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L-proline supplementation improves nitric oxide bioavailability and counteracts the blood pressure rise induced by angiotensin II in rats

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ABSTRACT

We evaluated whether L-proline (Pro) supplementation improves redox status and nitric oxide (NO) bioavailability and prevents or delays angiotensin II (AngII)-induced hypertension.

Male Sprague-Dawley rats were distributed to four experimental groups: Pro + AngII (Pro-Ang), Pro + Saline (Pro-Sal), Vehicle + AngII (Veh-Ang) and Veh + Saline (Veh-Sal). Pro solution (2 $g.kg^{-1}.day^{-1}$) or water (vehicle) were orally administered, from day 0 to day 21. AngII (200 $ng.kg^{-1}.min^{-1}$) or saline were infused (s.c.) from day 7 to day 21. Systolic blood pressure (SBP) was measured by the tail-cuff method. From day 20–21, animals were kept on metabolic cages for 24h-urine collection. On day 21, urine and blood were collected for further quantification of redox status biomarkers, NO-related markers (urinary nitrates and nitrites, U-NOx; plasma asymmetric dimethylarginine, P-ADMA), metabolic and renal parameters.

Pro prevented the AngII-induced SBP rise [mean (95% CI), Day 19: Pro-AngII, 137 (131; 143) *vs.* Veh-AngII, 157 (151; 163) mm Hg, $P < 0.001$]. Pro-AngII rats also had increased values of U-NOx, systemic and urinary total antioxidant status (TAS), urinary H_2O_2 and plasma urea, as well as reduced P-ADMA and unaltered urinary isoprostanes. Plasma Pro was inversely correlated with P-ADMA ($r = -0.52$, $p = 0.0009$) and positively correlated with urinary TAS ($r = 0.55$, $p = 0.0005$) which, in turn, was inversely correlated with P-ADMA ($r = -0.56$, $p = 0.0004$). Furthermore, urinary H_2O_2 values decreased across P-ADMA tertiles (p for linear trend = 0.023).

These results suggest that Pro reduces P-ADMA levels and improves redox status, thereby increasing NO bioavailability and counteracting the AngII-induced SBP rise. H_2O_2 and TAS modulation by Pro may contribute to the reduced P-ADMA concentration.

1. Introduction

Arterial hypertension is highly prevalent worldwide, contributing significantly to cardiovascular and renal morbidity and mortality [1–3]. Of note, the risk of developing cardiovascular and renal complications actually starts to increase at normal to high-normal blood pressure ranges [4–6] and the progression from these blood pressure categories to a hypertensive state appears to be determined not only by baseline blood pressure but also by urinary nitric oxide (NO) metabolites levels [7]. Indeed, experimental and human hypertension has been associated with reduced NO bioavailability due to decreased NO production by endothelial NO synthase (eNOS) and/or increased NO degradation by

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reactive oxygen species (ROS) [8–11].

The adoption of a healthy diet is an important strategy both in prevention and management of hypertension [2]. In the last decades, special emphasis has been given to food or beverage components that have the ability to reduce oxidative stress and improve NO bioavailability, for their putative positive impact on cardiovascular disease prevention and treatment [12–14]. L-proline (Pro) is an abundant amino acid in foods from animal (e.g. milk, meat, salmon) and plant origin (e.g. wheat, barley, corn), and also present in high concentrations in many beverages, such as wine, grape juice and beer [15–17]. Although Pro can act both as antioxidant and prooxidant agent, it appears to exert a protective net effect against redox dysfunction. The antioxidant effects of Pro have been reported to involve upregulation and stabilization of antioxidant enzymes, scavenging of ROS, metal chelation, balance of intracellular redox homeostasis and improvement of cell resistance to hydrogen peroxide (H_2O_2) [18–20]. Due to these properties that mitigate the impact of ROS on living organisms, Pro has been added to an elite list of non-enzymatic antioxidants [21]. Even the ROS generated during Pro metabolism have been shown to induce signalling pathways involved in cell protection [19]. Importantly, Pro also has the putative ability to increase NO production due to the interrelationship of Pro cycle with the L-arginine-urea cycle and recent studies have demonstrated that Pro is a major dietary precursor for arginine synthesis in humans [22–24].

The critical role of the renin-angiotensin-aldosterone system (RAAS) in the regulation of arterial pressure by renal and extrarenal mechanisms is widely recognized [25–29]. RAAS inhibitors are known to be effective antihypertensive agents even when there is no evidence of systemic RAAS activation, either in animal models, such as the Spontaneously Hypertensive Rat, and in human essential hypertension [25,26]. Indeed, both angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are among the most widely used antihypertensive drugs [2]. Therefore, the Ang II-infusion model has been considered a valuable model for dissecting the various mechanisms contributing to chronic hypertension [26].

Since NO counterbalances the effects of AngII on vascular tone and renal sodium excretion [30], and AngII-induced hypertension is associated with reduced NO bioavailability and increased ROS production [27,31,32], our study aimed to evaluate whether Pro supplementation improves redox status and NO bioavailability and prevents or delays the onset of AngII-induced hypertension.

2. Materials and methods

2.1. Animals and experimental design

Forty male Sprague-Dawley rats (250–350 g; Charles River Laboratories, Spain) fed water and food *ad libitum* were housed under controlled temperature (22 °C), relative humidity (40–50%) and photoperiod (12h/12h light-dark cycle) conditions. All animal care and experimental procedures complied with the European Community guidelines for the use of experimental animals (Directive 2010/63/EU) and were approved by the local Ethics Committee.

All animals were acclimatized for 7 days upon arrival to the animal facility and subsequently underwent a 7-day training period to adapt to the study procedures prior to the beginning of the experiments. Two series of experiments were performed. In each of them, rats were randomly distributed to four experimental groups (6 per group in the first series, and 4 per group in the second): Pro + AngII (Pro-Ang), Pro + saline (Pro-Sal), vehicle + AngII (Veh-Ang) and vehicle + saline (Veh-Sal). Anaesthesia-derived complications caused the death of three animals from series I, during the surgical implantation of osmotic minipumps, wherefore final group sizes were: Veh-Sal, n = 9, Pro-Sal, n = 8, Veh-Ang, n = 10, and Pro-Ang, n = 10. Pro (2 $g.kg^{-1}.day^{-1}$; PharmaGrade, Sigma-Aldrich, Madrid, Spain) aqueous solution or water (vehicle) were orally administered, from day 0 to day 21. Pro dose was selected according to previous studies evaluating the subchronic toxicity or the protective effects of oral Pro administration [33,34]. This dose was shown to exert protective effects on inflammatory activation and regenerative response and to increase survival rate in a rat model of liver injury [33]. Furthermore, in a subchronic oral toxicity study of Pro the no-observed-adverse-effect-level (NOAEL) for Pro was determined to be a dietary dose of 2772.9 $mg.kg^{-1}.day^{-1}$ for male rats [34]. Although these studies were conducted in a different strain (Fischer rats), we used this dose since we could not find studies evaluating the effect of supplementation with Pro (alone) in Sprague-Dawley rats. Pro solutions were freshly prepared each day, and their concentration was adjusted according to the individual beverage intake and weight gain, to ascertain constant dosage throughout the experiment. AngII (200 $ng.kg^{-1}.min^{-1}$; Sigma-Aldrich, Madrid, Spain) or saline (0.9% NaCl, sham animals) were subcutaneously infused from day 7 to day 21, using osmotic pumps (model 2002; Alza, Palo Alto, CA, USA) surgically implanted in the dorsal region under a mixture of hydrochloride salts of ketamine and xylazine (respectively 80 and 10 $mg.kg^{-1}$, i.p.) in series I, or ketamine and medetomidine (respectively 60 and 0.25 $mg.kg^{-1}$, i.p.) in series II. Animals were kept in separate cages during the experiment, to allow individual monitoring of the ingested drink, and on metabolic cages from day 20–21, for urine collection. Rats' food intake and general health status were also monitored throughout the experiment. On day 21, animals were anaesthetized with 5% isoflurane (IsoFlo, Abbot Laboratories, Chicago, IL, USA) and blood was withdrawn from the left ventricle with heparinized needles to tubes containing heparin. Plasma was obtained by centrifugation at 1000 × *g* for 20 min at 4 °C. Both urine and plasma samples were divided into aliquots. Kidneys were perfused with saline before excision and then dissected into cortex and medulla. All samples were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. A schematic representation of the protocol is shown in Fig. 1.

2.2. Systolic blood pressure measurements

Systolic blood pressure (SBP) was measured in conscious animals by the non-invasive tail-cuff method, using a photoelectric pulse detector (LE 5000, Letica, Barcelona, Spain). Rats underwent a 7-day training period to adapt to the measurement methodology before day 0, and then were evaluated several times throughout the study. Each measurement consisted of five determinations, whose mean was used for further calculations. Values analysed were obtained on three distinct occasions: before the beginning of the experiment (baseline, Day 0); one week after the beginning of the Pro supplementation, before surgical implantation of AngII osmotic pumps (1wk Pro, Day 6); and at the end of the experiment, after 2 weeks of AngII infusion and 3 weeks of Pro treatment (*2wk Ang-3wk Pro*,Day 19).

2.3. Metabolic and renal parameters quantification

Plasma concentrations of Pro, L-arginine and hydroxyproline were quantified by High Performance Liquid Chromatography (HPLC). Plasma and urinary levels of sodium, potassium and chloride ions were determined by indirect potentiometry. Creatinine levels were quantified by the Jaffe method in both rats' plasma and urine. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were quantified by the modified International Federation of Clinical Chemistry (IFCC) method. Concentrations of urea (urease method), uric acid (with uricase/peroxidase method), glucose (hexokinase method), total cholesterol (enzymatic method) and triglycerides (glycerol phosphate oxidase method, GPO) were also determined in the plasma. Total protein content in the urine was quantified by the pyrogallol red method and expressed as $mg{\cdot}kg^{-1}{\cdot}day^{-1}$.

