Dietary supplementation with essential amino acids boosts the beneficial effects of rosuvastatin on mouse kidney

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Abstract The effects of high-potency statins on renal function are controversial. To address the impact of statins on renal morpho-functional aspects, normotensive young mice were treated with rosuvastatin (Rvs). Moreover, because statins may impair mitochondrial function, mice received either dietary supplementation with an amino acid mixture enriched in essential amino acids (EAAm), which we previously demonstrated to increase mitochondrial biogenesis in muscle or an un-supplemented control diet for 1 month. Mitochondrial biogenesis and function, apoptosis, and insulin signaling pathway events were studied, primarily in cortical proximal tubules. By electron microscopy analysis, mitochondria were more abundant and more heterogeneous in size, with dense granules in the inner matrix, in Rvs- and Rvs plus EAAm-treated animals. Rvs administration increased protein kinase B and endothelial nitric oxide synthase phosphorylation, but the mammalian target of rapamycin signaling pathway was not affected. Rvs increased the expression of sirtuin 1, peroxisome proliferator-activated receptor γ coactivator-1α, cytochrome oxidase type IV, cytochrome c, and mitochondrial biogenesis markers. Levels of glucose-regulated protein 75 (Grp75), B-cell lymphoma 2, and cyclin-dependent kinase inhibitor 1 were increased in cortical proximal tubules, and expression of the endoplasmic reticulum–mitochondrial chaperone Grp78 was decreased. EAAm supplementation maintained or enhanced these changes. Rvs promotes mitochondrial biogenesis, with a probable anti-apoptotic effect. EAAm boosts these processes and may contribute to the efficient control of cellular energetics and survival in the mouse kidney. This suggests that appropriate nutritional interventions may enhance the beneficial actions of Rvs, and could potentially prevent chronic renal side effects.

Keywords Rosuvastatin · Essential amino acids · Kidney · Mitochondria · Mice

Abbreviations p-AKT Phosphorylated protein kinase B
Bcl-2 B-cell lymphoma 2
CDKN1A Cyclin-dependent kinase inhibitor 1 (p21)
COX IV Cytochrome c oxidase
Cyt c Cytochrome c
EAAm Essential amino acid mixture
p-eNOS Phosphorylated endothelial nitric oxide synthase
ER Endoplasmic reticulum
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
Grp75 Glucose-regulated protein 75
Grp78 Glucose-regulated protein 78
mtDNA Mitochondrial DNA
Introduction

According to the 2013 American College of Cardiology/American Heart Association Guideline on the Treatment of Blood Cholesterol, statins are the only class of drugs indicated for the prevention and treatment of atherosclerosis and cardiovascular disease (CVD) in subjects with increased risk (Stone et al. 2013). Statins are also known as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors. HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis, and its inhibition reduces pleiotropically the production of a number of molecules with inflammatory, endothelial, oxidant, and blood vessel tone properties (Susuc et al. 2003; Bae et al. 2010; Fiore et al. 2011). Adverse effects and intolerance of statins depend on the specific prescribed molecule and on patient characteristics (Mancini et al. 2013). Adverse muscle, hepatic, and gastrointestinal effects have been extensively reported in the literature (Mansi et al. 2013), but controversy exists regarding the effects of high-potency statins on renal function. Statin-induced hematuria and proteinuria have been well established, and microalbuminuria is thought to be secondary to statin interference with the tubular reabsorption of albumin (van der Tol et al. 2012; Robles et al. 2013). In 36 studies of rosuvastatin (Rvs) involving more than 40,000 patients, renal impairment or renal failure was reported in only 536 participants (Stein et al. 2012). In addition, two meta-analyses that assessed the benefits and harms of statin use in patients with renal disease showed no deterioration, and rather a trend toward improvement or maintenance of renal function, with decreased CVD and mortality, in patients with chronic kidney disease (Upadhyay et al. 2012; Palmer et al. 2012).

Different potential mechanisms have been suggested as causing or contributing to statin side effects (Knauer et al. 2010). Mitochondrial mechanisms have been repeatedly implicated, particularly in muscle damage (Golomb and Evans 2008). Statins lead to dose-dependent reductions in coenzyme Q₁₀ (De Pinieux et al. 1996), a key mitochondrial antioxidant and electron transport carrier that can bypass existing mitochondrial respiratory chain defects (Rosenfeldt et al. 2002). Several studies demonstrate that statins predispose to mitochondrial defects (Gambelli et al. 2004; Schick et al. 2007) in all users and, to a greater degree, in vulnerable individuals. A decreased Q₁₀ content was accompanied by a decreased maximal oxidative phosphorylation (OXPHOS) capacity in simvastatin-treated patients (Larsen et al. 2013). Moreover, dose-dependent reductions in coenzyme Q₁₀ can reduce cell energy, promote oxidation or apoptosis, and unmask silent mitochondrial defects (Lenaz et al. 2002). It is plausible that these findings partly explain the muscle pain and exercise intolerance that many patients experience with their statin treatment.

