

## RESEARCH PAPER

# Arginase inhibition alleviates hypertension in the metabolic syndrome

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metabolic syndrome; aorta; hypertension; arginase; citrulline; norvaline

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## BACKGROUND AND PURPOSE

We have previously shown that arginase inhibition alleviates hypertension associated with in a diabetic animal model. Here, we investigated the protective effect of arginase inhibition on hypertension in metabolic syndrome.

## EXPERIMENTAL APPROACH

Metabolic syndrome was induced in rats by administration of fructose (10% in drinking water) for 12 weeks to induce vascular dysfunction. Three arginase inhibitors (citrulline, norvaline and ornithine) were administered daily in the last 6 weeks of study before and tail BP was recorded in conscious animals. Concentration response curves for phenylephrine (PE), KCl and ACh in addition to ACh-induced NO generation were obtained in thoracic aorta rings. Serum glucose, insulin, uric acid and lipid profile were determined as well as reactive oxygen species (ROS) and arginase activity.

## KEY RESULTS

Arginase activity was elevated in metabolic syndrome while significantly inhibited by citrulline, norvaline or ornithine treatment. Metabolic syndrome was associated with elevations in systolic and diastolic BP, while arginase inhibition significantly reduced elevations in diastolic and systolic BP. Metabolic syndrome increased vasoconstriction responses of aorta to PE and KCl and decreased vasorelaxation to ACh, while arginase inhibition completely prevented impaired responses to ACh. In addition, arginase inhibition prevented impaired NO generation and exaggerated ROS formation in metabolic syndrome. Furthermore, arginase inhibition significantly reduced hyperinsulinaemia and hypertriglyceridaemia without affecting hyperuricaemia or hypercholesterolaemia associated with metabolic syndrome.

## CONCLUSIONS AND IMPLICATIONS

Arginase inhibition alleviates hypertension in metabolic syndrome directly through endothelial-dependent relaxation/NO signalling protection and indirectly through inhibition of insulin resistance and hypertriglyceridaemia.

## Abbreviations

PE, phenylephrine; ROS, reactive oxygen species

## Introduction

Using the World Health Organization definition and data from the National Health and Nutrition Examination Survey, the age-adjusted prevalence of the metabolic syndrome in the United States is estimated at 24% and increases to 44% in adults who are over 60 years (Ford *et al.*, 2002). The metabolic

syndrome is characterized by the variable coexistence of hyperinsulinaemia, obesity, dyslipidaemia and hypertension. The pathogenesis of the syndrome has multiple origins. Obesity and sedentary lifestyle coupled with diet as well as still largely unknown genetic factors clearly interact to produce the syndrome (Kolovou *et al.*, 2005). Insulin resistance, which is a hallmark of metabolic disorders, including

type 2 diabetes mellitus, is also characterized by endothelial dysfunction (Kim *et al.*, 2006). Insulin, beside its essential metabolic action, it has also important vascular action which includes NO production from endothelium leading to vasodilatation and augmentation of glucose disposal in skeletal muscle (Kim *et al.*, 2006). Accordingly, insulin resistance is at most a modest determinant of hypertension and blood pressure (Saad *et al.*, 2004).

Reduced availability of L-arginine to endothelial NOS (eNOS) has been implicated in vascular dysfunction in diabetes and a variety of other disease conditions. Arginase, which metabolizes L-arginine to urea and ornithine, competes directly with NOS for L-arginine. Hence, increases in arginase activity can decrease tissue and cellular arginine levels, reducing its availability to eNOS (Romero *et al.*, 2008). This may lead to decreased NO production and increased production of superoxide by eNOS (Berkowitz *et al.*, 2003). Enhanced arginase activity has been implicated in a number of conditions characterized by vascular dysfunction, including diabetic erectile dysfunction (Bivalacqua *et al.*, 2001), pulmonary hypertension (Morris *et al.*, 2003) and coronary artery dysfunction (Zhang *et al.*, 2004). During diabetes, impaired vascular function is closely associated with oxidative stress and vascular inflammation (Romero *et al.*, 2008; El-Bassossy *et al.*, 2011a), both of which have been associated with increases in arginase activity and expression (Bivalacqua *et al.*, 2001). In addition, increased arginase activity has been reported in diabetic patients and can be reduced by insulin treatment (Kashyap *et al.*, 2008). Furthermore, arginase inhibition restores *in vivo* coronary microvascular function in type 2 diabetic Goto Kakizaki rats (Gronros *et al.*, 2011) and protects from renal injury in a streptozotocin model of diabetes (Morris *et al.*, 2011).

In addition to the production of urea, arginase is also involved in the biosynthesis of polyamines and the amino acids, ornithine, proline and glutamate (Cederbaum *et al.*, 2004). As such, it is not surprising that amino acids such as ornithine, norvaline and citrulline inhibit arginase (Hunter and Downs, 1945).

Therefore, the aim of the present work was to examine the potentially protective effect of the arginase inhibition in the metabolic syndrome and illustrate the mechanism of this protective effect.

## Materials and methods

### Animals

Studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010), and carried out in strict accordance with institutional animal care and use guidelines consistent with UK standards of care. Seventy-two male Wistar rats (6 weeks age; Zagazig University, Zagazig, Egypt) were used in the present study and housed in standard light/dark cycles with access to standard rat diet *ad libitum*.