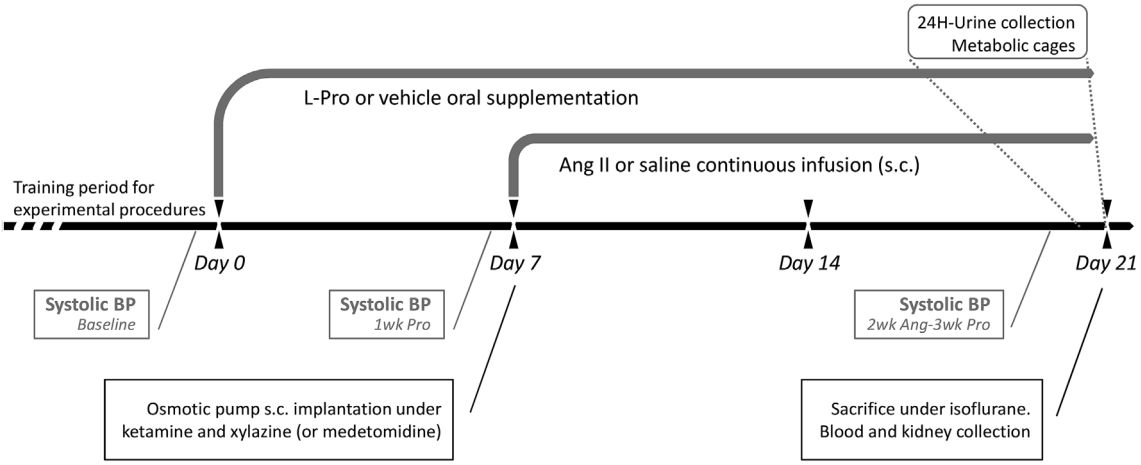


Fig. 1. Schematic overview of the experimental procedures. The experimental procedures were divided in two experimental series. Each series was treated as a complete randomized block.

2.4. Quantification of oxidative stress biomarkers

2.4.1. Urinary and renal H_2O_2

H_2O_2 was measured by a microplate fluorimetric assay using a specific kit (Amplex Red Hydrogen Peroxide Assay Kit, Molecular Probes, Alfagene, Carcavelos, Portugal) according to the manufacturer's protocol. Urinary excretion of H_2O_2 (U-H_2O_2) was evaluated in aliquots of 24-h urine samples and renal production of H_2O_2 was evaluated in kidney cortex and medulla, as previously reported [32,35]. Protein concentration of renal tissues was quantified using a Bradford assay kit (Bio-Rad, Hercules, CA, USA).

2.4.2. Urinary isoprostanes

Urinary excretion of isoprostanes (U-Isop) was quantified by a competitive enzyme immunoassay (Urinary Isoprostane ELISA Kit, Oxford Biomedical Research, Inc., Rochester Hills, MI, USA) in 24-h urine containing butylated hydroxytoluene (BHT; 0.005%, m/v) added before storage.

2.4.3. Plasma and urinary total antioxidant status (TAS)

TAS was evaluated in plasma (P-TAS) and 24-h urine samples (U-TAS) by a spectrophotometric assay using a specific kit (Antioxidant Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA).

2.5. Renal antioxidant enzymes activities

Catalase (Cat) and glutathione peroxidase (GPx) activities were determined in renal medulla and cortex, as previously described [35–37]. Briefly, renal medullary and cortical fragments were homogenized in cold phosphate buffer (50 mmol L^{-1} pH 7.4, 0.1% Triton X-100) with a Polytron homogenizer. Then, homogenates were centrifuged at 15700 × *g* for 10 min at 4 °C and the collected supernatants were assayed for enzyme activity. Cat activity was evaluated by monitoring the rate of H_2O_2 decomposition at 240 nm, at 25 °C, for 40 s. One unit (U) of Cat was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per min. Results were calculated using a molar extinction coefficient of 0.0394 L $mmol^{-1}$ cm^{-1} and expressed as U·mg $protein^{-1}$. GPx activity was assayed by measuring the conversion of NADPH to $NADP^+$ at 340 nm, following the reduction of GSSG to GSH in the presence of glutathione reductase, at 37 °C. Enzyme activity was calculated using the molar extinction coefficient of $NADP^+$ at 340 nm, 6.22 L $mmol^{-1}$ cm^{-1}, and expressed as nmol NADPH $oxidized{\cdot}min^{-1}$.mg $protein^{-1}$. The supernatant protein concentration was quantified using a Bradford assay kit (Bio-Rad, Hercules, CA, USA).

2.6. Quantification of biomarkers of NO production and bioavailability

2.6.1. Urinary nitrates and nitrites

Urinary excretion of nitrates and nitrites (U-NOx) was evaluated as a measure of NO formation and bioavailability [7,38,39]. The total concentration of nitrates and nitrites in 24-h urine samples was quantified by a colorimetric assay (Nitrate/Nitrite Colorimetric Assay kit, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.6.2. Plasma asymmetric dimethylarginine (ADMA) and L-arginine-to-ADMA concentration ratio

ADMA, present in plasma and cells, has been shown to inhibit NO generation by NO synthases (NOS) and to contribute to oxidative stress and endothelial dysfunction [40–42]. Therefore, we used plasma ADMA as an indirect biomarker of NO production and bioavailability. ADMA concentration in the plasma (P-ADMA) was determined by an enzyme-linked immunosorbent assay (ELISA) using a specific kit (ADMA Ultrasensitive ELISA Assay kit, Eagle Biosciences, Nashua, NH, USA). Since the state of activation or inhibition of NOS appears to depend on the concentrations of its substrate and inhibitor [42], the plasma L-arginine-to-ADMA concentration ratio (Arginine-to-ADMA ratio) was also calculated.

2.7. Data analysis

Statistical analyses were performed with the InVivoStat 3.7, a free statistical analysis system for *in vivo* data that uses R as its statistics engine [43]. Results are expressed as predicted means (95% confidence interval, CI), unless otherwise stated; *n* corresponds to the number of rats per group; *P* values below 0.05 were considered significant.

According to the experimental design (randomized block design with two between-subjects factors – Pro and AngII treatments – and the experimental series as a blocking factor), single measure parametric analysis was used, testing for *Pro*- and *AngII*-effects, *AngII* × *Pro* interaction and blocking effects. Data sets presenting deviations from the parametric assumptions (homogeneity of variance and normally distributed residuals) were analysed as either base 10 log-transformed (U-H_2O_2, U-Isop, U-TAS and proteinuria; plasma AST and urine volume) or rank-transformed (U-NOx) data. Multiple comparisons were performed using the least significant difference (LSD) test/planned comparisons.

For data collected at several time points (SBP, body mass, water and food ingestion) a repeated measures (RM) parametric analysis (mixed-model approach [44]) was used, with between-subjects factors as described above (*Pro* and *AngII*) and within-subjects factor *Time*. *P*-values were adjusted with Holm's multiple comparison procedure, according to the following pre-planned comparisons, within each day: Pro-Sal *vs*. Veh-Sal, Veh-Ang *vs*. Veh-Sal, Pro-Ang *vs*. Veh-Ang, Pro-Sal, and Veh-Sal. Daily Pro intake was analysed with a RM parametric analysis (between-subjects factor *AngII*, blocking factor and within-subjects factor *Time*).

Correlations were analysed using the GraphPad Prism 7 software (La Jolla, CA, USA). Pearson's single regression analysis was used to estimate correlations between sets of parametric data (or log-transformed normalized data). Tertile analysis of P-ADMA, as independent variable, and U-H_2O_2 (log-transformed), as dependent variable, was also performed. The tertiles of P-ADMA were defined based on all rats used. Trend analysis was conducted using GraphPad Prism 7 software (La Jolla, CA, USA) with a one-way ANOVA followed by a *post hoc* test for linear trend.

Although for the plasma parameters we had a final number of 37 blood samples (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-Ang, n = 10), we only had 36 samples for most urinary biomarkers of redox status and NO bioavailability (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-Ang, n = 9), due to the exclusion of one degraded urine sample, collected without BHT.

3. Results

3.1. Body mass and intake of Pro, food and water

There were no significant differences in the ingested dose of Pro between Sal and AngII rats, although the dose on days 1 and 2 was significantly above the mean of days 3–21 (Fig S1, Supplementary Material), corresponding to the initial adaptation period of the rats to the new beverage. No significant effects of either Pro or AngII treatments were detected on rats' body mass (Fig S2, Supplementary Material), although L-Pro supplementation, but not AngII infusion, was associated with lower solid food intake (Fig S3, Supplementary Material), which shows that the energetic content of the Pro cannot be neglected. Both Pro and AngII treatments significantly increased animals' water intake (Fig S4, Supplementary Material).

3.2. Effect of Pro supplementation on SBP in Sal- and AngII-infused rats

The preventive protocol of Pro supplementation was effective in counteracting the SBP rise induced by a 2-week continuous infusion of AngII (Day 19: Pro-Ang, 137 (131; 143) *vs.* Veh-Ang, 157 (151; 163) mm Hg, $P < 0.001$; and Pro-Ang 137 (131; 143) *vs.* Veh-Sal, 125 (119; 131) mm Hg, $P > 0.05$) (Fig. 2), although not causing any changes in SBP of normotensives (Day 19: Pro-Sal, 126 (120; 133) *vs.* Veh-Sal, 125 (119; 131) mm Hg, $P > 0.05$) (Fig. 2).