Extensive evidence supports the efficacy of dietary supplementation with a balanced amino acid mixture enriched in essential amino acids (EAAm) in clinical disorders characterized by deficits of energy production, including ageing, type 2 diabetes, heart failure, alcohol consumption, and renal failure (Cano et al. 2006; Valerio et al. 2011; Corsetti et al. 2012). It is widely accepted that the kidneys play a major role in amino acid metabolism and, partially, in the regulation of amino acid plasma levels. About 97% of the filtered amino acids are actively reabsorbed by renal tubules, and amino acids affect several kidney physiological processes depending on their quality and quantity (Sillernagl 1988). Among others, arginine, the substrate of nitric oxide synthase (NOS) and thus the precursor of NO synthesis (Andrew and Myer 1999), plays key roles in renal vessel and tubular functions (Rajapakske and Mattson 2013). Arginine ingestion or systemic administration increases renal blood flow and glomerular filtration rate in humans (Kano et al. 1992), possibly by displacement of asymmetric-di-methyl-arginine (ADMA), the endogenous inhibitor of endothelial nitric oxide synthase (eNOS) (MacAllister and Vallance 1994). Furthermore, renal eNOS is present not only in endothelial cells but also in the cells of the collecting ducts and macula densa, where NO regulates tubulo-glomerular feedback and renin secretion (MacAllister and Vallance 1994). The mTOR-AKT-eNOS pathway is known to be the cellular sensor of amino acid availability (Zoncu et al. 2011). Signaling via mTOR has been implicated in proliferative kidney diseases with an increased phosphorylation of downstream targets, including ribosomal protein S6 (Shillingford et al. 2006).

Notably, the EAs leucine, lysine and phenylalanine act similarly to insulin and suppress proteolysis of circulating and renal peptides. Supplementation with this EAAm was found to slow renal senescence in rodents (Corsetti, et al. 2010) and in hemodialysis patients (Bolasco et al. 2011). We have recently demonstrated that this specific amino acid formula restores mitochondrial function and antioxidant responses through eNOS expression, with reduced inflammatory processes, in the organs and tissues of aged rodents (D’Antona et al. 2010; Corsetti et al. 2011).
Although lacking arginine, the EAAm promotes arginine synthesis (Rondanelli et al. 2011). The present work aimed to study firstly the effects of long-term treatment with Rvs on the morphological and immunohistochemical aspects of the kidney in young mice, and secondly the impact of EAAm supplementation on the renal effects of Rvs, mainly focusing on mitochondrial adaptive processes.

Materials and methods

Animals and treatments

C57BL/6 male mice (2 months old, n = 36, Charles River, Calco, Italy) were housed in a quiet room with controlled temperature and humidity. A 12 h/12 h light/dark cycle was maintained (lights on from 7 a.m. to 7 p.m.). The experimental protocol was conducted in accordance with European Community guidelines and the Italian Ministry of Health, complied with The National Animal Protection Guidelines, and was approved by the Institutional Animal Ethical Committee.

Animals were given unrestricted access to a standard diet (4.3 % fat, 18.8 % protein, 76.9 % carbohydrate, Laboratorio Dottori Piccioni; for information about dietary ingredients and actual composition of the diet see Table 1) and tap water, and were treated for up to 1 month with Rvs (20 mg/kg/day, n = 10 animals), Rvs plus EAAm (1.5 mg/g body weight, n = 10 animals), or EAAm alone (1.5 mg/g body weight, n = 8 animals) via drinking water. A control group received drinking water without any drugs or supplement (CTRL; n = 8 animals). EAAm (composition, relative percentage, and dietary intake of each amino acid are reported in Table 2) was dissolved in tap water in quantities determined by calculating average daily drinking for 2 weeks before starting treatment (approximately 6 ml as in Bachmanov et al. 2002) and stored at 4°C before daily administration. EAAm concentrations were previously found to be active in rodents and mimic the recommended daily dose for humans (see Pellegrino et al. 2005; D’Antona et al. 2010). The Rvs dosage selected in this study is comparable to those adopted in other studies conducted to prove its ability to lower blood cholesterol (Enomoto et al. 2009).

Body weight, kidney weight, and average daily food and water consumption are reported in Table 3. Interestingly, the dietary EAAm supplementation induced a non-statistically significant reduction of food consumption in Rvs-treated and Rvs-untreated mice in comparison to the respective controls. This reduction led to slight changes in daily protein intake (CTRL: 0.76 ± 0.07 g/day; EAAm:

| Table 1 Dietary ingredients, amino acid content and actual composition of the diet |
|---------------------------------|----------------|--------|
| **Macronutrient composition**   | **Nutrient %** | **Amino acid g/100 g** |
| Raw proteins                   | 18.8           | Arginine 1.15 |
|                                |                | Histidine 0.48 |
|                                |                | Isoleucine 0.80 |
|                                |                | Leucine 1.50  |
|                                |                | Lysine 1.05   |
|                                |                | Methionine + cysteine 0.75 |
|                                |                | Phenylalanine 0.90 |
|                                |                | Threonine 0.75 |
|                                |                | Tryptophan 0.23 |
| Crude fat                      | 4.3            | Valine 1.00  |
| Crude cellulose                | 3.8            |                |
| Crude ash                      | 7.7            |                |
| Humidity                       | 12             |                |

| **Micronutrient composition (mg/kg)** |
|--------------------------------------|
| Vitamin A                            | 12,000 UI      |
| Vitamin B1                           | 8              |
| Vitamin B2                           | 10             |
| Vitamin B6                           | 10             |
| Pantothenic acid                     | 15             |
| Vitamin K                            | 1              |
| Vitamin PP                           | 40             |
| Vitamin B12                          | 0.02           |
| Folic acid                           | 2              |
| Choline chloride                     | 1,000          |
| Biotin                               | 0.1            |
| Iron                                 | 100            |
| Cobalt                               | 0.25           |
| Copper                               | 3              |
| Manganese                            | 55             |
| Iodine                               | 0.8            |
| Zinc                                 | 50             |
| DL-methionine                        | 500            |

