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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, partly because of their highly diffuse and broad reactivity and the lack of specific RCS-scavenging therapies. Naturally occurring histidine dipeptides (e.g., anserine and carnosine) show 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 (absorption, distribution, metabolism, excretion, and toxicity) profile and was determined to have the greatest potency and selectivity toward α,β-unsaturated aldehydes (e.g., 4-hydroxynonenal, HNE, ACR) among all others reported thus far. 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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Ethan J. Anderson,1,2 Giulio Vistoli,1 Lalage A. Katunga,2 Katsuhiko Funai,4 Luca Regazzoni,3 T. Blake Monroe,1,2 Ettore Gilardoni,3 Luca Cannizzaro,3 Mara Colzani,1 Danilo De Maddis,1 Giuseppe Rossoni,5 Renato Canevotti,6 Stefania Gagliardi,6 Marina Carini,3 and Giancarlo Aldini3

1Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, Iowa, USA. 2Department of Pharmacology and Toxicology, East Carolina University, Greenville, North Carolina, USA. 3Department of Pharmaceutical Sciences, University of Milan, Milan, Italy. 4Diabetes and Metabolism Research Center, University of Utah, Salt Lake City, Utah, USA. 5Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy. 6Flamma S.p.A., Chignolo d’Isola, Bergamo, Italy.

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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 (reactive carbonyl species [RCS]) are spontaneously formed during the preparation of high-fat/ high-sugar–containing foods under high heat (1) and are formed in vivo as a byproduct of oxidative stress. These RCS alter 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 breakdown products, lipid-derived aldehydes are another significant source of carbonyl stress in vivo. Individuals who consume diets rich in vegetable and corn oil have very high endogenous levels of n-6 polyunsaturated fatty acids (PUFAs). The α,β-unsaturated carbonyls derived from n-6 PUFA oxidation have particularly diverse biological effects. Of these, 4-hydroxynonalen (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). The 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 by-products 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 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 analogs, is a potent endogenous scavenger of RCS and 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 the clinical applicability of l-carnosine as an oral drug therapy, however. The largest of these is that high serum and tissue carnosinase activity in humans abrogates the 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 propionylamino)-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 that characterize a drug-like molecule (i.e., chemical and metabolic stability, bioavailability, and safety), our a priori rationale was that an RCS-sequestering therapeutic compound should: (a) be stable in plasma; (b) effectively scavenge (i.e., trap) circulating RCS; and (c) be highly reactive and selective toward damaging RCS (29). l-carnosine fulfills some of the above-mentioned requirements (30). However, it lacks an important basic requirement to be a suitable RCS-sequestrating agent, because it is unstable in the circulation as a result of 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 toward HNE, while preserving its selectivity; (b) maintain the active transport by human H+/peptide cotransporter-1 (hPepT1); and (c) eliminate 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 (Figure 1B); and (c) has a marginal role in hPepT1 transport (Figure 1C). Reduction of the carboxyl group has only a modest impact on the PepT1 interaction (Figure 1C), since both the carboxyl and 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 (Figure 1B), and its reduction to the hydroxyl function has a dramatic impact on the stability of the CN1-carnosinol complex, as confirmed by the reported drop in the interaction energy (–22.770 vs. –14.862 kcal/mol).

Given these modeling results, the carboxyl reduction to yield carnosinol should meet all the above requirements and should
toward transition metal ions such as Cu²⁺, which are roughly com-
monly affect the ionization constant or the chelating activity
JCI94307DS1). Furthermore, carboxyl reduction did not sig-
nificant by lipophilic RCS (Supplemental Table 1; supplemental
material available online with this article; https://doi.org/10.1172/
JCI94307DS1). The proposed reaction mecha-
nism (Figure 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 (CI) (Figure 2B).
The mechanism of carnosinol reaction toward
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The increased potency of carnosinol com-
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stereoelectronic parameters as predicted by quantum mechan-
ic simulations (Supplemental 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 catalyz-
es the formation of the Michael adduct.