3.3. Effect of Pro supplementation on systemic metabolic parameters and on renal function in Sal- and AngII-infused rats

The effect of Pro supplementation on plasma metabolic parameters and on renal function is presented on Table 1. Systemic Pro levels were higher in Pro-supplemented, as opposed to non-supplemented groups ($P < 0.001$). Plasma urea was increased only upon treatment with both Pro and AngII ($P < 0.01$ *vs.* Veh-Ang). Proteinuria was elevated by Pro both in AngII- or saline-treated animals ($P < 0.01$ and $P < 0.05$ *vs.* Veh-Sal, respectively). Urine volume increased upon treatment with Pro ($P < 0.01$ *vs.* Veh-Sal) or AngII ($P < 0.05$ *vs.* Veh-Sal) alone or concomitant Pro and AngII treatments ($P < 0.001$ *vs.* Veh-Sal).

3.4. Effect of Pro supplementation on oxidative stress biomarkers

3.4.1. Effect of Pro on U-H_2O_2 and U-Isop in Sal- and AngII-infused rats

U-H_2O_2 (Fig. 3A and B) increased upon Pro supplementation only in Pro-Ang group ($P < 0.01$ *vs.* Veh-Ang). No significant changes in U-Isop were detected between groups (Fig. 3C and D).

3.4.2. Effect of Pro on renal production of H_2O_2 and activity of H_2O_2-metabolizing enzymes

Analysis of the renal production and enzymatic metabolism of H_2O_2 (Table 2) did not reveal any differences between groups.

3.4.3. Effect of Pro on systemic and urinary TAS in Sal- and AngII-infused rats

Plasma TAS was increased by Pro treatment, both in AngII- and saline-treated rats ($P < 0.05$ and $P < 0.01$ *vs.* Veh-Sal, respectively). Urinary TAS behaved similarly ($P < 0.001$ and $P < 0.05$ *vs.* Veh-Sal, respectively), although combined treatment was associated with a further increase of these values ($P < 0.01$ *vs.* Veh-Ang and $P < 0.05$ *vs.* Pro-Sal) (Fig. 4A, B and C).

3.5. Effect of Pro supplementation on biomarkers of $^{\bullet}$NO availability

3.5.1. Effect of Pro on U-NOx in Sal- and AngII-infused rats

U-NOx (Fig. 5A and B) was increased by concomitant treatment with Pro and AngII ($P < 0.05$ *vs.* Pro-Sal and $P < 0.01$ *vs.* Veh-Ang). Pro-treatment alone did not increase U-NOx values.

3.5.2. Effect of Pro on systemic ADMA levels and on L-arginine/ADMA ratio in Sal- and AngII-infused rats

The concomitant treatment with Pro and AngII decreased plasma ADMA levels (P-ADMA: $P < 0.01$ *vs.* Veh-Ang and $P < 0.01$ *vs.* Veh-Sal) and increased L-Arginine/ADMA ratio (P-Arginine/ADMA: $P < 0.05$ *vs.* Veh-Ang and Pro-Sal) (Fig. 6A and B).

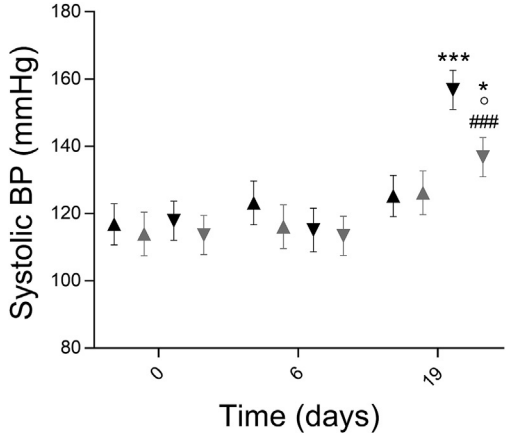
3.6. Correlation analysis

Plasma Pro values showed a negative correlation with plasma ADMA concentrations (Fig. 7A) and a positive correlation with U-TAS (Fig. 7B and C). Furthermore, P-ADMA exhibited negative correlations with U-TAS values (Fig. 7D and E) and linear trend analysis showed that U-H_2O_2 values decreased across P-ADMA tertiles (Fig. 7F and G).

4. Discussion

The major findings of our study are that Pro supplementation significantly increases the urinary excretion of NO metabolites and counteracts the blood pressure rise only in AngII-infused animals, but enhances the antioxidant capacity, both in controls and in AngII-infused rats. Noteworthy, the Pro-induced improvement of NO bioavailability appears to involve an inhibitory effect of Pro on the plasma concentration of ADMA, an endogenous inhibitor of NOS [40].

In humans, the urinary excretion of NO metabolites has been highlighted as an important predictor of the progression to hypertension, with urinary NO metabolites levels being significantly lower in progressors as compared to normotensive controls [7]. Indeed, NO exerts several actions relevant to blood pressure control. Examples of NO vasculoprotective effects include vasodilation, inhibition of smooth muscle cell proliferation, anticoagulation, inhibition of leukocyte adhesion and antioxidant capacity [30]. Treatments that increase NO bioavailability have been shown to reduce blood pressure in animal models and humans. For example, the administration of L-arginine, the precursor for NO production, significantly increased NO and ameliorated AngII-induced hypertension and salt-dependent hypertension in rats [45,46]. Studies in hypertensive patients have also demonstrated a blood pressure lowering effect of L-arginine [47,48]. Conversely,



Repeated Measures Analysis			
Within-subject effects		***Between-subject effects***	
Factor	**P**	**Factor**	**P**
Time	< 0.0001	*Block*	0.009
*Pro * Time*	0.3	*Pro*	0.008
*Ang * Time*	< 0.0001	*Ang*	0.01
*Pro * Ang * Time*	0.004	*Pro * Ang*	0.2

▲ Sal-Veh
▲ Sal-Pro
▼ Ang-Veh
▼ Ang-Pro

###P<0.001 *vs* Veh-Ang
°P<0.05 *vs* Pro-Sal
***P<0.001 *vs* Veh-Sal
*P<0.05 *vs* Veh-Sal

Fig. 2. Mean (95% CI) systolic blood pressure for the different experimental groups (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-Ang, n = 10) at days 0, 6 and 19 (*Baseline*, 1*wk Pro* and *2wk Ang-3wk Pro*, respectively), as described in the experimental scheme (Fig. 1). Data were analysed with a repeated measures parametric analysis (mixed-model approach), with 2 between-subjects factors (Pro and Ang) and blocking factor, also used for the single-data point measurements, and a within-subjects factor (Time). *P*-values obtained from the analysis for each factor and factor interactions are presented in the table beside the graph. Results from multiple comparison procedures, performed as described in section 2.7, are indicated on the graph. The analysed data had three missing values on day 6: one from group Veh-Sal and two from group Pro-Ang.

inhibition of NO synthesis significantly increases blood pressure [8,11,49,50]. In our study, we hypothesized that supplementation with Pro would improve NO bioavailability due to its interrelationship with arginine-urea cycle [22]. Indeed, Pro has recently been recognized as a dietary precursor for arginine synthesis [23,24]. Although systemic L-arginine concentration was not altered in Pro-treated animals, we observed a marked increase in urinary excretion of NO metabolites in AngII group receiving Pro, as well as an increase in plasma urea concentration, thus suggesting that Pro served as a precursor for the synthesis of arginine, which in turn was used for the production of NO and urea.

Besides evaluating the impact of Pro on NO metabolites, arginine and urea values, we also analysed its effect on the systemic concentration of ADMA, an endogenous inhibitor of NOS that results from the methylation of arginine residues in intracellular proteins by protein arginine methyltransferases (PRMT) [40,41]. In addition to competitive NOS inhibition, ADMA may also reduce the activity of cationic amino acid transporters, thus limiting the supply of the substrate L-arginine, from the plasma, to intracellular NOS. This effect further contributes to NOS uncoupling, oxidative stress and hence inhibition of NO generation [42]. Of note, recent studies suggest that the AngII-induced increase of ADMA with consequent reduction of NO production importantly contributes to the pathogenesis of arterial hypertension [50,51]. Interestingly, we found a significant reduction in plasma ADMA concentration and an increase in the plasma arginine-to-ADMA ratio in AngII-infused rats treated with Pro. Furthermore, Pro concentration was inversely correlated with plasma ADMA. Although the relationship between Pro and ADMA has not been previously investigated, a study evaluating arginine metabolites values in systemic inflammatory states showed that plasma Pro levels were inversely correlated with plasma high sensitivity C reactive protein concentration, which in turn correlated positively with systemic ADMA values [52]. These findings, suggestive of an inverse regulation of Pro and ADMA, are in accordance with our results.

Pro metabolism by Pro oxidase generates ROS, such as superoxide radical and H_2O_2 [19]. In our study, AngII-infused rats treated with Pro

Table 1
Metabolic parameters and renal function on day 21 in the experimental groups.