Amino acid contents are reported as g/100 g mice food. Methionine was supplemented to the basic mixture.

| Table 2 Composition, relative percentage and dietary intake of EAAm |
|----------------|----------------|----------------|
| Amino acid     | Molecular mass (g/mol) | Percentage (%) | Dietary intake (g/kg/day) |
| Leucine        | 131.2           | 30.5           | 0.457               |
| Lysine         | 142.2           | 13.2           | 0.198               |
| Isoleucine     | 131.2           | 15.6           | 0.234               |
| Valine         | 117.1           | 19.6           | 0.294               |
| Threonine      | 119.1           | 10.8           | 0.162               |
| Cysteine       | 121.2           | 4.4            | 0.066               |
| Histidine      | 155.2           | 2.7            | 0.040               |
| Phenylalanine  | 165.2           | 1.6            | 0.024               |
| Methionine     | 149.2           | 1              | 0.015               |
| Tyrosine       | 181.2           | 0.4            | 0.006               |
| Tryptophan     | 204.2           | 0.2            | 0.003               |
umn thermostatted at 40°C.

A diode array detector. The procedure used was as follows: 262 nm excitation and 324 nm emission for FMOC, using at 338 excitation and 390 nm emission for OPA and previously described slightly modified (Aquilani et al. 2000).

Amino acid analysis was performed with the method de-
ABC-peroxidase staining system kit (Santa Cruz), and dehydrated and mounted with Distyrene Plasticizer Xylene (DPX). The reaction product was visualized using 0.3 % H2O2 and 3,3′-diaminobenzidine (DAB) at room temperature with positive staining appearing as a brownish color. Some sections were incubated with isotype-matched IgGs instead of primary antibodies as controls. Similar results were obtained when experiments were performed using the peroxidase–anti-peroxidase detection system, confirming that the presence of endogenous biotin did not lead to misinterpretation of the immunostaining data. Each set of experiments was performed in triplicate under the same experimental conditions. The intensity of histochemical and immunohistochemical staining was measured using an optical Olympus BX50 microscope equipped with an image analysis program (Image Pro Plus™ 4.5.1, Immagini & Computer, Milan, Italy) and was then analyzed quantitatively. The integrated optical density (IOD) was calculated for arbitrary areas by observing ten fields in each sample with a 40× objective. Data were pooled, mean values were calculated, and the results of each group were statistically compared.

Gene expression and mtDNA quantification

RNA was isolated from kidneys using the RNA easy Mini Kit (Qiagen, Milan, Italy) and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Italy), as described by D’Antona et al. (2010). For the evaluation of mtDNA, total DNA was extracted with QIAamp DNA extraction kit (Qiagen). The mRNA levels and mtDNA amount were measured by quantitative Real-Time PCR in triplicate, with iTaq Universal SYBR Green SuperMix (Bio-Rad Laboratories) on a CFX Connect Real-Time PCR System (Bio-Rad Laboratories). Primers were designed using Primer3 (version 0.4.0) software and are shown in Table 4. The cycle number at which the various transcripts were detectable (threshold cycle, CT) was compared with housekeeping CT, referred to as ΔCT. The gene relative level was expressed as 2\(^{-ΔΔCT}\), in which ΔΔCT corresponded to the difference between the ΔCT of either treatment group and the ΔCT of the control group.

Immunoblot analysis of mitochondrial markers

Protein extracts were obtained from kidneys using T-PER Mammalian Protein Extraction Reagent (Pierce, Thermo Scientific, Rockford, USA), as indicated by the manufacturer, in the presence of a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, Milan, Italy). Protein content was determined with bicinchoninic acid protein assays (BCA, Pierce, Euroclone, Milan, Italy). An appropriate amount of protein was run on SDS-PAGE under reducing conditions for immunoblotting. The separated proteins were then semi-dry transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and proteins of interest were revealed with specific antibodies: anti-p-S6 (Ser235/236), anti-S6, anti-p-AKT (Ser473), anti-AKT, anti-p-eNOS (Ser1177), anti-eNOS, anti-COX IV, anti-Cyt c, and anti-Grp78 (all from Cell Signaling, Euroclone, Milan, Italy); anti-SIRT1, anti-Grp75, and anti-Bcl-2 (all from Santa Cruz); and anti-PGC-1α (Abcam, Cambridge, UK) each at 1:1,000 dilution. Anti-β-Actin (1:10,000; Cell Signaling) and anti-Vinculin (1:10,000; Sigma-Aldrich) were used as loading controls. Immunostaining was detected using horseradish peroxidase conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature (Tedesco et al. 2010). Amounts of each protein were measured using SuperSignal Substrate (Pierce) and densitometrically quantified with an IMAGEJ software image analyzer.