### Study protocol

Animals were randomly divided into five experimental groups (eight animals each): control, metabolic syndrome

(M), citrulline-treated metabolic syndrome (M-citrulline), norvaline-treated metabolic syndrome (M-norvaline) and ornithine-treated metabolic syndrome (M-ornithine). In addition, the effect of arginase inhibitors on control was studied in three groups: citrulline-treated control, norvaline-treated control and ornithine-treated control. Metabolic syndrome was maintained by giving fructose 10% in drinking water *ad libitum* daily for 12 weeks. After 6 weeks, treated rats were received citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) and ornithine (200 mg·kg<sup>-1</sup>) treatment as solution (0.8–1 mL) in distilled water by orogastric gavage for 6 weeks of study while control and resistant groups receive water as a vehicle instead. The doses of citrulline, norvaline and ornithine were chosen based upon their reported arginase inhibiting activity (Kang *et al.*, 2000; Romero *et al.*, 2008) and confirmed by arginase activity measurement.

At the end of the study and 12 h after the last gavage, blood pressure was measured. Rats were then terminally anaesthetized with diethyl ether and blood was collected from the retro-orbital plexus and centrifuged (3000× g, 4°C, 20 min) to separate serum that was analysed for glucose, uric acid, lipid profile and arginase activity. Then, through opening the abdomen, descending thoracic aorta was carefully excised and placed in a Petri dish filled with cold Krebs–Henseleit buffer containing (in mM): NaCl 118.1, KCl 4.69, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, glucose 11.7, MgSO<sub>4</sub> 0.5 and CaCl<sub>2</sub> 2.5. The aorta was cleaned of excess connective tissue and fat and cut into rings of approximately 3 mm in length. For each animal, one aortic ring was suspended in an organ bath for studying vascular reactivity while the second was used to study ACh-induced NO generation and the third for measuring the reactive oxygen species (ROS) content. In order to study the acute effect on vascular reactivity, aorta from resistant animals were incubated in organ bath with arginase inhibitors; citrulline, norvaline or ornithine (all at 1 mM) for 1 h before studying vascular reactivity. These concentrations and time were chosen based upon those used in previous reports (Fuentes *et al.*, 1994; Huynh *et al.*, 2009) plus our preliminary data. In order to investigate the effect of hyperuricaemia on vascular reactivity, aortae or kidney isolated from normal animals were incubated with uric acid (200 μM), arginase inhibitors or L-arginine for 1 h before studying the effect on vascular reactivity or arginase activity. The uric acid incubation concentration was chosen based upon the serum level of uric acid in animals with metabolic syndrome observed in our study.

### Arginase activity

Arginase activity was measured in serum of chronically treated animals and in the incubation buffer of isolated aorta after *in vitro* treatment by the colorimetric determination of the arginase enzyme product, urea (Mendez *et al.*, 2002). Briefly, 100 μL serum or the incubation buffer was incubated for 1 h with reaction mixture containing the activator (manganese sulphate) and substrate (arginine) in carbonate buffer (pH 9.5). The produced urea was condensed with diacetylmonoxime in an acid medium in the presence of ferric chloride (oxidant) and carbazide (accelerator). Then, the absorbance was measured at 525 nm by 6105 UV/Vis spectrophotometer (Jenway®, Essex, UK). For the *in vitro*

experiments, the measured activities were normalized to the wet weight of the aortic rings.

### Serum analysis

Serum glucose was determined colorimetrically using a Randox reagent kit (Antrim, UK). Triglycerides (TGs) and total cholesterol were estimated enzymatically using Boehringer Mannheim® colorimetric kit (Mannheim, Germany). Uric acid was measured colorimetrically by uricase method; uric acid is converted into hydrogen peroxide, which forms a red-coloured quinoneimine dye measured at 520 nm. (Fossati *et al.*, 1980).

Serum insulin level was assayed by sandwich ELISA (Millipore, Cairo, Egypt) that uses microtiter plate coated with mouse monoclonal anti-rat insulin antibodies. The estimated insulin resistance index (HOMA-IR) was calculated using the serum fasting glucose and insulin levels according to the following equation (Matthews *et al.*, 1985):  $\text{HOMA-IR} = \text{glucose concentration (mmol}\cdot\text{L}^{-1}) \times \text{insulin } (\mu\text{U}\cdot\text{L}^{-1})/22.5$ .

### BP measurement

BP was measured indirectly in a conscious and slightly restrained rat by the tail cuff method (El-Bassossy *et al.*, 2012b) using non-invasive blood pressure system attached to a computerized data acquisition system (ADI Instruments, Cairo, Egypt). For these measurements, rats were conditioned to the restraint and the warming chamber for 10–20 min·day<sup>-1</sup> for at least 3 days before measurements. BP measurements were performed from 7:00 a.m. to 12:00 p.m. by the same investigator. After 5–10 min of stabilization in a warming chamber (35°C), a typical run involved 10 repetitions of the automated inflation–deflation cycle. The mean of six readings within a 5–10 mmHg range was taken as the BP.