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 that was recent-
ly 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
carboxylation specifically involves Lys6 and His68. Shown in Figure
3 is the MS spectrum of ubiquitin incubated in the absence and pres-
ence of HNE, inducing the formation of an HNE Michael adduct
characterized by a molecular weight (MW) shifted by 156 kDa with
respect to native ubiquitin. Carnosinol dose-dependently inhibited
ubiquitin carboxylation. The greater activity with respect to hydral-
azine when measured via HPLC as compared with MS can be easily
explained by considering that hydralazine forms a reversible Schiff
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The percentage of inhibition of HNE-induced ubiquitin carboxy-
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reported in Table 1. Note again that hydralazine depletes pyridoxal
levels completely, a feature of this drug that undoubtedly contrib-
utes to the adverse risk profiles related to vitamin B deficiency.

We then used both HPLC and MS to test carnosinol seques-
tering activity toward MGO and MDA (Supplemental Figures 2
and 3 and Table 2). We found carnosinol to be 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 methylglyoxal-
lysin dimer –like (MOLD-like) structure was identified by MS as
the major reaction product between carnosinol and MGO (Sup-
lemental Figure 2). The sequestering activity toward MDA was
found superimposable with respect to carnosine, and the N-prope-

Carnosinol is a selective and potent RCS-sequestering agent

We first performed in vitro testing of the sequestering activity of
carnosinol toward the most widely studied RCS involved in oxi-
dative-based diseases and belonging to the chemical classes of
α,β-unsaturated aldehydes, i.e., 4-hydroxynonenal (HNE), ACR
dialdehydes, i.e., malondialdehyde (MDA) and glyoxal (GO), and
ketoaldehydes, i.e., MGO.

Sequestering of HNE. The sequestering activity of carnosinol
toward HNE and ACR was first evaluated and compared with
L-carnosine and for other small molecules known to possess sim-
ilar properties (32). We directly compared carnosinol reactivity
toward ACR and HNE with the reactivity of L-carnosine toward
these aldehydes. Both compounds effectively reduced free alde-
hyde levels via conjugate formation over time, but interestingly,
carnosinol showed a more rapid depletion of both ACR and HNE
during the incubation compared with L-carnosine (Supplemental Table 2).
We then determined selectivity 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 hours and consider a quencher/RCS ratio of 1:1 for
HNE and 10:1 for pyridoxal (30). Carnosinol was the most reac-
tive compound with respect to L-carnosine and other well-known
RCS-sequestering agents such as aminoguanidine and pyridox-
amine. We found carnosinol to be less effective only by comparison with hydralazine, a commonly
prescribed antihypertensive medication with non-
selective RCS reactivity, as shown by its effects
with pyridoxal. In contrast, we found that carnos-
ol did not react with pyridoxal, and hence it can
be considered a selective RCS-sequestering agent,
like the parent compound L-carnosine.

We first performed in vitro testing of the sequestering activity of
carnosinol toward a 24-hour incubation, with a molar ratio of quencher/aldehyde equal to 1:1 (HNE).

The proposed reaction mechanism (Figure 2A) is similar to that already clarified
for L-carnosine and based on the formation of an
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<td>MS (Q% ± SD)</td>
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<tr>
<td>l-carnosine</td>
<td>37.3 ± 2.3</td>
<td>25.4 ± 3.7</td>
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<td>Carnosinol</td>
<td>61.5 ± 1.9</td>
<td>38.1 ± 4.0</td>
</tr>
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<td>Pyridoxamine</td>
<td>2.1 ± 0.5</td>
<td>0.0 ± 3.4</td>
</tr>
<tr>
<td>Hydralazine</td>
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<td>15.2 ± 3.3</td>
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<td>Aminoguanidine</td>
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HPLC Q% values express the percentages of the reacted target aldehyde in the presence of the tested quenchers after a 24-hour incubation, with 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 a 24-hour incubation, with a molar ratio of quencher/aldehyde equal to 1:1 (HNE).

