavage to rats.
during the last three weeks of the study while carnosinol was dissolved in the water diet. During all the experiments, rats had free access to water and food.

For mouse models of diet-induced obesity, C57BL6/J female mice (Jackson Laboratory) were crossed to male GPx4+/− mice and pups were genotyped by polymerase chain reaction (PCR) using primers described in (15). At 8-12 weeks, WT and GPx4+/− male age-matched littersmates were randomly assigned to groups and individually housed. Mice were fed either control (CNTL-TD110367) or high fat-high sucrose (HFHS-TD110365) diet from Harlan-Teklad Laboratories (Madison, WI) ad libitum for 25 weeks. The composition of this diet was a special formulation consisting of mixed saturated and n-6 PUFA (44.6% kcal/g fat), with high sucrose (34% kcal/g) content (54). After 8 weeks of HFHS diet, half of the HFHS diet cohort (WT and GPx4+/−) were administered carnosinol (45 mg/kg/day) in their drinking water until study termination at 20 weeks. This dose was calculated based on a series of days where water consumption was meticulously recorded, and dose of carnosinol was maintained and adjusted to each mouse throughout duration of the study, according to its body weight.

Statistical analysis

Data from our in vitro analyses are presented as mean ± S.D. Physiological and biochemical data in rodent models are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism v.7 (GraphPad Prism, La Jolla, Ca.). For fructose-fed rat model, one way analysis-of-variance (ANOVA) was performed on continuous variables followed by Newman-Keuls post hoc test comparing all groups, with α< 0.05 considered statistically significant. For the HFHS mouse model experiments, a 2-way ANOVA analysis was used to assess genotype (WT vs. GPx4+/-) x treatment (Ctl diet vs. HFHS vs. HFHS & carnosinol), following by a Tukey’s multiple comparisons test. This allowed us to compare main effects of treatment within each genotype, and to ascertain if there were interactions with genotype + treatment. α< 0.05 was considered statistically significant.

Study Approval

All aspects of this study involving the care and use of laboratory animals received institutional approval in accordance with Italian law (D. L.vo 116/92), and with AAALAC guidelines in the United States. Studies in rat model were approved by the Animal Ethics Committee of University of Milan, Italy and communicated to the Italian Ministry of Health, corresponding to article seven of the D.L. 116/92. Mouse model studies were performed with approval from the institutional animal care and use committee of East Carolina University (Greenville, NC) and were in compliance with the National Institutes of Health’s Guide for Care and Use of Laboratory Animals.
**Author Contributions**

G.V., G.A. and E.J.A. had equivalent intellectual contribution to this manuscript, and are the guarantors of all the data contained herein. G.V. (*in silico* modeling) and G.A. (bioanalysis and testing) were principally responsible for design of Carnosinol. E.J.A. was principally responsible for overall study design, experimental model development and pharmacological testing of Carnosinol, and writing the manuscript. L.A.K., K.F., T.B.M., E.G., L.C., M.C., D.D.M., G.R. and M.C. designed and conducted experiments, and analyzed data, and assisted with manuscript preparation. L.R. and E.G. had a key role with LC-MS carnosinol-aldehyde adduct determination. R.C. and S.G. contributed to Carnosinol synthesis.

**Acknowledgments**

Carnosinol was provided by Flamma S.p.A. This project was supported by funds from MIUR (Ministry of Education, University and Research; PRIN 2009), from Regione Lombardia-MIUR (L.297–Art. 12/BioTech DM27909 and decree n. 6737 July, 2nd 2009), and from the National Institutes of Health grants R01HL122863 and R21AG057006 to E.J.A. Authors would like to thank Cherese Beatty and the other undergraduate students at East Carolina University who assisted with animal experiments and tissue histology. G.V. gratefully acknowledges Concetta De Stefano (University of Messina) for the support in determining the chelation constants.

**Conflict of Interest Disclosure**

Giancarlo Aldini, Giulio Vistoli, Marina Carini, and Marica Orioli of University of Milan, and Flamma S.p.A. are co-inventors of the patent “Amino alcohol derivatives and their therapeutic activities” (EP 2519507 B1, US 8623900 B2) which includes Carnosinol. Renato Canevotti and Stefania Gagliardi are employees of Flamma S.p.A., the manufacturer of Carnosinol.
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC (Q%±S.D.)</th>
<th>MS (Q%±S.D.)</th>
<th>Pyridoxal (Q%±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-carnosine</td>
<td>37.3 ± 2.3</td>
<td>25.4 ± 3.7</td>
<td>0.1 ± 0.9</td>
</tr>
<tr>
<td>Carnosinol</td>
<td>61.5 ±1.9</td>
<td>38.1± 4.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>2.1 ± 0.5</td>
<td>0.0 ± 3.4</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>87.2 ± 3.2</td>
<td>15.2 ± 3.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>2.3 ± 0.7</td>
<td>3.9 ± 3.2</td>
<td>12.8 ± 0.1</td>
</tr>
</tbody>
</table>