### Vascular reactivity

Vascular reactivity was studied using the isolated artery technique (El-Bassossy *et al.*, 2009). Thoracic aorta rings were suspended under 8 mN resting tension in individual 30 mL organ chambers containing Krebs–Henseleit buffer at 37°C and aerated with 95% oxygen, 5% carbon dioxide. Ring tension was determined by use of an isometric force transducer (ADI Instruments, Cairo, Egypt). Force displacement was recorded with a PowerLab Data Interface Module connected to a PC running Chart software v7 (ADI Instruments). Rings were equilibrated for 60 min during which time the bath solution was changed every 30 min. Before beginning the experiment, vessel viability was assessed by exposing arteries to KCl (80 mM). This was repeated until stable responses were achieved (usually two exposures). For studying the vasoconstrictor responsiveness of aorta, cumulative concentrations of phenylephrine (PE, 10<sup>-9</sup> to 10<sup>-5</sup> M) or KCl (10 to 100 mM) were added to the organ bath and the response was recorded. For studying the vasodilator responsiveness of aorta, rings were first pre-contracted with submaximal concentrations of PE (3 × 10<sup>-7</sup> M in the case of the diabetic group and 10<sup>-6</sup> M in other groups). The submaximal concentration of PE was chosen to give similar precontraction in all studied groups. The cumulative concentrations of acetylcholine (ACh, 10<sup>-9</sup> to 10<sup>-5</sup> M) were then added to the organ bath and the response was recorded. At the end of

experiment, aortic rings were removed and weighted. The recorded responses were normalized to the wet weight of the used aortic rings.

### Microvascular reactivity

In order to support our vascular reactivity findings, part of the experiments was repeated in a microvascular system using the isolated perfused kidney. The rat kidney was isolated and perfused as previously described (El-Mas *et al.*, 2011) with slight modifications. Briefly, rats were killed by cervical dislocation followed by decapitation, the abdomen was opened by a midline incision and the left kidney was exposed. The left renal artery was dissected free from the surrounding fat. Loose ties were made around the renal artery. A bevelled 18-gauge needle connected to a 5 mL syringe filled with heparinized saline (100 U·mL<sup>-1</sup>) was used for cannulation. The left renal artery was cannulated via an incision made in the aorta. The cannula was immediately secured with ligatures and the kidney was flushed with heparinized saline and rapidly excised from its surrounding tissues. The kidney was transferred into a jacketed glass chamber maintained at 37°C and continuously perfused with Krebs–Henseleit buffer aerated with 95% oxygen, 5% carbon dioxide. Kidney perfusion was adjusted at a constant flow rate of 5 mL·min<sup>-1</sup> using a Masterflex® peristaltic pump (Cole-palmer, Jubail, Saudi Arabia) and the venous effluent was allowed to drain freely. The kidney perfusion pressure was continuously monitored by means of a Gould–Statham pressure transducer distal to the pump and recorded using a PowerLab Data Interface Module connected to a PC running Chart software v7 (ADI Instruments). As the renal flow was kept constant, changes in perfusion pressure were indicative of alterations in renal microvascular resistance. A 30 min equilibration period was allowed at the beginning of the experiment to ensure stabilization of the kidney perfusion pressure. For studying the renal microvascular relaxation, isolated kidneys were first continuously perfused with PE (10 μM). After another 30 min equilibration, vasodilatory responses of the renal vasculature to bolus doses injections of ACh (0.1, 1, 100, 500 nmol) were established. After washout, uric acid (200 μM) was added to the perfusion medium for another 1 h. Then, the renovascular dilatation to ACh was repeated. After wash out, citrulline was added to the perfusion medium for 30 min and the renovascular dilatation to ACh was investigated.

### ACh-induced NO generation

The real-time generation of intracellular NO following ACh stimulation was investigated with the fluorescence probes 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) according to the method described by El-Bassossy *et al.* (2011b; 2012a). The aorta rings isolated from chronically treated animals were loaded for 30 min at 37°C with 5 mM of DAF-FM diacetate plus 0.1% Pluronic F-127 in Krebs–Henseleit buffer containing 25 mM HEPES. Aorta rings were washed twice and then cut and placed in a chamber designed especially for fluorescence measurement in aortic segments with the endothelial side up. 100 μL of Krebs–Henseleit buffer containing HEPES were added quickly to the chamber. DAF fluorescence was measured using a LS45 fluo-

rescence spectrophotometer (PerkinElmer®, Cairo, Egypt) with remote fibre optic, which allows measurement of tissue fluorescence in the sample chamber. Readings ( $\lambda_{ex} = 485$ ,  $\lambda_{em} = 515$  nm) were taken before and every 5 s after ACh ( $10^{-4}$  M) addition. The NO donor diethyl amine NONOate ( $100$  nM) was used as a positive control for DAF.

### Intracellular of ROS generation

The intracellular levels of ROS were determined by the fluorescent probe dichlorofluorescein diacetate (DCF-DA) according to the method described previously by (Ugochukwu and Cobourne, 2003) with slight modification. Frozen aortae were homogenized in 20 parts (w/v) of 0.32 M sucrose solution in ice and the centrifuged at 2000 rpm at 4°C for 15 min. Then, 100  $\mu$ L of the supernatant was added to 1900  $\mu$ L 18:1 40 mM·L<sup>-1</sup> Tris (pH 7.4): HEPES buffer (10 mM HEPES, 120 mM NaCl, 2.5 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 6 mM glucose, and 1 mM CaCl<sub>2</sub>) containing 5  $\mu$ M DCF-DA plus 0.1% Pluronic F-127. The fluorescence was measured ( $\lambda_{ex} = 485$ ,  $\lambda_{em} = 515$  nm) before and after 2 h of incubation at 37°C. The difference in fluorescence per hour was used as indicative for ROS formation.