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 reduces atherogenesis (27, 34, 35), dyslipidemia, and renal dys-
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cardiometabolic and inflammatory parameters using a short-term
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Total food and water intake was similar between the groups. Sys-
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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, carnosin-
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4D, E–F) in fructose-fed rats. Importantly, the beneficial effects
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Dose–dependent effects of carnosinol on systemic inflammation and
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Prior studies by our group and others have demonstrated that
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glycerides and cholesterol (Figure 4, G and H), improved glycemic control (Figure 4, I and J), and mitigated liver toxicity (Figure 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 (Figure 4, L and M), kidney, and liver of HNE adducts (Supplemental Figure 4). Liver and renal fibrosis were not significantly affected by fructose feeding or drug treatment (data not shown), although the urinary creatinine clearance rate decreased 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 selenoenzyme that exclusively neutralizes lipid peroxides in membranes and lipoproteins (37). Homozygous null GPx4 mice die around gestational day 7, underscoring the critical role for this enzyme in development. GPx4-haploinsufficient (GPx4+/−) mice have approximately 40% of the WT 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 displayed exacerbated metabolic derangements and cardiomyopathy when fed an n-6 PUFA-enriched, high-fat/high-sucrose (HFHS) diet (15). These cardiometabolic 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 have higher levels of HNE adducts and lower GPx4 enzyme levels in their myocardial tissue compared with nondiabetic patients (15), a finding that corroborates previous observations concerning HNE adducts in diabetic patients (12–14, 16). Thus, GPx4+/− mice are ideal for the 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 the drinking water) in a cohort of HFHS diet–induced obese WT mice. Given our previous findings in GPx4+/− mice on a HFHS diet, a cohort of GPx4+/− mice was used in parallel with WT mice to allow for assessment of carnosinol in a clinically relevant, translational model of severe carbonyl stress. Drug was admin-

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC (Q% ± SD)</th>
<th>MS (Q% ± SD)</th>
<th>MDA UC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-carnosine</td>
<td>12.8 ± 1.6</td>
<td>41.1 ± 0.6</td>
<td>4 ± 0.4</td>
</tr>
<tr>
<td>Carnosinol</td>
<td>36.4 ± 1.6</td>
<td>13.0 ± 2.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>2.5 ± 1.4</td>
<td>0.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>48.6 ± 4.2</td>
<td>14.2 ± 2.0</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>34.7 ± 2.7</td>
<td>11.3 ± 2.0</td>
<td>9.4 ± 0.7</td>
</tr>
</tbody>
</table>

HPLC Q%, fraction (percentage of total) of aldehyde quenched after a 24-hour incubation; MS Q%, percentage of carbonylated ubiquitin formation inhibited by the tested compounds; UC_{50}, 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 and the dose-dependent activity toward MDA by MS. Data for the quenching activity of l-carnosine, pyridoxamine, hydralazine, and aminoguanidine toward MDA and MGO are from ref. 30.
istered to mice starting after 8 weeks on the HFHS diet, and a control group was fed normal chow for the duration of the study (Supplemental Figure 5A). As expected, the HFHS diet increased body weights and adiposity in both WT and GPx4 +/– mice (Supplemental Figure 5, B and C), and carnosinol had no effect on these parameters, although fasting serum triglyceride and cholesterol levels were decreased in the carnosinol-treated mice (Supplemental Table 4). We detected no effect of carnosinol on whole-body energy expenditure (Supplemental Figure 6) or food or water intake (data not shown) in mice compared with those on a HFHS diet alone. Carnosinol treatment led to enhanced glucose disposal following oral glucose challenge in obese WT, but not GPx4 +/–, mice (Figure 5, A–C). This improved glucose disposal may be attributed in part to increased skeletal muscle insulin sensitivity, as carnosinol normalized insulin-stimulated 2-deoxyglucose (2-DG) uptake in extensor digitorum longus (EDL) to levels similar to those in control-fed lean mice (Figure 5D), although this effect was not seen in soleus (Figure 5E). We observed no improvement in insulin sensitivity with carnosinol in soleus tissue from obese WT mice, although we observed a modest improvement in obese GPx4 +/– soleus (Figure 5F). Reactive aldehyde derivatives of lipid peroxidation have recently come into focus as novel redox signaling agents that, paradoxically, have beneficial and pathological roles, depending on the concentration and tissues and 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