HPLC Q% values express the percentages of the target aldehyde reacted in the presence of the tested quenchers after 24 h incubation by using a molar ratio of quencher/aldehyde equal to 1:1 (HNE) or 10:1 (pyridoxal). MS Q% values express the percentage of inhibition of carbonylated ubiquitin induced by HNE after 24 h incubation and using a molar ratio of quencher/aldehyde equal to 1:1 (HNE).
Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>MGO</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC (Q%±S.D.)</td>
<td>MS (Q%±S.D.)</td>
</tr>
<tr>
<td>L-carnosine</td>
<td>12.8±1.6</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Carnosinol</td>
<td>36.4±1.6</td>
<td>13.6±2.1</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>2.5±1.4</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>48.6±4.2</td>
<td>14.2±2.0</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>34.7±2.7</td>
<td>11.3±2.0</td>
</tr>
</tbody>
</table>

HPLC Q% = fraction (% total) of aldehyde quenched after 24 hour incubation. MS Q% = % of carbonylated ubiquitin formation inhibited by the tested compounds. UC₅₀ = concentration required to inhibit MDA induced ubiquitin carbonylation by 50%. Carnosinol activity was compared with that of carnosine and of well-known RCS sequestering agents. MGO activity was evaluated by HPLC and MS assays while the dose-dependent activity towards MDA by MS. Data for the quenching activity of L-Carnosine, pyridoxamine, hydralazine and aminoguanidine towards MDA and MGO are from (30).
Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Hi Fructose</th>
<th>Hi Fruc + Carnosinol (10 mg/kg)</th>
<th>Hi Fruc + Rosiglitazone (10 mg/kg)</th>
<th>Hi Fruc + Carnosinol (45 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>13.24± 0.43</td>
<td>17.26± 0.87</td>
<td>16.47± 1.51</td>
<td>13.97± 0.67</td>
<td>14.81± 0.85</td>
</tr>
<tr>
<td>Liver weight (g/100 g body weight)</td>
<td>3.26± 0.07</td>
<td>3.85± 0.14</td>
<td>3.78± 0.28</td>
<td>3.37± 0.11</td>
<td>3.51± 0.13</td>
</tr>
<tr>
<td>Hepatic total lipids (mg/g liver)</td>
<td>27.4± 2.5</td>
<td>41.1± 3.0†</td>
<td>38.8± 3.9†</td>
<td>34.9± 3.7</td>
<td>31.9± 2.6</td>
</tr>
<tr>
<td>Hepatic triglycerides (µmol/g liver)</td>
<td>20.8± 2.2</td>
<td>43.6± 3.0†</td>
<td>38.4± 3.8†</td>
<td>30.3± 3.1§</td>
<td>28.5± 2.4§</td>
</tr>
<tr>
<td>Hepatic total cholesterol (µmol/g liver)</td>
<td>5.3± 0.5</td>
<td>9.8± 0.8†</td>
<td>9.1± 0.7†</td>
<td>7.5± 0.6</td>
<td>6.4± 0.5§</td>
</tr>
<tr>
<td>Right kidney weight (g)</td>
<td>1.26± 0.07</td>
<td>1.40± 0.06</td>
<td>1.36± 0.08</td>
<td>1.28± 0.09</td>
<td>1.30± 0.08</td>
</tr>
<tr>
<td>Left kidney weight (g)</td>
<td>1.23± 0.07</td>
<td>1.35± 0.06</td>
<td>1.32± 0.07</td>
<td>1.25± 0.07</td>
<td>1.27± 0.06</td>
</tr>
<tr>
<td>Urinary volume (ml/day)</td>
<td>11.9± 1.1</td>
<td>15.8± 1.5</td>
<td>11.5± 1.4</td>
<td>13.0± 1.7</td>
<td>10.6± 2.0</td>
</tr>
<tr>
<td>Urinary albumin (mg/day)</td>
<td>34.1± 3.1</td>
<td>297.8± 28.2†</td>
<td>265.4± 24.8†</td>
<td>87.4± 7.6†</td>
<td>102.1± 8.9†</td>
</tr>
<tr>
<td>Urinary protein (mg/day)</td>
<td>72.8± 6.0</td>
<td>378.6± 22.9†</td>
<td>320.4± 24.9†</td>
<td>153.4± 13.5†§</td>
<td>175.8± 11.0†§</td>
</tr>
<tr>
<td>Urinary creatinine (mg/day)</td>
<td>138.9± 9.9</td>
<td>98.4± 6.8†</td>
<td>108.8± 6.2</td>
<td>121.4± 9.5</td>
<td>132.6± 10.3§</td>
</tr>
<tr>
<td>Urinary 8-isoprostane (ng/day)</td>
<td>58.6± 5.0</td>
<td>149.4± 7.9†</td>
<td>130.2± 13.5†</td>
<td>136.5± 12.6†</td>
<td>108.4± 7.6†§</td>
</tr>
</tbody>
</table>

Data are shown as Mean ± S.E.M, from N=6 per group; †P<0.01 vs. Control Diet; §P<0.01 vs. Hi Fructose; *P<0.01 vs. Hi Fruc + Car (10 mg/kg)
References