	Veh-Sal	Pro-Sal	Veh-Ang	Pro-Ang
Plasma				
L-Proline ($mg \cdot dL^{-1}$)	2.8 (1.7; 3.9)	6.1 (4.9; 7.3)***	2.5 (1.4; 3.6)	6.4 (5.3; 7.4)###,***
L-Arginine ($mg \cdot dL^{-1}$)	2.7 (2.5; 2.9)	2.9 (2.7; 3.2)	2.9 (2.6; 3.1)	2.9 (2.7; 3.1)
Urea ($mg \cdot dL^{-1}$)	28 (24; 31)	30 (26; 34)	29 (25; 32)	35 (32; 39)##,**,°
Uric acid ($mg \cdot dL^{-1}$)	0.98 (0.75; 1.22)	1.03 (0.77; 1.28)	1.11 (0.88; 1.34)	0.94 (0.71; 1.17)
Hydroxyproline ($mg \cdot dL^{-1}$)	0.62 (0.49; 0.74)	0.57 (0.44; 0.70)	0.57 (0.45; 0.68)	0.58 (0.47; 0.70)
Glucose $\times 10^{-1}$ ($mg \cdot dL^{-1}$)	23 (22; 25)	23 (22; 25)	22 (21; 24)	22 (21; 24)
Total cholesterol ($mg \cdot dL^{-1}$)	43 (39; 48)	48 (43; 53)	45 (41; 50)	44 (39; 48)
Triglycerides ($mg \cdot dL^{-1}$)	74 (54; 95)	79 (58; 101)	78 (58; 97)	87 (67; 106)
Aspartate transaminase[a] (AST) $\times 10^{-1}$ ($UI \cdot L^{-1}$)	12 (10; 13)	12 (10; 14)	14 (13; 17)*	14 (12; 16)
Alanine transaminase (ALT) ($UI \cdot L^{-1}$)	31 (28; 34)	34 (31; 38)	32 (29; 35)	33 (30; 36)
Renal function				
Creatinine clearance ($mL \cdot min^{-1} \cdot kg^{-1}$)	9.0 (7.5; 10.5)	10.2 (8.6; 11.7)	10.9 (9.5; 12.3)	9.7 (8.3; 11.1)
Proteinuria[a] ($mg \cdot kg^{-1} \cdot day^{-1}$)	28 (22; 34)	38 (30; 47)*	34 (28; 42)	43 (35; 53)**
Urine volume[a] ($mL \cdot kg^{-1} \cdot day^{-1}$)	28 (21; 39)	53 (38; 75)**	49 (36; 39)*	64 (47; 87)***
Fractional excretion of Na^+ (%)	0.27 (0.22; 0.33)	0.27 (0.21; 0.33)	0.29 (0.24; 0.34)	0.32 (0.26; 0.37)
Fractional excretion of Cl^- (%)	0.59 (0.49; 0.69)	0.58 (0.48; 0.69)	0.61 (0.52; 0.71)	0.69 (0.59; 0.78)
Fractional excretion of K^+ (%)	13 (11; 15)	12 (10; 15)	12 (10; 14)	15 (13; 17)

Data are shown as means (95% CI), n = 9 (Veh-Sal), n = 8 (Pro-Sal), n = 10 (Veh-Ang) and n = 9–10 (Pro-Ang). *$P < 0.05$ *vs* Veh-Sal; **$P < 0.01$ *vs* Veh-Sal; ***$P < 0.001$ *vs* Veh-Sal; ##$P < 0.01$ *vs* Veh-Ang; ###$P < 0.001$ *vs* Veh-Ang; °$P < 0.05$ *vs* Pro-Sal.

[a] Although urine volume, proteinuria and aspartate transaminase data were log-transformed prior to analysis, in order to stabilize the variance, their means are presented in the original scale, after back transformation.

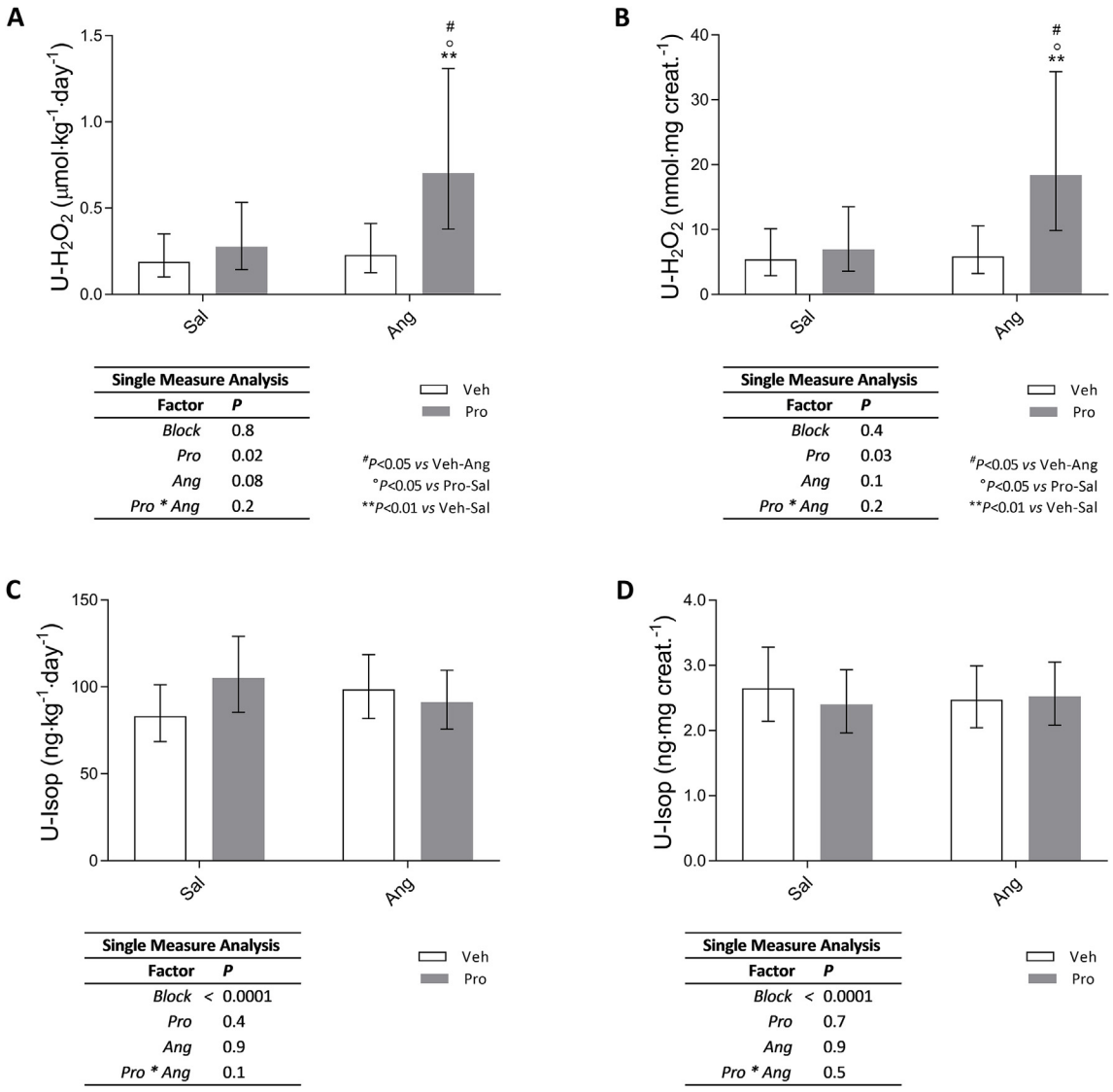


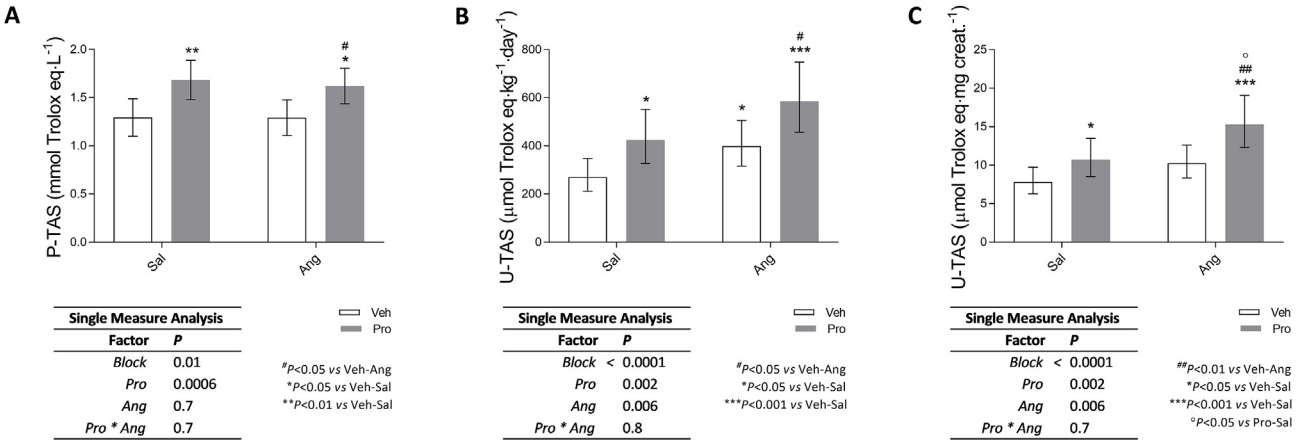
Fig. 3. Mean (95% CI) urinary excretion of H_2O_2 (U-H_2O_2) (A, B) and isoprostanes (U-Isop) (C, D) for the different experimental groups (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-AngII, n = 9–10) at the end of the experiment (day 21). Both parameters are presented as values *per* kg *per* day (A, C) and values *per* mg creatinine (B, D). Log-transformed U-H_2O_2 and U-Isop data were analysed with single measure parametric analysis (ANOVA), with 2 experimental factors (Pro and Ang) and a blocking factor. *P*-values obtained from the analysis for each factor and interaction of factors are presented in tables below the graphs. Results from multiple comparison procedures, performed as described in section 2.7, are indicated on the graph.