| Figure 4. Dose-dependent mitigation of inflammation and metabolic disease parameters by carnosinol in HF diet-fed rats. (A) Study design for HF diet–induced metabolic disease in a rat model. The effect of either a HF diet alone or in combination with low-dose (10 mg/kg) or high-dose (45 mg/kg) carnosinol or rosiglitazone in the drinking water on overall changes in body weight (B) and the levels of serum AGEs (C), TNF-α (D), IL-6 (E), C-reactive protein (F), triglycerides (G), cholesterol (H), glucose (I), and insulin (J). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (K), along with liver triglycerides (L) and liver cholesterol (M) levels, are also shown. †P < 0.01 versus control diet; ‡P < 0.01 versus HF diet alone; *P < 0.01 versus HF plus carnosinol (10 mg/kg); 1-way ANOVA using a Newman-Keuls post hoc test for multiple comparisons (n = 6). |
via HNE adduct formation (43, 44). Lipid peroxidation and HNE adduct formation have also emerged as potential causal factors in hyperinsulinemia and the 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 (Figure 5, F and G) and pancreas (Figure 5, H and I) increased with a HFHS diet, particularly in GPx4+/– mice. Carnosinol effectively mitigated the accumulation of these adducts in both WT and GPx4+/– mice on a HFHS diet, suggesting that this may be one aspect of the mechanism by which carnosinol improves systemic glycemic control in obese mice.

Liver inflammation and fibrosis are mitigated by carnosinol in mouse models of severe carboxyl stress--and diet-induced obesity
Nonalcoholic steatohepatitis (NASH) is among a cluster of obesity-related pathologies and is closely linked with insulin resistance. NASH is distinct from fatty liver disease, in that intralobular inflammation and fibrosis are 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 proinflammatory 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 tissue, 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 on a HFHS diet, but carnosinol significantly decreased RAGE expression in both groups (Figure 6A). Expression of the proinflammatory cytokines TNF-α and IL-6 was significantly higher in the livers of HFHS-fed mice, and carnosinol mitigated the expression of TNF-α, but not IL-6, in this tissue (Figure 6, B and 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, we observed that the HFHS diet increased liver triglyceride content in WT and GPx4+/– mice, with substantially greater lipid deposition occurring in the obese GPx4+/– mice (Figure 6, D and E). Interestingly, we found that the increase in liver triglyceride deposition with a HFHS diet was not accompanied by changes in serum triglycerides (Supplemental Table 4), although this could be attributable to necropsy being performed while the mice were in a fasted state. Although total triglyceride content in liver did not significantly change with carnosinol treatment in both WT and GPx4+/– mice on a HFHS diet, suggesting that this may be one aspect of the mechanism by which carnosinol improves systemic glycemic control in obese mice.

Table 3. Hepatic and renal parameters in a HF-fed rat model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control diet</th>
<th>HF</th>
<th>HF + carnosinol (10 mg/kg)</th>
<th>HF + rosiglitazone (10 mg/kg)</th>
<th>HF + 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>43.6 ± 3.0a</td>
<td>38.8 ± 3.9a</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 ± 3.0a</td>
<td>38.4 ± 3.8a</td>
<td>30.3 ± 3.1a</td>
<td>28.5 ± 2.4a</td>
</tr>
<tr>
<td>Hepatic total cholesterol (μmol/g liver)</td>
<td>5.3 ± 0.5</td>
<td>9.8 ± 0.8a</td>
<td>9.1 ± 0.7a</td>
<td>7.5 ± 0.6</td>
<td>6.4 ± 0.57</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 ± 28.2a</td>
<td>265.4 ± 24.8a</td>
<td>87.4 ± 7.6a</td>
<td>102.1 ± 8.9a</td>
</tr>
<tr>
<td>Urinary protein (mg/day)</td>
<td>72.8 ± 6.0</td>
<td>378.6 ± 22.9a</td>
<td>320.4 ± 24.9a</td>
<td>153.4 ± 13.5a</td>
<td>175.8 ± 11.0a</td>
</tr>
<tr>
<td>Urinary creatinine (mg/day)</td>
<td>138.9 ± 9.9</td>
<td>98.4 ± 6.8a</td>
<td>108.8 ± 6.2</td>
<td>121.4 ± 9.5</td>
<td>132.6 ± 10.3a</td>
</tr>
<tr>
<td>Urinary 8-isoprostane (ng/day)</td>
<td>58.6 ± 5.0</td>
<td>149.4 ± 7.9a</td>
<td>130.2 ± 13.5a</td>
<td>136.5 ± 12.6a</td>
<td>108.4 ± 7.6a</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM (n = 6 per group). *P < 0.01 versus control diet; **P < 0.01 versus HF.
Tandem mass spectra of the signal at 269.16 from traces in Figure 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 a 2-hour incubation in liver homogenate, with an estimated half-life of 3 hours according to a first-order decay model. We did not observe this instability with the carnosinol-ACR adduct in liver homogenate.