Figure 1: In silico models and biochemistry of carnosinol metabolism. A) Structure-activity relationships for L-carnosine and comparison with carnosinol. B) The putative complex between carnosinol and CN1, highlighting the missing ion pair with Arg350 when compared to the corresponding complex for L-carnosine. C) The putative complex between carnosinol and hPepT1, revealing that the inserted hydroxyl group elicits H-bonds similar to that already observed for L-carnosine.
Figure 2: Carnosinol is a selective sequestering agent of HNE. A) Proposed reaction mechanism of carnosinol (m/z 213) with HNE (M.W. 156). Carnosinol, like L-carnosine, reacts with HNE through a two-step mechanism. The reaction starts with the formation of a reversible Schiff base (an α,β-unsaturated imine, CI) to yield the macrocyclic adduct through an intramolecular Michael addition, which hydrolyzes to form the stable hemiacetal derivative CII. B) mass spectrum of the reaction mixture of carnosinol with HNE incubated for 24 h at 37°C characterized by the peaks at m/z 213 attributed to the carnosinol, m/z 351 attributed to the Schiff Base (CI) and m/z 369 attributed to Michael adduct (CII).
Figure 3: Carnosinol inhibits HNE-induced ubiquitin carbonylation. A) MS spectrum of ubiquitin control sample focusing on the z=11 multicharged ion peak at m/z=779; B) 11+ peaks obtained upon incubating ubiquitin with HNE, corresponding to unmodified (m/z=779) and HNE-modified (m/z=793) forms of the protein; C and D) 11+ peaks obtained upon incubating ubiquitin with HNE and carnosinol at two different molar ratios (HNE:carnosinol 1:0.5 mol: mol, panel C; and 1:1 mol: mol, panel D) showing reduced intensity for the peak corresponding to the modified protein (m/z=793).
Figure 4. Dose-dependent mitigation of inflammation and metabolic disease parameters by carnosinol in high-fructose fed rats. Shown in (A) is the study design for high fructose (HF)-induced metabolic disease in a rat model. The effect of either HF diet alone, or combined with low (10 mg/kg) or high (45 mg/kg) dose carnosinol and rosiglitazone in drinking water is shown on overall change in body weight (B), levels of serum AGEs (C), TNFα (D), IL-6 (E), C-reactive Protein (F), triglycerides (G), cholesterol (H), glucose (I), and insulin (J). Serum ALT & AST (K), along with liver triglycerides (L) and liver cholesterol (M) are also shown. †P<0.01 vs. Control Diet; §P<0.01 vs. HF diet alone; *P<0.01 vs. HF+ Car (10 mg/kg) with one-way ANOVA, using a Newman-Keuls post hoc test for multiple comparisons (N=6).
Figure 5. Therapeutic effect of carnosinol on metabolic homeostasis and carbonyl stress in mouse models of diet-induced obesity. Effect of HFHS diet with and without carnosinol treatment is shown for glucose tolerance (A-C, N=8) and insulin sensitivity of EDL (D) and soleus muscle (E) at termination of study for each group (N=6). Representative immunoblots for HNE-adducts in whole tissue homogenates prepared from mixed gastrocnemius skeletal muscle (F) and pancreas (H) are shown for n=3 mice in each group, along with the corresponding densitometry (G, I). †P<0.01 vs. Control Diet within each respective genotype. A 2-way ANOVA followed by Tukey’s multiple comparisons test was used to test for main effect of treatment within each genotype.
Figure 6. Carnosinol effect on liver inflammation and steatosis in mouse models of diet-induced obesity. Expression of pro-inflammatory genes RAGE (A), TNFα (B), and IL-6 (C) in the livers of the mice from each treatment group are shown using quantitative RT-PCR. Representative images (100X) showing liver histology in mice from each treatment group are shown using H & E (D), oil red O staining of triglycerides (E), and picrosirius red under polarized light for collagen/fibrosis (F). Both insoluble (G) and soluble (H) forms of liver hydroxyproline is quantified, along with expression of collagen 1a1 using qRT-PCR (H). Quantified data are shown as mean ± S.E.M., N=6 in each group. †P<0.01 vs. Control Diet within each respective genotype. A 2-way ANOVA followed by Tukey’s multiple comparisons test was used to test for main effect of treatment within each genotype.
Figure 7. Pharmacodynamics and aldehyde-scavenging capacity of Carnosinol. LC-MS analyses were performed on pooled liver homogenates prepared from WT and GPx4<sup>−/−</sup> mice in each of the treatment groups. Single ion chromatograms of carnosinol-acrolein adduct (269.16082 m/z, ±1ppm) are shown in (A). The bottom trace in panel A is the reference standard used in this analysis prepared by spiking rat liver homogenate with 0.5 µM carnosinol-acrolein adduct. Shown in (B) is LC-MS analysis showing the single ion chromatogram of carnosinol-HNE adducts in human serum spiked with carnosinol & HNE (top panel), carnosinol only (middle panel), and serum only. The green box value denotes the corresponding concentration of these adducts.