Table 2
Renal production of H_2O_2 and activity of H_2O_2-metabolizing enzymes.

	Veh-Sal	Pro-Sal	Veh-Ang	Pro-Ang
Renal medulla				
H_2O_2 (nmol·mg protein^{-1})	0.90 (0.65; 1.15)	0.71 (0.44; 0.97)	0.88 (0.64; 1.12)	0.95 (0.71; 1.19)
GPx (nmol oxidized NADPH·min^{-1}·mg protein^{-1})	49 (29; 69)	71 (50; 92)	74 (55; 93)	56 (37; 75)
Catalase (U·mg protein^{-1})	58 (42; 74)	56 (39; 73)	76 (61; 92)	61 (46; 76)
Renal cortex				
H_2O_2 (nmol·mg protein^{-1})	0.93 (0.60; 1.26)	0.57 (0.22; 0.92)	0.91 (0.60; 1.23)	0.89 (0.58; 1.21)
GPx × 10^{-1} (nmol oxidized NADPH·min^{-1}·mg protein^{-1})	12 (10; 14)	11 (9; 13)	14 (12; 16)	13 (11; 15)
Catalase (U·mg protein^{-1})	105 (90; 121)	93 (77; 110)	89 (75; 104)	104 (90; 120)

Data are shown as mean (95% CI), n = 9 (Veh-Sal), n = 8 (Pro-Sal), n = 10 (Veh-Ang) and n = 10 (Pro-Ang).

excreted significantly more H_2O_2 in their urine. Since AngII has been shown to stimulate H_2O_2 generation [31,32,53], the simultaneous administration of AngII and Pro probably exacerbated H_2O_2 production. Just recently, H_2O_2 was shown to inhibit the activity of PRMT1 in a concentration-dependent manner [54]. Of note, we observed that H_2O_2 values progressively decreased across ADMA tertiles. As PRMT1 is responsible for ADMA formation [40,54], we hypothesize that increased H_2O_2 generation in Pro-Ang II rats might contribute, at least in part, to the reduction in plasma ADMA values. Interestingly, a recent study showed that women with polycystic ovary syndrome have lower NO,



Single Measure Analysis	
Factor	P
Block	0.01
Pro	0.0006
Ang	0.7
*Pro * Ang*	0.7

#P<0.05 *vs* Veh-Ang
*P<0.05 *vs* Veh-Sal
**P<0.01 *vs* Veh-Sal

Single Measure Analysis	
Factor	P
Block	< 0.0001
Pro	0.002
Ang	0.006
*Pro * Ang*	0.8

#P<0.05 *vs* Veh-Ang
*P<0.05 *vs* Veh-Sal
***P<0.001 *vs* Veh-Sal

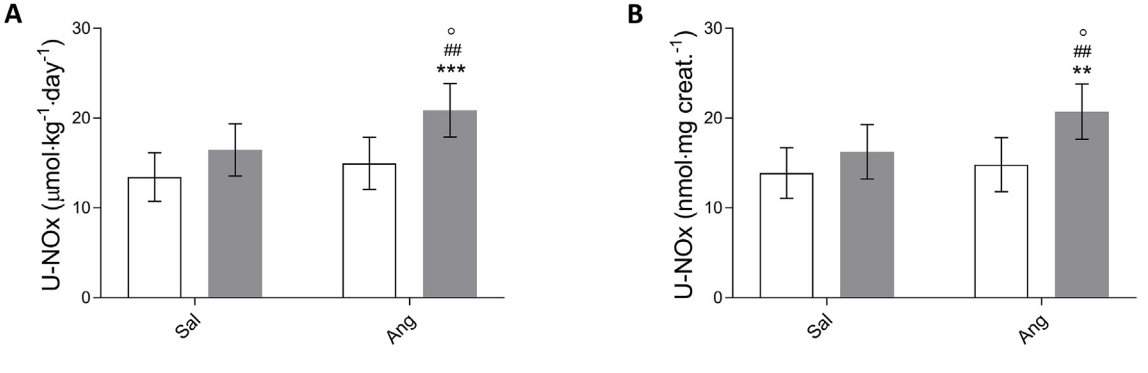
Single Measure Analysis	
Factor	P
Block	< 0.0001
Pro	0.002
Ang	0.006
*Pro * Ang*	0.7

##P<0.01 *vs* Veh-Ang
*P<0.05 *vs* Veh-Sal
***P<0.001 *vs* Veh-Sal
°P<0.05 *vs* Pro-Sal

Fig. 4. Mean (95% CI) of TAS values in the plasma (P-TAS) (A) and in the urine (U-TAS) (B, C) for the different experimental groups (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-Ang, n = 9–10) at the end of the experiment (day 21). The urinary parameter is presented as values *per* kg *per* day (B) and values *per* mg creatinine (C). Log-transformed U-TAS data were analysed with single measure parametric analysis (ANOVA), with 2 experimental factors (Pro and Ang) and a blocking factor. *P*-values obtained from the analysis for each factor and interaction of factors are presented in tables below the graphs. Results from multiple comparison procedures, performed as described in section 2.7, are indicated on the graph.

higher ADMA and lower H_2O_2 values than controls [55]. Despite the H_2O_2 increase, Pro treatment did not elicit elevation of urinary isoprostanes, which are acknowledged biomarkers of oxidative damage [56]. Moreover, Pro supplementation increased the antioxidant capacity, both in Sal- and AngII-treated animals. Indeed, Pro has several antioxidant properties, such as the ability to scavenge ROS, the protection and upregulation of antioxidant enzymes and the maintenance of proper levels of key redox molecules, such as glutathione and $NADP^+$/NADPH [19]. These antioxidant properties of Pro, along with its positive impact on NO bioavailability, probably counteracted the deleterious effects that could result from an increased H_2O_2 generation. The simultaneous rise of H_2O_2 and TAS, apparently contradictory, has already been reported by Güney et al. in the liver of melatonin-treated young rats [57]. These authors suggested that the total antioxidant capacity increase was an adaptive response to the H_2O_2 rise, probably resultant from the melatonin-induced increase in superoxide dismutase (SOD) activity [57]. Pro has also been shown to elevate SOD activity [58]. Furthermore, as above mentioned, the concomitant administration of Pro and AngII possibly amplified H_2O_2 generation. Thus, an adaptive mechanism to the raised H_2O_2 values could have also contributed to the increased TAS in Pro-Ang rats. Importantly, we detected a significant positive correlation between Pro and antioxidant status and a significant negative correlation between ADMA and urinary antioxidant status. Since the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme responsible for ADMA degradation, has been shown to be inhibited by oxidative stress and rescued by antioxidant treatment [59], it is also possible that the increase in antioxidant status induced by Pro also accounts for the reduction of ADMA concentration.

Several studies have reported a relationship between elevated circulating ADMA concentration and increased cardiovascular risk. ADMA values are associated with reduced urinary excretion of NO metabolites, endothelial dysfunction and intima-media thickening in hypertensive patients [60–62] and correlate positively with blood pressure, even in subjects without any known vascular disease [62,63]. Furthermore, the systemic infusion of ADMA caused significant sodium retention, and



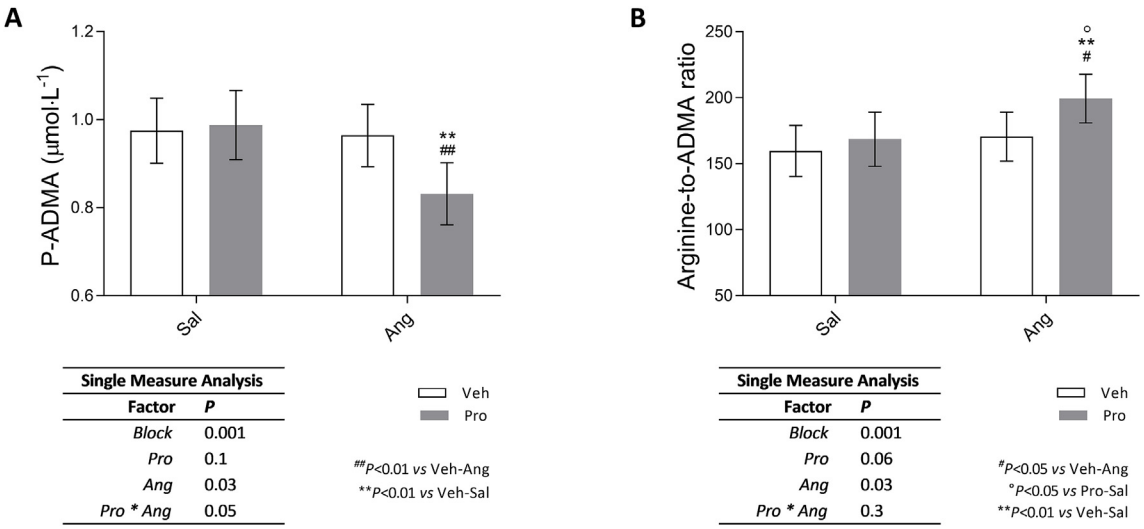
Single Measure Analysis	
Factor	P
Block	< 0.0001
Pro	0.004
Ang	0.04
*Pro * Ang*	0.3

##P<0.01 *vs* Veh-Ang
***P<0.001 *vs* Veh-Sal
°P<0.05 *vs* Pro-Sal

Single Measure Analysis	
Factor	P
Block	< 0.0001
Pro	0.009
Ang	0.07
*Pro * Ang*	0.2

##P<0.01 *vs* Veh-Ang
**P<0.01 *vs* Veh-Sal
°P<0.05 *vs* Pro-Sal

Fig. 5. Mean (95% CI) urinary excretion of nitrates and nitrites (U-NOx) (A, B) for the different experimental groups (Veh-Sal, n = 8; Pro-Sal, n = 7; Veh-Ang, n = 7; Pro-Ang, n = 7) at the end of the experiment (day 21). The parameter is presented as values *per* kg *per* day (A) and values *per* mg creatinine (B). Rank-transformed U-NOx data were analysed with single measure parametric analysis (ANOVA), with 2 experimental factors (Pro and Ang) and a blocking factor. *P*-values obtained from the analysis for each factor and interaction of factors are presented in tables below the graphs. Results from multiple comparison procedures, performed as described in section 2.7, are indicated on the graph.