Table 4 Primers used for quantitative PCR analysis

| Gene        | ID          | Primer sequences | PCR product | \(T_a\) (°C) |
|-------------|-------------|------------------|-------------|--------------|
| PGC1-α      | NM_008904.2 | Sense 5'-ACTATGAATCAAGCCACTACAGAC-3' | 148 bp     | 60          |
|             |             | Antisense 5'-TTTCCAGGCTTATTACGACG-3' |             |              |
| Tfam        | NM_009360.4 | Sense 5'-AACAGATAGTCCTGTCTGGGAAA-3' | 99 bp      | 60          |
|             |             | Antisense 5'-TGTGACGTGCTACAGGGATCT-3' |             |              |
| NRF-1       | NM_001164226.1 | Sense 5'-TTGCACCTGCGTCTGAGC-3' | 127 bp     | 60          |
|             |             | Antisense 5'-GGGCACTTCAGGTTTTCTC-3' |             |              |
| CDKN1A      | NM_007669.4 | Sense 5'-ACATGAAAACCTCCTAGACCGG-3' | 131 bp     | 60          |
|             |             | Antisense 5'-TCACTGCTGATCCCGTGAGG-3' |             |              |
| mtDNA       | NC_005089.1 | Sense 5'-AACTTTGCGATTGGAAGG-3' | 183 bp     | 60          |
|             |             | Antisense 5'-ACACATTGGGTTAGGAACA-3' |             |              |

\(T_a\) annealing temperature

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Table 4 Primers used for quantitative PCR analysis

| Gene | ID          | Primer sequences | PCR product | \(T_a\) (°C) |
|------|-------------|------------------|-------------|--------------|
| PGC1-α | NM_008904.2 | Sense 5'-ACTATGAATCAAGCCACTACAGAC-3' | 148 bp | 60          |
|       |             | Antisense 5'-TTTCCAGGCTTATTACGACG-3' |             |              |
| Tfam  | NM_009360.4 | Sense 5'-AACAGATAGTCCTGTCTGGGAAA-3' | 99 bp | 60          |
|       |             | Antisense 5'-TGTGACGTGCTACAGGGATCT-3' |             |              |
| NRF-1 | NM_001164226.1 | Sense 5'-TTGCACCTGCGTCTGAGC-3' | 127 bp | 60          |
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\(T_a\) annealing temperature

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Plasma amino acid profile after Rvs and/or amino acid treatment in mice. Plasma concentrations of individual amino acids (pmol/μl) were measured in control mice fed ad libitum (CTRL; n = 8), dietary supplemented with amino acid mixture (EAAm n = 8), treated with rosuvastatin (Rvs n = 8) and rosuvastatin plus EAAm (Rvs + EAAm n = 8). Sum AA, total amino acids. Statistical significance was tested by two-way ANOVA followed by Bonferroni post hoc test for multiple comparison.

|                  | Control Mean ± SEM | EAAm Mean ± SEM | Rvs Mean ± SEM | Rvs + EAAm Mean ± SEM |
|------------------|--------------------|-----------------|---------------|-----------------------|
| Alanine          | 372 ± 16           | 351 ± 15\textsuperscript{**} | 453 ± 23\textsuperscript{*} | 451 ± 29\textsuperscript{*, †} |
| Arginine         | 243 ± 27           | 220 ± 22        | 266 ± 46      | 196 ± 17              |
| Asparagine       | 21 ± 1             | 19 ± 1          | 19 ± 1        | 21 ± 2                |
| Aspartic acid    | 16 ± 2             | 19 ± 3          | 18 ± 3        | 23 ± 3                |
| Citrulline       | 70 ± 6             | 65 ± 5          | 69 ± 7        | 58 ± 7                |
| Cysteine         | 14 ± 1             | 16 ± 1          | 16 ± 1        | 16 ± 1                |
| Glutamic acid    | 67 ± 4             | 78 ± 5          | 75 ± 4        | 73 ± 4                |
| Glutamine        | 627 ± 35           | 546 ± 24        | 594 ± 19      | 600 ± 14              |
| Glycine          | 203 ± 4            | 258 ± 13\textsuperscript{**} | 224 ± 15     | 270 ± 12\textsuperscript{***} |
| Histidine        | 46 ± 1             | 47 ± 2          | 37 ± 5        | 39 ± 4                |
| 1Met-histidine   | 8 ± 1              | 8 ± 1           | 9 ± 1         | 9 ± 1                 |
| Isoleucine       | 66 ± 10            | 85 ± 6          | 72 ± 8        | 89 ± 5                |
| Leucine          | 122 ± 9            | 167 ± 18\textsuperscript{*} | 153 ± 10     | 151 ± 10              |
| Lysine           | 89 ± 22            | 162 ± 22\textsuperscript{*, †} | 93 ± 15      | 120 ± 14              |
| Methionine       | 59 ± 4             | 55 ± 7          | 56 ± 9        | 42 ± 2                |
| Ornithine        | 54 ± 14            | 58 ± 12         | 69 ± 11       | 80 ± 14               |
| Phenylalanine    | 53 ± 5             | 69 ± 9          | 70 ± 11       | 73 ± 4                |
| Proline          | 90 ± 5             | 100 ± 7         | 105 ± 9       | 107 ± 13              |
| Hydroxyproline   | 102 ± 36           | 104 ± 41        | 100 ± 14      | 87 ± 10               |
| Serine           | 123 ± 6            | 118 ± 8         | 120 ± 8       | 122 ± 8               |
| Threonine        | 123 ± 10           | 159 ± 9\textsuperscript{*} | 164 ± 8*     | 160 ± 9*              |
| Tryptophan       | 93 ± 7             | 93 ± 7          | 95 ± 7        | 95 ± 7                |
| Tyrosine         | 86 ± 7             | 85 ± 8          | 92 ± 10       | 92 ± 7                |
| Valine           | 142 ± 11           | 155 ± 14        | 218 ± 17\textsuperscript{**} | 215 ± 18\textsuperscript{**} |
| Sum AA           | 2,861 ± 82         | 3,025 ± 120     | 3,142 ± 63    | 3,203 ± 79            |

Statistical analysis

Morphometric data were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) unless otherwise stated. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons (GraphPad Prism, CA). Statistical significance was set at p < 0.05.