To further characterize carnosinol reactivity and the stability of aldehyde adducts, we tested the HNE-scavenging capacity of carnosinol in human serum by spiking the serum with a known Table 5. We found that carnosinol levels in liver and kidney were lower in the HFHS-fed GPx4+/- mice than in the WT mice. Importantly, the lower concentration of free carnosinol in these mice corresponded with a higher concentration of carnosinol-ACR adducts in the liver as compared with concentrations in HFHS-fed WT mice treated with carnosinol (Figure 7A). Specifically, a signal at 269.16082 (i.e., the expected m/z value for the carnosinol-ACR Michael adduct) was detectable at the same retention time observed in a reference sample prepared by spiking an aliquot of carnosinol-ACR adduct in rat liver homogenate at a final concentration of 0.5 μM (Figure 7A, bottom). The narrow mass range considered for extracting the chromatograms in Figure 7A (i.e., 1 ppm mass tolerance) and the reproducible retention time if compared with a spiked sample are conclusive evidence of the formation of a carnosinol metabolite after supplementation.

Figure 5. Therapeutic effect of carnosinol on metabolic homeostasis and carbonyl stress in mouse models of diet-induced obesity. The effect of a 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) tissue upon termination of the 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) tissue (n = 3 mice per group), along with the corresponding densitometric analysis (G and I). *P < 0.01 versus control diet within each respective genotype. A 2-way ANOVA followed by Tukey’s multiple comparisons test was used to test for the main effect of the treatment within each genotype.

tal Table 5). We found that carnosinol levels in liver and kidney were lower in the HFHS-fed GPx4+/- mice than in the WT mice.

Importantly, the lower concentration of free carnosinol in these mice corresponded with a higher concentration of carnosinol-ACR adducts in the liver as compared with concentrations in HFHS-fed WT mice treated with carnosinol (Figure 7A). Specifically, a signal at 269.16082 (i.e., the expected m/z value for the carnosinol-ACR Michael adduct) was detectable at the same retention time observed in a reference sample prepared by spiking an aliquot of carnosinol-ACR adduct in rat liver homogenate at a final concentration of 0.5 μM (Figure 7A, bottom). The narrow mass range considered for extracting the chromatograms in Figure 7A (i.e., 1 ppm mass tolerance) and the reproducible retention time if compared with a spiked sample are conclusive evidence of the formation of a carnosinol metabolite after supplementation.
The concentration of preformed carnosinol and HNE as well as with carnosinol alone. Using our high-resolution LC-MS approach, we found that not only was carnosinol-HNE detectable, but unlike in tissues, the adduct was highly stable in human serum, with more than 85% of the carnosinol-HNE adduct remaining after a 2-hour incubation (Figure 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 carbon-yl stress plays a causal role in the metabolic disorders of obesity has remained unclear, however. In the present study, we sought to investigate the pathological role of RCS in obesity with a rational design and pharmacological evaluation of carnosinol, a chemical analog of l-carnosine with high oral bioavailability and a 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

Figure 6. Carnosinol effect on liver inflammation and steatosis in mouse models of diet-induced obesity. Expression of the proinflammatory genes RAGE (A), TNF-α (B), and IL-6 (C) in the livers of mice from each treatment group was determined by quantitative reverse transcription PCR (qRT-PCR). Representative images of liver histology showing H&E staining (D), oil red O staining of triglycerides (E), and Picrosirius red staining under polarized light for collagen/fibrosis (F) in mice from each treatment group. Original magnification, ×100; scale bars: 10 μm. Both insoluble (G) and soluble (H) forms of liver hydroxyproline were quantified, along with expression of collagen 1a1 (Col1a1), determined by qRT-PCR (I). Quantified data are shown as the mean ± SEM (n = 6/group). †P < 0.01 versus control diet for each respective genotype. A 2-way ANOVA followed by Tukey’s multiple comparisons test was used to test for the main effect of treatment within each genotype.
represents a promising lead compound in a new class of RCS-scavenging agents derived from the histidyl dipeptide l-carnosine.