### Drugs and chemicals

The following drugs and chemicals were used: STZ, ACh, PE, SNP, L-Norvaline, L-Ornithine, L-citrulline (Sigma-Aldrich, Munich, Germany); uric acid (Oxford Laboratory, Mumbai, India). All the used chemicals were dissolved in double distilled water.

### Statistical analysis

All data are expressed as mean  $\pm$  SEM. Statistical analysis was performed by ANOVA followed by Newman–Keuls' *post hoc* test using a computer-based curve fitting program (Prism 5, Graphpad, San Diego, CA, USA). Correlation was calculated using Pearson's correlation coefficient.

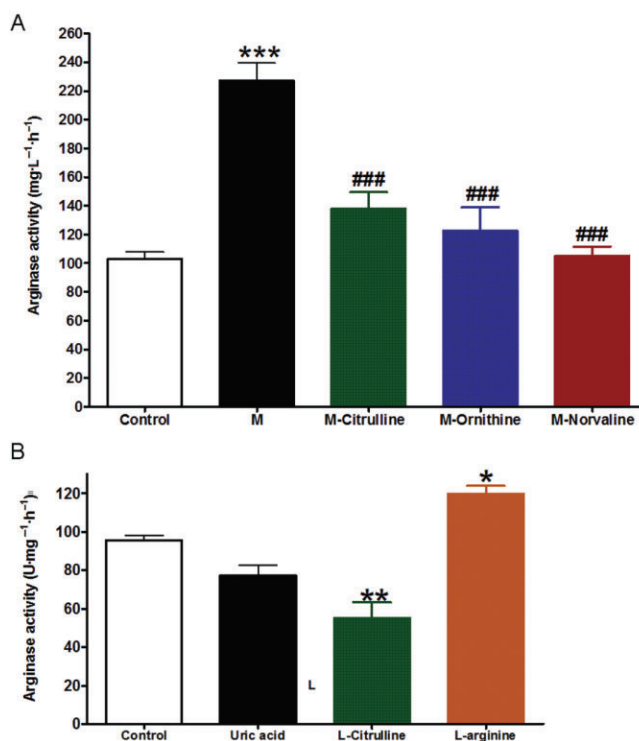
## Results

### Arginase activity

Fructose administration (10% in drinking water) was associated by a significant elevation in serum arginase activity compared to control ( $P < 0.001$ , Figure 1A) while this activation of arginase enzyme was significantly inhibited by all the arginase inhibitors used: citrulline, norvaline and ornithine ( $P < 0.001$ , Figure 1A). *In vitro* incubation of aortae isolated from normal animals with uric acid (200  $\mu$ M, 1 h) did not significantly affected arginase activity. However, incubation with citrulline (1 mM, 1 h) significantly inhibited arginase activity in isolated aorta compared with control ( $P < 0.01$ , Figure 1B). On the other hand, incubation with L-arginine (1 mM, 1 h) led to a significant increase in arginase activity compared with control ( $P < 0.05$ , Figure 1B).

### Serum parameters

Fructose administration for 12 weeks led to a significant elevation in blood glucose and insulin levels and the insulin resistance index (all at  $P < 0.001$ ) compared with control



**Figure 1**

Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and daily oral administration (last 6 weeks) of citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) or ornithine (200 mg·kg<sup>-1</sup>) on serum arginase activity (A) or the effect of *in vitro* incubation with uric acid (400  $\mu$ M, 1 h), citrulline (1 mM, 1 h) or arginine (1 mM, 1 h) on aortic arginase activity (B). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding M group values; by one-way ANOVA and Newman–Keuls *post hoc* test.

(Table 1). Arginase inhibition by citrulline, norvaline or ornithine significantly inhibited the developed hyperglycaemia, hyperinsulinaemia and insulin resistance associated with fructose administration (all at  $P < 0.001$ , Table 1). Fructose administration was also associated with hypertriglyceridaemia ( $P < 0.001$ ), hypercholesterolaemia ( $P < 0.001$ ) and hyperuricaemia ( $P < 0.001$ ). While arginase inhibition by citrulline, norvaline or ornithine completely prevented the developed hypertriglyceridaemia (all at  $P < 0.001$ ), it did not significantly affect the developed hypercholesterolaemia or hyperuricaemia (Table 1). In addition, there were strong statistically significant correlations between arginase activity (as shown in Figure 1) and each of the following: insulin resistance index ( $r = 0.71$ ,  $P < 0.01$ ) and triglycerides ( $r = 0.81$ ,  $P < 0.001$ ) in all experimental groups.