Results

Plasma amino acid levels

Table 5 shows the plasma amino acid levels in the different groups of mice. In particular, glycine, leucine, lysine, and threonine were higher, in plasma of EAAm-treated mice compared to untreated animals. Accordingly to the results obtained by Trupp et al. (2012) in humans treated with simvastatin, Rvs increased threonine, alanine and valine levels compared to untreated animals. Finally, changes were seen in Rvs plus EAAm group, with increased levels of alanine, glycine, threonine, and valine compared to untreated animals. Interestingly, the only significant interaction between Rvs and EAAm treatment was observed for alanine. The plasma levels of this amino acid were higher in comparison with EAAm and Rvs alone thus suggesting an additive effect of the treatments. Overall, the effects of EAAm and Rvs on plasma amino acids suggest that changes in certain circulating amino acids may contribute to the observed effects in the mouse kidney.

Histology and ultrastructure analysis

Body weight, feeding, and water consumption in each group of mice are shown in Table 3. No differences were evident between Rvs- and Rvs plus EAAm-treated mice, or between these treated animals and the untreated controls. Kidney weight, a parameter related to renal function, did not differ between groups. Histological evaluation after staining with hematoxylin/eosin, sirius red (for collagen detection), or PAS (for detecting polysaccharides, including glycogen, and mucosubstances, including glycoproteins, glycolipids and mucins) did not reveal damage.
fibrosis, or other evident changes in any renal compartment (Fig. 1 and data not shown). The number of renal glomeruli \( N_{\text{glo}}/\text{mm}^2 \) and the ratios between cross-sectional glomerular areas and total area \( A_{\text{glo}}/A_{\text{tot}} \) were not statistically different between the experimental groups (Table 6).

Ultrastructural analysis of the proximal tubular cells, which are characterized by a high OXPHOS mitochondrial activity (Szeto et al. 2011) and which are actively involved in Rvs excretion (Verhulst et al. 2008), showed abundant, round-shaped mitochondria, particularly in the EAAm-supplemented group (Fig. 2). Heterogeneous and elongated mitochondria, with multiform cristae and electron-dense granules in the matrix, were more evident around the nuclei of these cells both in Rvs- and Rvs plus EAAm-treated mice when compared to untreated ones (Fig. 2).

Rosuvastatin alone or in combination with EAAm promotes mitochondrial biogenesis and function in mouse kidneys

The effects of Rvs alone or in combination with EAAm on mitochondrial biogenesis were investigated in kidney homogenates from treated and untreated animals.

Table 6: Glomeruli morphometry

|                | Control | EAAm | Rvs | Rvs + EAAm |
|----------------|---------|------|-----|------------|
| \( N_{\text{glo}}/\text{mm}^2 \) | 15.12 ± 1.51 | 16.4 ± 0.9 | 15.01 ± 1.77 | 15.98 ± 1.48 |
| \( A_{\text{glo}}/A_{\text{tot}} \) | 0.06 ± 0.02 | 0.06 ± 0.01 | 0.04 ± 0.02 | 0.05 ± 0.02 |

\( N_{\text{glo}}/\text{mm}^2 \), density of glomeruli; \( A_{\text{glo}}/A_{\text{tot}} \), ratio between glomerular area and total parenchymal area. Values were expressed as mean ± SEM. Measurements were done in 8–10 animals per group.

Figure 1: Renal histological analysis. PAS staining in untreated (CTRL) (a), EAAm (b), Rvs (c), and Rvs + EAAm (d) groups. Histological evaluation revealed no detectable renal damage, fibrosis or other alteration in any of the groups or renal compartments. Experiments were performed in 8–10 animals per group. EAAm amino acid mixture enriched in essential amino acids, Rvs rosuvastatin. *Bar 50 \( \mu \text{m} \).

Figure 2: Electron microscopy analysis of mouse kidneys. a Untreated mice (CTRL). b EAAm-treated mice; the mitochondria were abundant, round and with dense matrix. *Bar 5 \( \mu \text{m} \). c Rvs-treated animals; mitochondria were more heterogeneous, elongated and scattered around the nucleus. *Bar 5 \( \mu \text{m} \). Inset higher magnification; irregular cristae and electron-dense granules in the mitochondrial matrix resembling calcium deposits (black arrows) were evident. *Bar 2 \( \mu \text{m} \). d Rvs + EAAm treatment. Similar features as in panel c. Experiments were performed in 8–10 animals per group. EAAm amino acid mixture enriched in essential amino acids, Rvs rosuvastatin, N nucleus. *Bar 5 \( \mu \text{m} \).

Figure 3a, b shows that neither treatment changed the mRNA levels of peroxisome proliferator-activated receptor \( \gamma \) coactivator 1\( \alpha \) (PGC-1\( \alpha \)), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), nor the amount of mtDNA (an index of mitochondrial mass) in the kidneys. Both Rvs and EAAm increased the protein levels of sirtuin 1 (SIRT1), a highly conserved class III histone deacetylase sensitive to nutritional status that is able to activate PGC-1\( \alpha \) (Fig. 3c). Protein levels of PGC-1\( \alpha \), which acts as a regulator of mitochondrial biogenesis, were also increased (Fig. 3c). Notably, Rvs plus EAAm showed an additive effect on SIRT1 and PGC-1\( \alpha \) protein levels (Fig. 3c). Similar results were obtained with COX IV and Cyt c, respiratory proteins that are an indirect index of mitochondrial function. EAAm alone had a small effect on Cyt c levels (Fig. 3d). These results suggest that Rvs...
promotes the expression of some markers of mitochondrial biogenesis and that EAAm supplementation reinforces this effect.