Single Measure Analysis	
Factor	*P*
Block	0.001
Pro	0.1
Ang	0.03
*Pro * Ang*	0.05

Single Measure Analysis	
Factor	*P*
Block	0.001
Pro	0.06
Ang	0.03
*Pro * Ang*	0.3

Fig. 6. Mean (95% CI) plasma values of ADMA (A) and L-Arginine/ADMA ratio (B) for the different experimental groups (Veh-Sal, n = 9; Pro-Sal, n = 8; Veh-Ang, n = 10; Pro-Ang, n = 10) at the end of the experiment (day 21). Data were analysed with single measure parametric analysis (ANOVA), with 2 experimental factors (Pro and Ang) and a blocking factor. *P*-values obtained from the analysis for each factor and interaction of factors are presented in tables below the graphs. Results from multiple comparison procedures, performed as described in section 2.7, are indicated on the graph.

increased systemic vascular resistance and blood pressure in humans [49], wherefore the modulation of ADMA concentration appears to be an important strategy to increase NO bioavailability, improve blood pressure control and decrease cardiovascular risk [64,65]. Thus, the reduction of ADMA and its association to increased H_2O_2 values and raised antioxidant status in AngII-rats treated with Pro provide new clues for the development of therapeutic strategies to reduce cardiovascular dysfunction.

Chronic Pro treatment has been recently described to promote oxidative damage and metabolic changes in rats [58,66]. However, the plasma concentrations of Pro reached in our Pro-treated animals were lower (0.4–0.6 mmol L^{-1}) than those referred in the aforementioned studies (1.0–2.0 mmol L^{-1}). The dose of Pro used in our study does not seem to cause major metabolic derangements, as evidenced by unaltered levels of glucose, total cholesterol, triglycerides, uric acid and unchanged activity of the hepatic enzymes, AST and ALT. Regarding the effects on renal function, Pro treatment induced an increase in proteinuria, both in saline- and in AngII-infused rats, but did not alter creatinine clearance, used as a glomerular filtration rate (GFR) index. The rise in proteinuria might result from a Pro-induced increase in protein synthesis [67], since a high protein loading has been associated with higher proteinuria [68]. Increased proteinuria could also be due to the production of L-arginine from Pro [23,24], since L-arginine has been suggested to increase albuminuria and proteinuria by reducing tubular protein reabsorption [46,69]. Of note, proteinuria in Pro-treated rats was only significantly different from Veh-Sal rats. The absence of significant differences between Veh-Ang and Pro-Ang rats is in accordance with a study by Rajapakse et al., in which the combined administration of L-arginine and AngII did not alter either albuminuria or proteinuria. Importantly, these authors underlined that, although L-arginine could prevent glomerular damage, it was unable to decrease albuminuria [46]. Nevertheless, a prolonged treatment with Pro may be detrimental, since the long-term increase of protein loading in the kidneys appears to cause renal damage by inducing hyperfiltration, glomerular hypertrophy and glomerular sclerosis [68]. Another problem related with Pro supplementation may be the increase in plasma urea, which in our study was significant only in the Pro-Ang group. Recent studies evidenced that elevated urea concentration may contribute to apoptosis of vascular smooth muscle cells, endothelial damage, adipocyte dysfunction, renal fibrosis, anaemia, breakdown of the intestinal barrier and disordered gut microbiome, thus promoting cardiovascular, metabolic and renal diseases and contributing to an overall accelerated aging phenotype [70]. Furthermore, since urea is the end-product of protein and nitrogen metabolism [70], a higher protein synthesis will contribute to raised urea concentration. Thus, the increases in urea and protein synthesis triggered by a prolonged treatment with Pro may limit its benefits on blood pressure control.

5. Conclusion

The impact of moderate-dose supplementation with Pro on cardiovascular function appears to be beneficial, rather than harmful, as reflected by decreased ADMA levels, increased NO bioavailability, increased antioxidant capacity and reduced blood pressure in AngII-infused rats. Importantly, the inhibitory effect of Pro on ADMA, probably related with the modulation of H_2O_2 and antioxidant status, may open new possibilities to counteract the deleterious impact of ADMA accumulation on cardiovascular function. Nevertheless, caution must be taken regarding the Pro-induced increases on urea and proteinuria.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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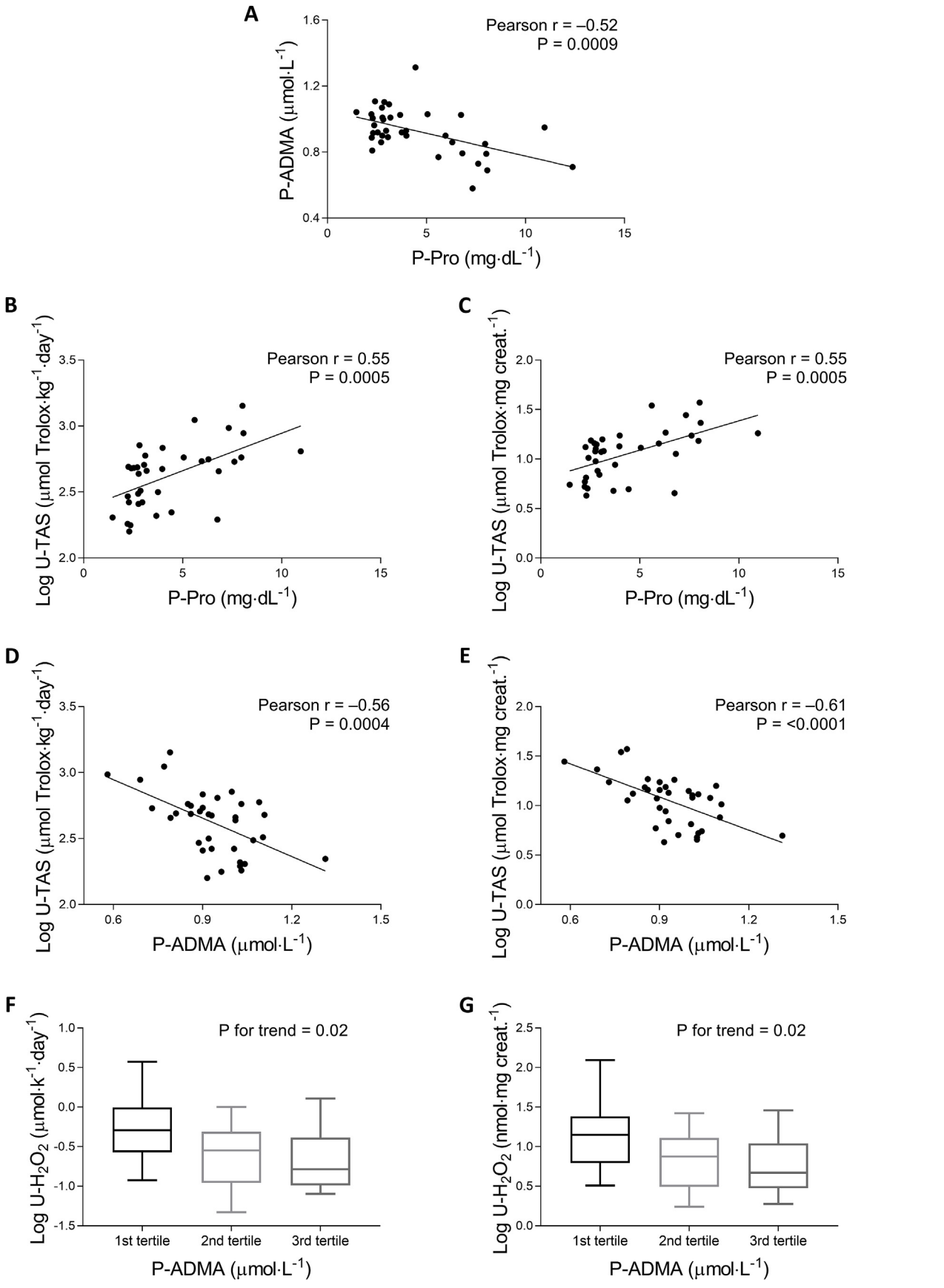

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Fig. 7. Correlations/associations between biomarkers: Pearson's correlations between observed values of plasma Pro and ADMA (A), plasma Pro and U-TAS (logarithm base 10; B, C) and plasma ADMA and U-TAS (logarithm base 10; D, E). Distribution of observed values of U-H_2O_2 (logarithm base 10) by tertiles of P-ADMA (F, G). Tertiles of P-ADMA (≤0.891; 0.900–1.006; > 1.010) were defined based on data from all animals. U-H_2O_2 data are expressed as medians and percentiles 25 and 75. *P*-values for linear trend across groups were calculated using a one-way ANOVA followed by a *post hoc* test for linear trend. Urinary values of TAS and H_2O_2 are presented as values *per* kg *per* day (B, D and F) and values *per* mg creatinine (C, E and G).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.10.007.