### Blood pressure

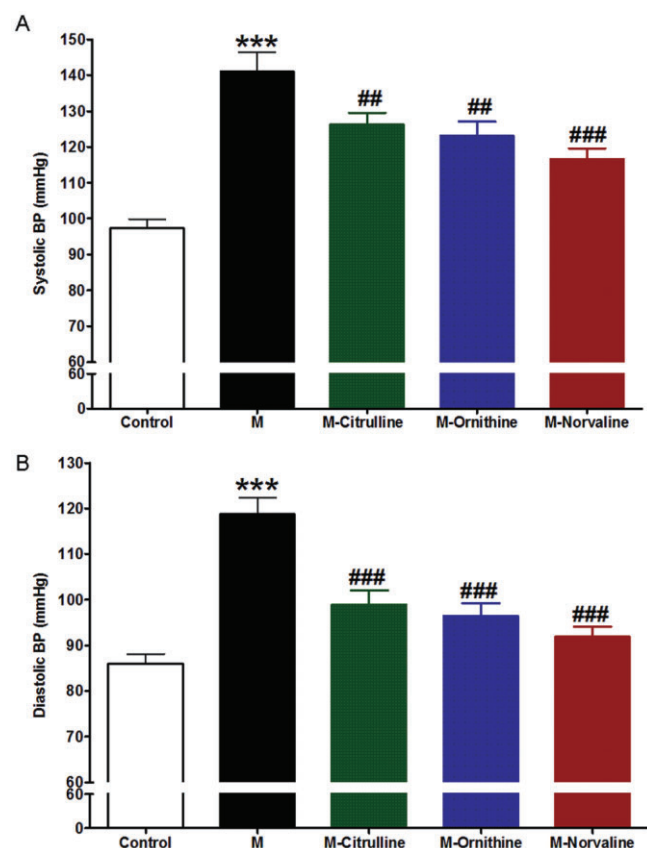
Fructose administration was associated with significant elevations in systolic and diastolic BP (both at  $P < 0.001$ , Figure 2). The elevation in systolic BP was significantly inhibited by citrulline ( $P < 0.01$ ), ornithine ( $P < 0.01$ ) and norvaline ( $P < 0.001$ ) treatment while the elevated diastolic BP was signifi-

**Table 1**

Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and daily oral administration (last 6 weeks) of citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) or ornithine (200 mg·kg<sup>-1</sup>) on serum levels of glucose and insulin, insulin resistance (IR) index, triglycerides, total cholesterol and uric acid

Treatment	Glucose (mg·ml <sup>-1</sup> )	Insulin (μg·l <sup>-1</sup> )	IR index	Triglycerides (mg·ml <sup>-1</sup> )	Total cholesterol (mg·ml <sup>-1</sup> )	Uric acid (mM)
Control	6.1 ± 0.6	3.4 ± 0.4	0.5 ± 0.1	9.4 ± 0.8	5.7 ± 0.7	0.4 ± 0.03
M	12.0 ± 0.7***	13.0 ± 0.9***	3.9 ± 0.6***	16.5 ± 2.3***	13.8 ± 1.0***	3.2 ± 0.1***
M-Citrulline	6.9 ± 0.6###	5.2 ± 0.6###	0.9 ± 0.1###	7.6 ± 0.5###	13.6 ± 0.9	3.1 ± 0.2
M-Ornithine	6.8 ± 1.1###	5.4 ± 1.1###	1.0 ± 0.3###	4.7 ± 0.4###	13.3 ± 1.7	2.3 ± 0.2
M-Norvaline	8.8 ± 0.4##	5.6 ± 1.3###	1.2 ± 0.2###	7.7 ± 0.8###	11.9 ± 1.0	2.8 ± 0.2

Values are expressed as the mean ± SE of mean; *n* = 8 animals; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, compared with the corresponding control group values; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 compared with the corresponding metabolic syndrome group values; by one-way ANOVA and Newman–Keuls *post hoc* test.

**Figure 2**

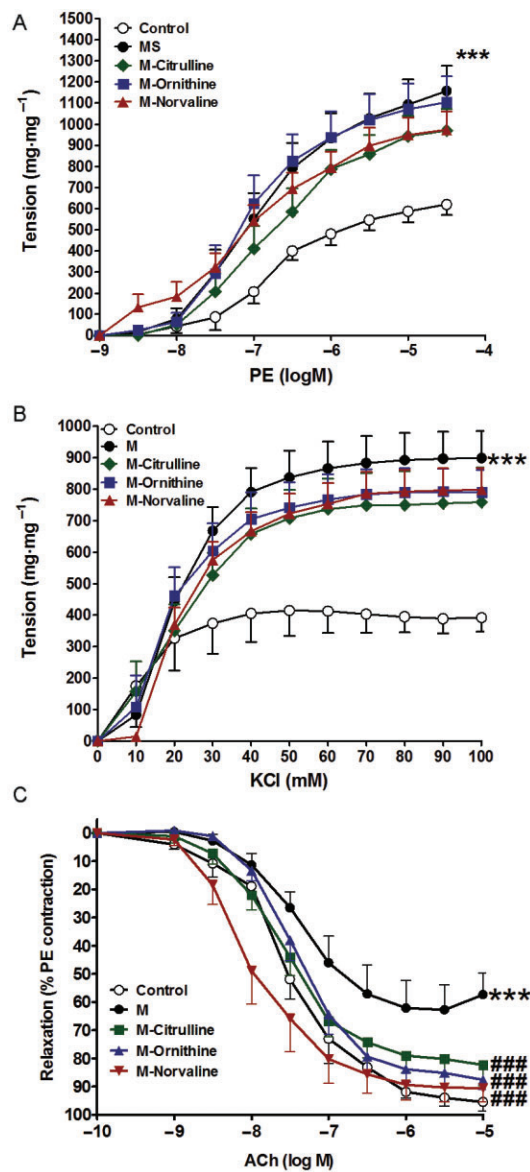
Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and daily oral administration (last 6 weeks) of citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) or ornithine (200 mg·kg<sup>-1</sup>) on the systolic (A) and diastolic (B) blood pressure. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, compared with the corresponding control group values; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 compared with the corresponding M group values; by one-way ANOVA and Newman–Keuls *post hoc* test.

cantly inhibited by all the arginase inhibitors used (all at *P* < 0.001, Figure 2). In addition, diastolic BP significantly correlated with arginase activity (*r* = 0.66, *P* < 0.001).