Rosuvastatin stimulates AKT and eNOS phosphorylation in mouse kidneys

To investigate the signaling mechanism(s) involved in the effects of Rvs and EAAm on renal mitochondria, we analyzed the mTOR-AKT-eNOS pathway. To determine whether Rvs and EAAm affected mTOR signaling in mice, kidney lysates were prepared and the amounts of phosphorylated S6 (p-S6) were determined by immunoblot analysis (Fig. 4a). The level of p-S6, normalized to total S6 (pS6/S6), was unchanged by either Rvs or EAAm, alone or in combination. In accordance with previous results (Bussolati et al. 2005; Ito et al. 2010), Rvs activated protein kinase AKT through increased Ser473-AKT phosphorylation (Fig. 4a). This effect was not strengthened by EAAm, which alone was unable to modify AKT phosphorylation (Fig. 4a). Neither Rvs nor EAAm, or their combination, promoted phosphorylation of AKT in Thr308 (data not shown). Activated AKT phosphorylates multiple targets, including eNOS. Ser117 phosphorylation of eNOS increases nitric oxide production (Kureishi et al. 2000). Accordingly, Rvs increased Ser117 p-eNOS levels in the kidney, while EAAm did not change either basal or Rvs-induced eNOS phosphorylation (Fig. 4a). Specific immunohistochemical analysis showed a moderate eNOS immunostaining uniformly distributed in the cortical

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Fig. 3 Molecular markers of mitochondrial biogenesis in mouse kidneys. a mRNA levels of mitochondrial biogenesis markers and b mtDNA amounts in the kidneys of untreated (CTRL) and treated groups analyzed by quantitative RT-PCR. The cycle number at which the transcripts were detectable was compared to that of GAPDH and expressed as relative expression versus controls, taken as 1.0. The experiments were performed in 8–10 animals per group and data are expressed as mean ± SEM. c, d Effects of treatments with Rvs and EAAm, either alone or in combination, on SIRT1, PGC-1α, COX IV, and Cyt c protein levels measured by immunoblot analysis. The Western blot images are representative of separate experiments done in 8–10 animals per group. The relative values were detected by densitometric analysis, relative to either vinculin or β-actin levels, with untreated (CTRL) mice given a value of 1.0. Data are expressed as mean ± SEM. *p < 0.05 vs untreated (CTRL) mice; #p < 0.05 vs Rvs-treated mice.
tubular cells of untreated and EAAm-supplemented mice (Fig. 4b; Table 7). The staining intensity was weakly decreased in the kidney of Rvs-treated animals, but eNOS was markedly increased when Rvs was used in combination with EAAm, particularly in the proximal tubular cells (Fig. 4b; Table 7). Faint and sparse staining for inducible and neuronal NOS (iNOS and nNOS) was observed in the kidneys of each group (data not shown).

Rosuvastatin regulates the physiology of mitochondria and endoplasmic reticulum in mouse kidneys

S473-phosphorylated AKT was recently localized to the endoplasmic reticulum (ER) subcompartment, termed the mitochondria-associated ER membrane (MAM) (Betz et al. 2013). In mammalian MAM, the ER and mitochondria are physically tethered to each other by the inositol trisphosphate (IP3) receptor (IP3R)/glucose-regulated protein 75 (Grp75)/voltage-dependent anion-selective channel 1 (VDAC1) trimeric complex (Betz et al. 2013). Grp75 expression was therefore investigated. Rvs, in contrast to EAAm alone, markedly increased Grp75 protein levels in the kidneys of treated mice (Fig. 5a). This increased expression was not changed by EAAm supplementation. These results were confirmed by immunohistochemical analysis. Grp75 immunostaining was faint and uniformly distributed in tubular and glomerular cells of mice in the control and EAAm alone groups (Fig. 5b), but was strongly increased by Rvs, mainly in the proximal tubular cells with glomeruli only occasionally stained (Fig. 5b). Although Grp75 staining was less intense when Rvs was given with EAAm supplementation, it remained much higher than in untreated and EAAm-supplemented mice (Fig. 5b; Table 7).

Statins reduce ER stress in isolated cell systems, and glucose-regulated protein 78 (Grp78) is involved in the homeostatic response to cellular redox damage (Breder et al. 2010; Molins et al. 2010). Grp78 expression was thus examined in the kidneys of treated and untreated mice. Chronic exposure to Rvs reduced Grp78 protein levels (Fig. 5c), while EAAm treatment did not change either basal or Rvs-decreased Grp78 expression. Immunostaining clearly identified Grp78 in the tubular cells of untreated animals (Fig. 5d); however, Rvs treatment induced sparse and moderate cytoplasmic staining of glomerular cells, while the proximal tubes were intensely stained (Fig. 5d). EAAm only partly modified these staining patterns (Fig. 5d; Table 7). These findings suggest that Rvs positively modulates the expression of proteins controlling mitochondrial and ER physiology, and that EAAm supplementation does not play a relevant role in favoring these processes.