Recently, several important studies involving experimental and clinical models have illustrated the unique pathological role of lipid-derived aldehydes, particularly HNE, in the development of cardiometabolic disease. A study in healthy lean men determined that 1 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 shown lipid peroxidation and HNE to be mediators of chronic inflammation and insulin resistance in adipose tissue (12, 19, 52) and skeletal muscle (42, 43) in obesity. From a mechanistic standpoint, the most important outstanding question involves the temporality and tissue dependency of RCS. Specifically, it is still unclear how long the oxidative stress must persist after the 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 is also noteworthy that in the carnosinol-treated mice, adipose tissue retains the highest concentration of the drug, behind liver and kidney tissue. Although 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 adipose tissue, and the resulting impact on metabolic parameters, may have played 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 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 a 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 in obesity.

A major determinant of lipid peroxidation now known to be intimately involved in regulating disease pathology is the expression and activity of the selenoenzyme GPx4. As 1 of only 3 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 a HFHS diet and that diabetic patients have diminished GPx4 content and elevated HNE adduct levels in their heart tissue compared with levels in age-matched nondiabet-
increased enzyme activity (and, consequently, lower serum l-carnosine levels) is linked to diabetic nephropathy (72). Transgenic db/db mice overexpressing human CN1 and having reduced serum carnosine levels exhibit higher fasting plasma glucose and HbA1c levels, to the extent that the glucosuria in these mice causes a 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 that human CN1 presents an attractive drug target for this patient population.

Our group envisioned 2 approaches that could circumvent the challenge posed by endogenous carnosinas: (a) inhibition of these enzymes during simultaneous oral l-carnosine therapy; and (b) design of stable carnosine peptide mimetics resistant to carnosinas. The former approach was not pursued because of a high potential for toxicity, as CN1 is critical for neurotransmitter production, 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 a significant RCS-scavenging effect in vitro and in vivo, but had poor intestinal absorption due to a low affinity for and transport by PepT1 (74). Other groups have successfully made carnosine derivatives that are resistant to carnosinase and initially showed therapeutic potential, but failed in preclinical testing largely because of decreased absorption (75, 76). An octylester derivative of D-carnosine showed enhanced intestinal absorption and therapeutic effects in a mouse model of cardiometabolic disease (34), but the translational applicability of this compound is low because of potency issues that likely stem from dosing limitations resulting from diminished absorption. Carnosinol is the most promising L-carnosine derivative that has been synthesized at this point. It is easily transportable by PepT1 and not metabolized by CN1 (Figure 1), it maintains the outstanding safety profile of L-carnosine, and it was determined to be more reactive toward HNE, MGO, and ACR compared with L-carnosine (Tables 1 and 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 and translational perspective. The present study supports the hypothesis that sugar- and lipid-derived RCS have a causal role in metabolic disorders associated with obesity and further demonstrates 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 the materials and methods used in this study can be found in the supplemental material.