### Vascular reactivity

Cumulative addition of PE (10<sup>-9</sup> to 10<sup>-5</sup> M) or KCl (10<sup>-2</sup> to 10<sup>-1</sup> M) to the organ bath resulted in concentration-dependent contractions of aorta in all the groups (Figure 3). Metabolic syndrome induced by fructose (10% in drinking water for 12 weeks) resulted in a large increase in aorta responsiveness to PE, reflected by a significant increase in apparent *E*<sub>max</sub> (*P* < 0.001, Figure 3A, Table 2). *In vivo* treatment of animals with the arginase inhibitors citrulline, ornithine or norvaline had no significant effect on the response of isolated aorta to PE (Figure 3A, Table 2). Metabolic syndrome was associated with an increase in the aortic responsiveness to KCl as shown by a significant increase in the apparent *E*<sub>max</sub> (*P* < 0.001, Figure 3B, Table 2). Similarly, *in vivo* arginase inhibition by citrulline, ornithine or norvaline did not significantly affect the response of isolated aorta to KCl (Figure 3B, Table 2). Cumulative addition of ACh (10<sup>-9</sup> to 10<sup>-5</sup> M) to the organ bath resulted in concentration-related decreases in the tension of aortic artery rings pre-contracted with PE (Figure 3C, Table 2). Metabolic syndrome was associated with a large decrease in aorta responsiveness to ACh, reflected by a significant decrease in apparent *E*<sub>max</sub> (*P* < 0.01) compared with the control (Figure 3C, Table 2). However, this impairment in isolated aorta response to ACh was prevented by *in vivo* treatment with citrulline, ornithine or norvaline (all at *P* < 0.001, Figure 3C, Table 2).

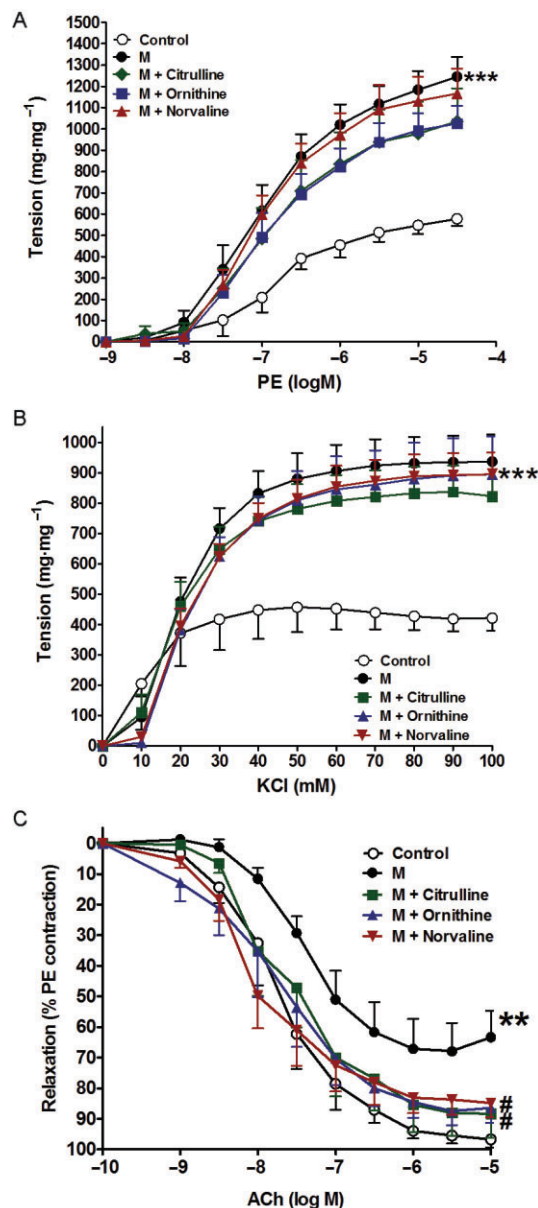
Incubation of isolated aorta from metabolic syndrome animals with any of the arginase inhibitors (citrulline, norvaline or ornithine, all at 1 mM for 1 h) did not affect the aortic hyperresponsiveness to PE (Figure 4A, Table 3). Similarly, none of the arginase inhibitors used affected the metabolic syndrome-induced aortic hyperresponsiveness to KCl (Figure 4B). In contrast, the impairment in response to ACh in aorta isolated from animals with metabolic syndrome (*P* < 0.01, Figure 4C, Table 3) was corrected by arginase inhibition with citrulline (*P* < 0.05) or ornithine (*P* < 0.05, Figure 4C, Table 3).



**Figure 3**

Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and daily oral administration (last 6 weeks) of citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) or ornithine (200 mg·kg<sup>-1</sup>) on the isolated aorta responsiveness to PE (A), KCl (B) and ACh (C). Symbols indicate mean ± SEM for  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding M group values; by one-way ANOVA and Newman-Keuls *post hoc* test.