References

[1] A.S. Go, D. Mozaffarian, V.L. Roger, E.J. Benjamin, J.D. Berry, W.B. Borden, D.M. Bravata, S. Dai, E.S. Ford, C.S. Fox, S. Franco, H.J. Fullerton, C. Gillespie, S.M. Hailpern, J.A. Heit, V.J. Howard, M.D. Huffman, B.M. Kissela, S.J. Kittner, D.T. Lackland, J.H. Lichtman, L.D. Lisabeth, D. Magid, G.M. Marcus, A. Marelli, D.B. Matchar, D.K. McGuire, E.R. Mohler, C.S. Moy, M.E. Mussolino, G. Nichol, N.P. Paynter, P.J. Schreiner, P.D. Sorlie, J. Stein, T.N. Turan, S.S. Virani, N.D. Wong, D. Woo, M.B. Turner, Heart disease and stroke statistics–2013 update: a report from the American Heart Association, Circulation 127 (2013) e6–e245.

[2] G. Mancia, R. Fagard, K. Narkiewicz, J. Redon, A. Zanchetti, M. Bohm, T. Christiaens, R. Cifkova, G. De Backer, A. Dominiczak, M. Galderisi, D.E. Grobbee, T. Jaarsma, P. Kirchhof, S.E. Kjeldsen, S. Laurent, A.J. Manolis, P.M. Nilsson, L.M. Ruilope, R.E. Schmieder, P.A. Sirnes, P. Sleight, M. Viigimaa, B. Waeber, F. Zannad, 2013 ESH/ESC guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of hypertension (ESH) and of the European Society of cardiology (ESC), J. Hypertens. 31 (2013) 1281–1357.

[3] M. Pereira, N. Lunet, A. Azevedo, H. Barros, Differences in prevalence, awareness, treatment and control of hypertension between developing and developed countries, J. Hypertens. 27 (2009) 963–975.

[4] S. Bo, G. Gruden, E. Charbonnier, M. Martorana, R. Gambino, M. Cassader, L. Gentile, P. Cavallo-Perin, M. Durazzo, High-normal blood pressure and impaired renal function. A prospective study in a population-based cohort, Minerva Med. 105 (2014) 211–219.

[5] S. Lewington, R. Clarke, N. Qizilbash, R. Peto, R. Collins, Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies, Lancet 360 (2002) 1903–1913.

[6] H. Ueda, M. Miyawaki, H. Hiraoka, High-normal blood pressure is associated with new-onset electrocardiographic left ventricular hypertrophy, J. Hum. Hypertens. 29 (2015) 9–13.

[7] M. Baumann, C. Schmaderer, T. Kuznetsova, R. Bartholome, J.F. Smits, T. Richart, H. Struijker-Boudier, J.A. Staessen, Urinary nitric oxide metabolites and individual blood pressure progression to overt hypertension, Eur. J. Cardiovasc. Prev. Rehabil. 18 (2011) 656–663.

[8] A. Albino-Teixeira, P. Soares-da-Silva, Isosorbide 5-mononitrate reverses high blood pressure in NG-nitro-L-arginine methyl ester treated rats, Gen. Pharmacol. 25 (1994) 1329–1336.

[9] M.C. Armas-Padilla, M.J. Armas-Hernandez, B. Sosa-Canache, R. Cammarata, B. Pacheco, J. Guerrero, A.R. Carvajal, R. Hernandez-Hernandez, Z.H. Israili, M. Valasco, Nitric oxide and malondialdehyde in human hypertension, Am. J. Therapeut. 14 (2007) 172–176.

[10] H. Cai, D.G. Harrison, Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress, Circ. Res. 87 (2000) 840–844.

[11] K. Sonoda, K. Ohtake, H. Uchida, J. Ito, M. Uchida, H. Natsume, H. Tamada, J. Kobayashi, Dietary nitrite supplementation attenuates cardiac remodeling in l-NAME-induced hypertensive rats, Nitric Oxide 67 (2017) 1–9.

[12] S.R. Bhatt, M.F. Lokhandwala, A.A. Banday, Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats, Eur. J. Pharmacol. 667 (2011) 258–264.

[13] M. Diebolt, B. Bucher, R. Andriantsitohaina, Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression, Hypertension 38 (2001) 159–165.

[14] K. Mahn, C. Borras, G.A. Knock, P. Taylor, I.Y. Khan, D. Sugden, L. Poston, J.P. Ward, R.M. Sharpe, J. Vina, P.I. Aaronson, G.E. Mann, Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo, FASEB J. 19 (2005) 1755–1757.

[15] S. Gorinstein, M. Zemser, F. Vargas-Albores, J.L. Ochoa, O. Paredes-Lopez, C. Scheler, J. Salnikow, O. Martin-Belloso, S. Trakhtenberg, Proteins and amino acids in beers, their contents and relationships with other analytical data, Food Chem. 67 (1999) 71–78.

[16] D. Long, K.L. Wilkinson, K. Poole, D.K. Taylor, T. Warren, A.M. Astorga, V. Jiranek, Rapid method for proline determination in grape juice and wine, J. Agric. Food Chem. 60 (2012) 4259–4264.

[17] G. Wu, F.W. Bazer, R.C. Burghardt, G.A. Johnson, S.W. Kim, D.A. Knabe, P. Li, X. Li, J.R. McKnight, M.C. Satterfield, T.E. Spencer, Proline and hydroxyproline metabolism: implications for animal and human nutrition, Amino Acids 40 (2011) 1053–1063.

[18] N. Krishnan, M.B. Dickman, D.F. Becker, Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress, Free Radic. Biol. Med. 44 (2008) 671–681.

[19] X. Liang, L. Zhang, S.K. Natarajan, D.F. Becker, Proline mechanisms of stress survival, Antioxid. Redox Signal 19 (2013) 998–1011.

[20] S.K. Natarajan, W. Zhu, X. Liang, L. Zhang, A.J. Demers, M.C. Zimmerman, M.A. Simpson, D.F. Becker, Proline dehydrogenase is essential for proline protection against hydrogen peroxide-induced cell death, Free Radic. Biol. Med. 53 (2012) 1181–1191.

[21] R. Rodriguez, R. Redman, Balancing the generation and elimination of reactive oxygen species, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 3175–3176.

[22] J.M. Phang, W. Liu, O. Zabirnyk, Proline metabolism and microenvironmental stress, Annu. Rev. Nutr. 30 (2010) 441–463.

[23] C. Tomlinson, M. Rafii, R.O. Ball, P.B. Pencharz, Arginine can be synthesized from enteral proline in healthy adult humans, J. Nutr. 141 (2011) 1432–1436.

[24] C. Tomlinson, M. Rafii, M. Sgro, R.O. Ball, P. Pencharz, Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates, Pediatr. Res. 69 (2011) 46–50.

[25] H. Kobori, M. Nangaku, L.G. Navar, A. Nishiyama, The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease, Pharmacol. Rev. 59 (2007) 251–287.

[26] Q. Li, W.E. Dale, E.M. Hasser, E.H. Blaine, Acute and chronic angiotensin hypertension: neural and nonneural components, time course, and dose dependency, Am. J. Physiol. 271 (1996) R200–R207.

[27] J.F. Reckelhoff, J.C. Romero, Role of oxidative stress in angiotensin-induced hypertension, Am. J. Physiol. Regul. Integr. Comp. Physiol. 284 (2003) R893–R912.

[28] G. Simon, G. Abraham, G. Cserep, Pressor and subpressor angiotensin II administration. Two experimental models of hypertension, Am. J. Hypertens. 8 (1995) 645–650.

[29] M.R. Weir, V.J. Dzau, The renin-angiotensin-aldosterone system: a specific target for hypertension management, Am. J. Hypertens. 12 (1999) 205S–213S.

[30] M.S. Zhou, I.H. Schulman, L. Raij, Nitric oxide, angiotensin II, and hypertension, Semin. Nephrol. 24 (2004) 366–378.

[31] J.M. Sasser, N.C. Moningka, M.W. Cunningham Jr., B. Croker, C. Baylis, Asymmetric dimethylarginine in angiotensin II-induced hypertension, Am. J. Physiol. Regul. Integr. Comp. Physiol. 298 (2010) R740–R746.

[32] T. Sousa, S. Oliveira, J. Afonso, M. Morato, D. Patinha, S. Fraga, F. Carvalho, A. Albino-Teixeira, Role of H(2)O(2) in hypertension, renin-angiotensin system activation and renal medullary disfunction caused by angiotensin II, Br. J. Pharmacol. 166 (2012) 2386–2401.

[33] Y. Obayashi, H. Arisaka, S. Yoshida, M. Mori, M. Takahashi, Proline protects liver from D-galactosamine hepatitis by activating the IL-6/STAT3 survival signaling pathway, Amino Acids 43 (2012) 2371–2380.

[34] Y. Tada, N. Yano, H. Takahashi, K. Yuzawa, H. Ando, Y. Kubo, A. Nagasawa, N. Ohashi, A. Ogata, D. Nakae, Toxicological evaluation of L-proline in a 90-day feeding study with Fischer 344 rats, Regul. Toxicol. Pharmacol. 58 (2010) 114–120.

[35] T. Sousa, D. Pinho, M. Morato, J. Marques-Lopes, E. Fernandes, J. Afonso, S. Oliveira, F. Carvalho, A. Albino-Teixeira, Role of superoxide and hydrogen peroxide in hypertension induced by an antagonist of adenosine receptors, Eur. J. Pharmacol. 588 (2008) 267–276.