Rosuvastatin and EAAm in combination promote an anti-apoptotic mechanism

Mitochondria and ER are involved in the apoptotic process, as are AKT, Grp75 and Grp78 (Tabas and Ron 2011; Logue...
et al. 2013). Rvs exerts a protective effect on injured podocytes through a p21-dependent anti-apoptotic pathway (Cormack-Aboud et al. 2009), and cytoplasmic p21 accumulation, due to AKT-dependent phosphorylation of nuclear p21, promotes a Bcl-2-dependent anti-apoptotic process (Vincent et al. 2012). For these reasons, the mRNA levels of CDKN1A, the gene encoding p21, were analyzed. Both Rvs and EAAm increased CDKN1A expression in the kidneys of treated mice (Fig. 6a). Rvs and EAAm in combination showed a strong synergistic effect on CDKN1A mRNA levels (Fig. 6a). Bcl-2 protein levels were increased by Rvs, but not by EAAm alone; however, the combination of Rvs and EAAm promoted higher Bcl-2 levels than Rvs alone (Fig. 6b). Immunohistochemical analysis showed faint Bcl-2 staining in all tubular cells in the untreated group, whereas Bcl-2 expression in tubular cells was increased in the kidneys of both Rvs- and EAAm-treated animals (Fig. 6c; Table 7). Bcl-2 staining was uniformly present in proximal tubular cells and was more marked in the Rvs plus EAAm group than in the Rvs group (Fig. 6c; Table 7).
Discussion

The present results show that long-term treatment with Rvs, at a dose comparable to that normally used in clinical trials to lower blood cholesterol levels, promotes the expression of markers of mitochondrial biogenesis in the kidneys of young mice. In particular, ultrastructural analysis identifies abundant and elongated mitochondria around the nuclei of cortical proximal tubular cells in mice treated with Rvs. Multiform cristae and electron-dense granules in mitochondrial matrix are evident, suggesting either intense functional activity or calcium accumulation within the organelles (Khraiwesh et al. 2013). The protein levels of SIRT1 and PGC-1α, two master regulators of mitochondrial biogenesis, and COX IV, an OXPHOS respiratory protein, are increased by Rvs in the kidneys. Dietary supplementation with EAAm, which modifies the plasma amino acid profile, potentiates these effects. Recent results have suggested that SIRT1 in proximal tubules protects against albuminuria in diabetes by maintaining nicotinamide mononucleotide concentrations around glomeruli, thus influencing podocyte function (Hasegawa et al. 2013). Previously, statins were reported to increase NO bioavailability in patients with hypercholesterolemia (John et al. 2001) and to exert beneficial effects on the activation and expression of eNOS in hypertensive rats (Kishi et al. 2008; Ohkawara et al. 2010). Moreover, Kureishi et al. (2000) demonstrated that simvastatin induces AKT-regulated eNOS activation (via Ser1177 phosphorylation), which increases NO production and consequently promotes endothelial cell survival. Similarly, EAAm was reported to promote mitochondrial biogenesis in cardiac and skeletal muscle by promoting eNOS activity (D’Antona et al. 2010) and AKT phosphorylation (Flati et al. 2010). Accordingly, the present results demonstrate that Rvs induces AKT phosphorylation at Ser473 and increases eNOS phosphorylation at Ser1177 in mouse kidney. Moderate eNOS immunostaining is uniformly distributed in cortical tubular cells and is evident in untreated and Rvs-treated or EAAm-supplemented mice. However, simultaneous administration of Rvs and EAAm markedly increases eNOS staining in the kidneys, particularly in the proximal tubules.

Controversial recent evidence suggests that statins may both activate or suppress the mTOR pathway in cancer or non-cancer cells (Roudier et al. 2006; Finlay et al. 2007). To this effect, Woodard et al. (2008) showed that fluvastatin induced apoptosis potently and limited the proliferation of renal cell carcinoma (RCC) cells in vitro; these effects were mediated by the suppression of AKT phosphorylation/activation, resulting in inhibition of mTOR and p70 S6 kinase. In the current study, S6 phosphorylation was not altered by Rvs and EAAm, alone or in combination. This result is consistent with a previous study in which dietary leucine supplementation did not modify the mTOR signaling in the kidney of neonatal pigs (Suryawan et al. 2012). Together these findings support a direct role for statins and amino acids in modulating the eNOS-dependent NO signaling pathway in renal tubular and endothelial cells through an AKT-dependent mTOR-independent pathway. In particular, the EAAAs have been shown to increase synthesis of arginine through reinforcement of the anaplerotic export of aspartate, necessary for recycling of citrulline to arginine by arginino-succinate synthetase. Complex mechanisms, including the stimulatory effect of Rvs on eNOS activity and increased arginine availability from EAAm supplementation, may contribute to the processes observed in the present work.

Considering the positive effect of NO on mitochondrial biogenesis and function (Nisoli et al. 2003, 2004; Nisoli and Carruba 2006), the present results imply that Rvs, particularly when combined with EAAs, may have relevant beneficial effects on kidneys, and particularly on the proximal tubular cells. Notably, these cells do not use glucose for their energy production (Balaban and Mandel 1988) but rely primarily on fatty acid oxidation. Fatty acid oxidation is mostly performed by mitochondria, which therefore have a central role in these cells. Mitochondrial production of ATP, which is essential for generating the energy-dependent ion gradients that drive renal tubular reabsorption, is impaired in acute kidney injury (Hall et al. 2011). This can cause massive and life-threatening losses of fluids, electrolytes, and low-molecular weight nutrients, a dysfunction known as renal Fanconi’s syndrome (Che et al. 2014). Recently, a rare mutation was identified in autosomal dominant Fanconi’s syndrome that creates a new mitochondrial targeting motif in the N-terminal portion of enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH) (Klootwijk et al. 2014). EHHADH is involved in peroxisomal oxidation of fatty acids and is expressed in proximal tubules. Studies of proximal tubular cells expressing mutant EHHADH revealed that mis-targeting of a peroxisomal protein to mitochondria impaired mitochondrial oxidative phosphorylation, with marked defects in the transport of fluids and glucose across the epithelium, indicative of the central role of mitochondria in proximal tubular function (Klootwijk et al. 2014). Although hyperaminoaciduria is a hallmark of the condition (Baum 1998), it is tempting to speculate that Rvs and particularly Rvs in combination with EAAs could have beneficial effects on mitochondrial function in the renal tubular cells impaired in Fanconi’s syndrome.