In experiments designed to assess the effect of uric acid on tissue responsiveness, incubation of isolated aorta from a normal animal for 1 h with uric acid (200 μM) had no significant effect on the aortic response to PE (Figure 5A, Table 4) or to KCl (Figure 5B, Table 4) compared to control. However, it significantly impaired the relaxation response to ACh ( $P < 0.001$ , Figure 5C, Table 4) compared to control. This impaired response to ACh due to incubation with uric acid



**Figure 4**

Effect of *in vitro* incubation with citrulline (1 mM, 1 h), norvaline (1 mM, 1 h) or ornithine (1 mM, 1 h) on the isolated metabolic syndrome (M) aorta responsiveness to PE (A), KCl (B) and ACh (C). Symbols indicate mean ± SEM for  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding M group values; by one-way ANOVA and Newman-Keuls *post hoc* test.

was significantly prevented by co-incubation with any of the arginase inhibitors used (citrulline, ornithine and norvaline all at 1 mM and  $P < 0.05$ , Figure 5C, Table 4).

### Microvascular reactivity

Cumulative injection of ACh, in doses of 0.1 to 500 nmol, resulted in dose-related decreases in the perfusion pressure of isolated kidney pre-contracted with PE (Figure 5D). Perfusion

**Table 2**

Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and daily oral administration (last 6 weeks) of citrulline (50 mg·kg<sup>-1</sup>), norvaline (50 mg·kg<sup>-1</sup>) or ornithine (200 mg·kg<sup>-1</sup>) on the maximal response ( $E_{max}$ ) and pD<sub>2</sub> (-Log EC<sub>50</sub>) or EC<sub>50</sub> values of PE, KCl and ACh dose response curves

Treatment	KCl		PE		ACh	
	$E_{max}$	EC <sub>50</sub>	$E_{max}$	pD <sub>2</sub>	$E_{max}$	pD <sub>2</sub>
Control	401.8 ± 29.0	8.3 ± 13.7	598.2 ± 25.8	6.7 ± 0.1	94.3 ± 2.6	7.5 ± 0.1
M	882.7 ± 32.4***	19.4 ± 3.7	1091.0 ± 53.0***	7.0 ± 0.1	61.3 ± 4.1***	7.4 ± 0.2
M-Citrulline	757.1 ± 45.1	17.4 ± 10.1	933.9 ± 46.7	6.8 ± 0.1	81.2 ± 3.2###	7.6 ± 0.1
M-Ornithine	779.2 ± 33.3	16.7 ± 5.4	1063.0 ± 54.7	7.1 ± 0.2	86.3 ± 2.7###	7.4 ± 0.1
M-Norvaline	777.5 ± 25.4	20.6 ± 3.1	925.9 ± 38.4	7.1 ± 0.1	89.5 ± 3.9###	8.0 ± 0.1##

Values are expressed as the mean ± SE of mean;  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding MS group values; by one-way ANOVA and Newman-Keuls *post hoc* test.

**Table 3**

Effect of *in vitro* incubation with citrulline (1 mM, 1 h), norvaline (1 mM, 1 h) or ornithine (1 mM, 1 h) on the maximal response ( $E_{max}$ ) and pD<sub>2</sub> (-Log EC<sub>50</sub>) or EC<sub>50</sub> values of PE, KCl and ACh dose response curves of the isolated metabolic syndrome (M) aorta

Treatment	KCl		PE		ACh	
	$E_{max}$	EC <sub>50</sub>	$E_{max}$	pD <sub>2</sub>	$E_{max}$	pD <sub>2</sub>
Control	437.2 ± 29.4	8.3 ± 11.0	567.4 ± 37.3	6.8 ± 0.1	95.8 ± 3.9	7.7 ± 0.1
M	920.6 ± 32.0***	19.1 ± 3.4	1225.0 ± 70.9***	7.0 ± 0.1	66.5 ± 4.1**	7.4 ± 0.1
M + Citrulline	818.1 ± 31.4	17.6 ± 4.2	1016.0 ± 97.9	7.0 ± 0.2	88.4 ± 6.2#	7.7 ± 0.2
M + Ornithine	868.0 ± 39.4	21.5 ± 4.1	1014.0 ± 60.7	7.0 ± 0.1	89.1 ± 6.3#	7.8 ± 0.2
M + Norvaline	877.0 ± 25.8	21.1 ± 2.9	1142.0 ± 58.7	7.0 ± 0.1	83.1 ± 3.9	8.1 ± 0.1