Materials and reagents. Solvents for HPLC and LC-MS and all analytical-grade chemicals were purchased from Sigma-Aldrich. L-carnosine and carnosinol were gifts of Renato Canevotti and Stefania Gagliardia (Flamma S.p.A.). For molecular and biochemical endpoints, analytical-grade chemicals were purchased from Sigma-Aldrich. L-carnosine and carnosinol were gifts of Renato Canevotti and Stefania Gagliardia (Flamma S.p.A.). For molecular and biochemical endpoints, analytical-grade chemicals were purchased from Sigma-Aldrich.
Rodent models of obesity and metabolic syndrome. Both rat and mouse models of diet-induced obesity were used in this study. Eight-week-old male Sprague-Dawley rats (Harlan Laboratories) weighing 200 ± 20 g were used for this study. Rats were housed under constant environmental conditions and were fed standard laboratory rat chow or a 60% HF diet (Mucedola S.R.L.) and tap water ad libitum. In particular, the control diet contained 60% corn starch (carbohydrates), 20% casein (protein), 0.5% 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 7 days before use in the study. Subsequently, rats were randomly divided into 5 groups. The control group received a standard rat chow diet for 6 weeks, whereas the other 4 groups of mice were given a fructose-enriched diet for 6 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 rosiglitazone (GlaxoSmithKlein), and the fifth group continued with the fructose-enriched diet alone (HF group). Rosiglitazone was orally administered to rats by gastric gavage during the last 3 weeks of the study, while carnosinol was dissolved in the water. During all the experiments, rats had ad libitum access to food and water.

For mouse models of diet-induced obesity, C57BL6/J female mice (The Jackson Laboratory) were crossed with male Gpx4 +/– mice, and the pups were genotyped by PCR using previously described primers (15). At 8 to 12 weeks of age, WT and Gpx4 +/– male age-matched littermates were randomly assigned to groups and individually housed. Mice were fed either a control (TD110367) or a HFHS (TD110365) diet from Harlan-Teklad Laboratories 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 a high-sucrose (34% kcal/g) content (54). After 8 weeks of the HFHS diet, half of the mice in 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 on the basis of a series of days on which water consumption was meticulously recorded, and the dose of carnosinol was maintained and adjusted according to the body weight of each mouse throughout the duration of the study.

Statistics. Data from our in vitro analyses are presented as the mean ± SD. Physiological and biochemical data for the rodent models are presented as the mean ± SEM. Statistical analysis was performed using GraphPad Prism, version 7 (GraphPad Software). For the fructose-fed rat model, 1-way ANOVA was performed on continuous variables followed by a Newman-Keuls post hoc test comparing all groups, with a P value of less than 0.05 considered statistically significant. For the HFHS mouse model experiments, a 2-way ANOVA was used to assess genotype (WT vs. Gpx4 +/–) and treatment (control diet vs. HFHS vs. HFHS and carnosinol), following by a Tukey’s multiple comparisons test. This allowed us to compare the main effects of the treatment within each genotype and to ascertain whether there were interactions between the genotype and the treatment. A P value of less than 0.05 was considered statistically significant.

Study approval. All in vivo studies were performed in accordance with Italian law (D.L.vo 116/92). All aspects of this study involving the care and use of laboratory animals received IACUC approval from the University of Milan in accordance with Italian law (D. L.vo 116/92) and with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in the United States. Studies in rat models were approved by the animal ethics committee of the University of Milan and communicated to the Italian Ministry of Health, corresponding to article seven of D.L. 116/92. Studies using mouse models were performed with the approval of the IACUC of East Carolina University, in compliance with NIH guidelines for the care and use of laboratory animals.

Author contributions
GV, GA, and EJA made equivalent intellectual contributions to this manuscript and are the guarantors of all the data contained herein. GV (in silico modeling) and GA (bioanalysis and testing) were principally responsible for the design of carnosinol. EJA was principally responsible for the overall study design, experimental model development, and pharmacological testing of carnosinol and for writing the manuscript. LAK, KF, TBM, LR, EG, LC, M. Colzani, DDM, GR, and M. Carini designed and conducted experiments, analyzed data, and assisted with manuscript preparation. LR and EG designed and optimized the assay for LC-MS carnosinol-aldehyde adduct determination. RC and SG contributed to carnosinol synthesis.

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Address correspondence to: Ethan J. Anderson, Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, Fraternal Order of Eagles Diabetes Research Center, University of Iowa, 115 S. Grand Avenue, Iowa City, Iowa 52242, USA. Phone: 319.335.8157; Email: ethan-anderson@uiowa.edu. Or to: Giancarlo Aldini, Department of Pharmaceutical Sciences, University of Milan, Via Mangiagalli, 25, 20133, Milan, Italy. Email: giancarlo.aldini@unimi.it.