[36] D. Patinha, J. Afonso, T. Sousa, M. Morato, M. Morato, A. Albino-Teixeira, Diabetes-induced increase of renal medullary hydrogen peroxide and urinary angiotensinogen is similar in normotensive and hypertensive rats, Life Sci. 108 (2014) 71–79.

[37] D. Patinha, J. Afonso, T. Sousa, M. Morato, A. Albino-Teixeira, Activation of adenosine receptors improves renal antioxidant status in diabetic Wistar but not SHR rats, Ups. J. Med. Sci. 119 (2014) 10–18.

[38] T. Quaschning, S. Kocak, C. Bauer, H.H. Neumayer, J. Galle, B. Hocher, Increase in nitric oxide bioavailability improves endothelial function in endothelin-1 transgenic mice, Nephrol. Dial. Transplant. 18 (2003) 479–483.

[39] T. Sousa, E. Fernandes, C. Nunes, J. Laranjinha, F. Carvalho, D. Pinho, M. Morato, A. Albino-Teixeira, Scavenging of nitric oxide by an antagonist of adenosine receptors, J. Pharm. Pharmacol. 57 (2005) 399–404.

[40] A. Popolo, S. Adesso, A. Pinto, G. Autore, S. Marzocco, L-Arginine and its metabolites in kidney and cardiovascular disease, Amino Acids 46 (2014) 2271–2286.

[41] L. Sibal, S.C. Agarwal, P.D. Home, R.H. Boger, The role of asymmetric dimethylarginine (ADMA) in endothelial dysfunction and cardiovascular disease, Curr. Cardiol. Rev. 6 (2010) 82–90.

[42] T. Teerlink, Z. Luo, F. Palm, C.S. Wilcox, Cellular ADMA: regulation and action, Pharmacol. Res. 60 (2009) 448–460.

[43] R.A. Clark, M. Shoaib, K.N. Hewitt, S.C. Stanford, S.T. Bate, A comparison of InVivoStat with other statistical software packages for analysis of data generated from animal experiments, J. Psychopharmacol. 26 (2012) 1136–1142.

[44] P.F. Smith, A note on the advantages of using linear mixed model analysis with maximal likelihood estimation over repeated measures ANOVAs in psychopharmacology: comment on Clark et al, J. Psychopharmacol. 26 (2012) 1605–1607 2012.

[45] L. Hu, R.D. Manning Jr., Role of nitric oxide in regulation of long-term pressure-natriuresis relationship in Dahl rats, Am. J. Physiol. 268 (1995) H2375–H2383.

[46] N.W. Rajapakse, C. De Miguel, S. Das, D.L. Mattson, Exogenous L-arginine

ameliorates angiotensin II-induced hypertension and renal damage in rats, Hypertension 52 (2008) 1084–1090.
[47] Y. Higashi, T. Oshima, R. Ozono, M. Watanabe, H. Matsuura, G. Kajiyama, Effects of L-arginine infusion on renal hemodynamics in patients with mild essential hypertension, Hypertension 25 (1995) 898–902.
[48] A. Palloshi, G. Fragasso, P. Piatti, L.D. Monti, E. Setola, G. Valsecchi, E. Galluccio, S.L. Chierchia, A. Margonato, Effect of oral L-arginine on blood pressure and symptoms and endothelial function in patients with systemic hypertension, positive exercise tests, and normal coronary arteries, Am. J. Cardiol. 93 (2004) 933–935.
[49] J.T. Kielstein, B. Impraim, S. Simmel, S.M. Bode-Boger, D. Tsikas, J.C. Frolich, M.M. Hoeper, H. Haller, D. Fliser, Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans, Circulation 109 (2004) 172–177.
[50] X. Tan, J.K. Li, J.C. Sun, P.L. Jiao, Y.K. Wang, Z.T. Wu, B. Liu, W.Z. Wang, The asymmetric dimethylarginine-mediated inhibition of nitric oxide in the rostral ventrolateral medulla contributes to regulation of blood pressure in hypertensive rats, Nitric Oxide 67 (2017) 58–67.
[51] H. Wang, H. Jiang, H. Liu, X. Zhang, G. Ran, H. He, X. Liu, Modeling disease progression: angiotensin II indirectly inhibits nitric oxide production via ADMA accumulation in spontaneously hypertensive rats, Front. Physiol. 7 (2016) 555.
[52] C.M. Brown, J.O. Becker, P.M. Wise, A.N. Hoofnagle, Simultaneous determination of 6 L-arginine metabolites in human and mouse plasma by using hydrophilic-interaction chromatography and electrospray tandem mass spectrometry, Clin. Chem. 57 (2011) 701–709.
[53] S.M. Kim, Y.G. Kim, K.H. Jeong, S.H. Lee, T.W. Lee, C.G. Ihm, J.Y. Moon, Angiotensin II-induced mitochondrial Nox4 is a major endogenous source of oxidative stress in kidney tubular cells, PLoS One 7 (2012) e39739.
[54] Y. Morales, D.V. Nitzel, O.M. Price, S. Gui, J. Li, J. Qu, J.M. Hevel, Redox control of protein arginine methyltransferase 1 (PRMT1) activity, J. Biol. Chem. 290 (2015) 14915–14926.
[55] M.B. Krishna, A. Joseph, P.L. Thomas, B. Dsilva, S.M. Pillai, M. Laloraya, Impaired arginine metabolism coupled to a defective redox conduit contributes to low plasma nitric oxide in polycystic ovary syndrome, Cell. Physiol. Biochem. 43 (2017) 1880–1892.
[56] T. Sousa, J. Afonso, F. Carvalho, A. Albino-Teixeira, Lipid peroxidation and antioxidants in arterial hypertension, in: A. Catala (Ed.), Lipid Peroxidation, InTech, 2012, pp. 345–392.
[57] Ş. Güney, A. Cumaoğlu, G. Öztürk, K.G. Akbulut, Ç. Karasu, Comparison of melatonin effect on oxidant status and antioxidant capacity in liver and heart of young and aged rats, Int. J. Gerontol. 7 (2013) 45–49.
[58] A.G. Ferreira, A.A. da Cunha, F.R. Machado, C.D. Pederzolli, G.R. Dalazen, A.M. de Assis, M.L. Lamers, M.F. dos Santos, C.S. Dutra-Filho, A.T. Wyse, Experimental hyperprolinemia induces mild oxidative stress, metabolic changes, and tissue adaptation in rat liver, J. Cell. Biochem. 113 (2012) 174–183.
[59] J.P. Cooke, DDAH: a target for vascular therapy? Vasc. Med. 15 (2010) 235–238.
[60] M. Serg, P. Kampus, J. Kals, M. Zagura, P. Muda, T.P. Tuomainen, K. Zilmer, E. Salum, M. Zilmer, J. Eha, Association between asymmetric dimethylarginine and indices of vascular function in patients with essential hypertension, Blood Press. 20 (2011) 111–116.
[61] A. Surdacki, M. Nowicki, J. Sandmann, D. Tsikas, R.H. Boeger, S.M. Bode-Boeger, O. Kruszelnicka-Kwiatkowska, F. Kokot, J.S. Dubiel, J.C. Froelich, Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension, J. Cardiovasc. Pharmacol. 33 (1999) 652–658.
[62] D. Wang, S. Strandgaard, J. Iversen, C.S. Wilcox, Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension, Am. J. Physiol. Regul. Integr. Comp. Physiol. 296 (2009) R195–R200.
[63] H. Miyazaki, H. Matsuoka, J.P. Cooke, M. Usui, S. Ueda, S. Okuda, T. Imaizumi, Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis, Circulation 99 (1999) 1141–1146.
[64] J.O. Lundberg, M.T. Gladwin, E. Weitzberg, Strategies to increase nitric oxide signalling in cardiovascular disease, Nat. Rev. Drug Discov. 14 (2015) 623–641.
[65] L. Rochette, J. Lorin, M. Zeller, J.C. Guilland, L. Lorgis, Y. Cottin, C. Vergely, Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? Pharmacol. Ther. 140 (2013) 239–257.
[66] A.G. Ferreira, E.B. Scherer, A.A. da Cunha, V. Manfredini, G.B. Biancini, C.S. Vanzin, C.R. Vargas, A.T. Wyse, Hyperprolinemia induces DNA, protein and lipid damage in blood of rats: antioxidant protection, Int. J. Biochem. Cell Biol. 54 (2014) 20–25.
[67] J.A. Brunton, M.P. Baldwin, R.A. Hanna, R.F. Bertolo, Proline supplementation to parenteral nutrition results in greater rates of protein synthesis in the muscle, skin, and small intestine in neonatal Yucatan miniature piglets, J. Nutr. 142 (2012) 1004–1008.
[68] A.P. Wakefield, J.D. House, M.R. Ogborn, H.A. Weiler, H.M. Aukema, A diet with 35% of energy from protein leads to kidney damage in female Sprague-Dawley rats, Br. J. Nutr. 106 (2011) 656–663.
[69] E. Bello, C. Caramelo, M.D. López, M.J. Soldevilla, F.R. Gonzalez-Pacheco, A. Rovira, R.G. Delgado, J.M. Alcazar, N. Martell, J. González, L.M. Ruilope, S. Casado, Induction of microalbuminuria by L-Arginine infusion in healthy individuals: an insight into the mechanisms of proteinuria, Am. J. Kidney Dis. 33 (1999) 1018–1025.
[70] W.L. Lau, N.D. Vaziri, Urea, a true uremic toxin: the empire strikes back, Clin. Sci. (Lond.) 131 (2017) 3–12.