The present findings suggest that Rvs modulates the physiological relationship between the mitochondria and the ER. Rvs markedly increased the expression of Grp75 in the proximal tubular and glomerular cells. Grp75 is a
component of a trimeric complex (IP3-Grp75-VDAC1) that regulates physical tethering of the ER and mitochondria at MAMs (Betz et al. 2013), together with mitofusins 1 and 2 (Twig et al. 2008). The ER–mitochondria tethering complex affects mitochondrial dynamics and function. In particular, association with the ER forms microdomains on the outer mitochondrial membrane. These sites are enriched in mitochondrial division components, such as dynamin-related guanosine triphosphatases 1 (DRP1) and membrane protein mitochondrial fission factor (Mff) (Friedman et al. 2011; Youle and van der Bliek 2012). Under stress conditions, the microdomains may recruit and regulate the activation of pro-apoptotic Bcl-2 proteins such as Bax, which promote permeabilization of the mitochondrial outer membrane and the release of death mediators such as Cyt c (Hoppins and Nunnari 2012). Thus, the ER–mitochondria contact points regulate apoptosis under particular conditions, in addition to modulating lipid transfer and calcium flux between the organelles. These processes are essential for mitochondrial function and ER homeostasis. Diverse evidence supports the effect of statins in reducing ER stress, and the role of Grp78, an ER stress sensor, in the homeostatic response to cellular redox damage has been explored (Breder et al. 2010; Molins et al. 2010). In the present work, long-term treatment with Rvs reduces Grp78 protein levels in kidney homogenates. This occurs specifically in glomerular cells, in contrast to the proximal tubular cells where Grp78 was accumulated. These findings suggest that Rvs can communicate diverse survival messages to different kidney cell types.

Rvs was previously found to protect injured podocytes through a p21-dependent anti-apoptotic pathway (Cormack–Aboud et al. 2009). Accordingly, the present results show that the anti-apoptotic Bcl-2 protein levels are increased by Rvs, alone and in combination with EAAm. Immunostaining confirms that Bcl-2 is accumulated in the tubular cells. Vincent et al. (2012) have recently demonstrated that cytoplasmic p21 accumulation, resulting from AKT-dependent phosphorylation of nuclear p21, promotes a Bcl-2-dependent anti-apoptotic process in cancer cells. Accordingly, both Rvs and EAAm here increase CDKN1A expression in the kidneys of treated mice, and this effect is more marked with Rvs and EAAm in combination.

These results are consistent with recent studies showing that both Rvs treatment and EAAm supplementation had beneficial effects on kidneys in rodents (Corsetti et al. 2010) and humans (Vidt et al. 2006; Bolasco et al. 2011). Although data on the potential renal toxicity of high-potency statins are controversial (Tiwari 2006; Golomb and Evans 2008; Dodiya et al. 2011), two meta-analyses showed that statin therapy is safe and effective in preventing mortality and major cardiovascular events in patients with non-dialysis-dependent chronic kidney disease (CKD) (Upadhyay et al. 2012, 2013; Palmer et al. 2012). However, these studies provide very limited evidence to support the use of statins in patients on dialysis, and statin therapy was not found to be effective in reducing the risk of kidney failure or decline in kidney function (Upadhyay 2013). Conversely, a recent pilot study conducted in 15 CKD patients on hemodialysis treatment for at least 6 months has shown that 3 month EAAm supplementation increased serum albumin and total proteins, with reduced levels of inflammatory markers and improved anemia (Bolasco et al. 2011). Although these effects were studied in a small group of patients and might be due to ameliorated nutrient intake besides that of the amino acid mixture, EAAm supplementation might improve the efficacy of statin therapy in dialysis-dependent subjects.

Conclusions

The present results support the favorable effects of a high-potency statin, like Rvs, on the morpho-functional aspects of mouse kidney. In particular, long-term treatment with Rvs supports mitochondrial function, probably through the AKT-eNOS signaling pathway, and regulates survival of proximal tubular cells. An original formula of essential amino acids enhances the statin’s effects. This provides mechanistic confirmation of recent clinical evidence in kidney disease and suggests the potential use of essential amino acid supplementation in conjunction with statins in patients with renal disorders characterized by tubular cell dysfunction.

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Conflict of interest The authors declare no conflict of interest.

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References

Andrew PJ, Myer B (1999) Enzymatic function of nitric oxide synthases. Cardiovasc Res 43:521–531
Aquilani R, Viglio S, Iadarola P, Guarinaschelli C, Arrigoni N, Fugazza G, Catapano M, Boschi F, Dossena M, Pastoris O (2000) Peripheral plasma amino acid abnormalities in rehabilitation patients with severe brain injury. Arch Phys Med Rehabil 81:176–181
Dietary supplementation with essential amino acids