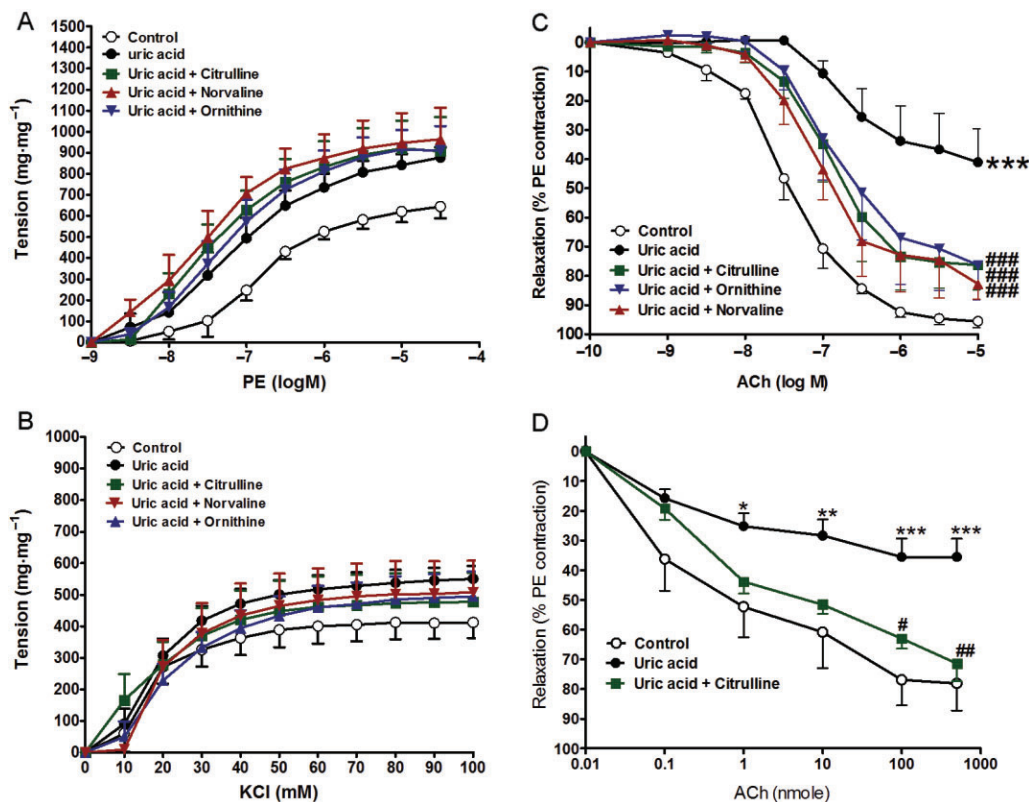
Values are expressed as the mean ± SE of mean;  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding MS group values; by One Way ANOVA and Newman-Keuls *post hoc* test.

**Table 4**

Effect of *in vitro* incubation with uric acid (400 μM, 1 h) with or without citrulline (1 mM, 1 h), norvaline (1 mM, 1 h) or ornithine (1 mM, 1 h) on the maximal response ( $E_{max}$ ) and pD<sub>2</sub> (-Log EC<sub>50</sub>) or EC<sub>50</sub> values of PE, KCl and ACh dose response curves of the isolated normal aorta

Treatment	KCl		PE		ACh	
	$E_{max}$	EC <sub>50</sub>	$E_{max}$	pD <sub>2</sub>	$E_{max}$	pD <sub>2</sub>
Control	437.2 ± 29.4	15.5 ± 5.5	609.3 ± 36.4	-6.8 ± 0.1	95.3 ± 2.2	7.4 ± 0.1
Uric acid	534.2 ± 19.3	15.4 ± 5.8	879.8 ± 66.4**	-7.2 ± 0.2	38.9 ± 5.4***	6.7 ± 0.2*
Uric acid + citrulline	478.6 ± 46.6	-2.2 ± 65.3	915.4 ± 72.1	-7.6 ± 0.3	76.9 ± 5.7###	6.9 ± 0.1
Uric acid + ornithine	484.3 ± 30.0	17.9 ± 10.4	920.1 ± 65.5	-7.3 ± 0.2	74.9 ± 8.5###	6.9 ± 0.2
Uric acid + norvaline	490.3 ± 33.7	19.2 ± 5.9	962.8 ± 79.3	-7.7 ± 0.4	78.8 ± 5.3###	7.1 ± 0.1

Values are expressed as the mean ± SE of mean;  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding uric acid group values; by one-way ANOVA and Newman-Keuls *post hoc* test.



**Figure 5**

Effect of *in vitro* incubation with uric acid (200  $\mu$ M, 1 h) with or without citrulline (1 mM, 1 h), norvaline (1 mM, 1 h) or ornithine (1 mM, 1 h) on the isolated normal aorta responsiveness to PE (A), KCl (B) and ACh (C) or on the isolated perfused kidney relaxation to ACh (D). Symbols indicate mean  $\pm$  SEM for  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding uric acid values; by one-way ANOVA and Newman-Keuls *post hoc* test.

of isolated normal kidney with buffer containing uric acid (200  $\mu$ M) for 1 h led to a significant reduction in the renovascular relaxation response to ACh ( $P < 0.001$ , Figure 5D). This impaired response to ACh due to incubation with uric acid was significantly inhibited by 30 min perfusion with buffer containing 1 mM citrulline ( $P < 0.01$ , Figure 5D).

### ACh-induced NO generation

Addition of ACh (10<sup>-4</sup>M) to DAF-loaded aorta from normal animals induced a strong increase in NO production, as assessed by increased fluorescence. However, metabolic syndrome significantly inhibited the ACh-stimulated NO release compared to control animals, reflected by a significantly reduced apparent  $E_{max}$  ( $P < 0.001$ , Figure 6A). Interestingly, this impaired aortic NO generation was completely prevented by arginase inhibition by citrulline ( $P < 0.001$ ), ornithine ( $P < 0.05$ ) or norvaline ( $P < 0.01$ , 6A).

### Intracellular ROS generation

Aorta isolated from animals with metabolic syndrome had a significantly increased ROS generation ( $P < 0.001$ , Figure 6B) compared to control. Interestingly all the arginase inhibitors (citrulline, norvaline and ornithine) significantly decreased ROS generation (all at  $P < 0.001$ , Figure 6B). Moreover, we found strong correlations between ROS generation and argi-

nase activity ( $r = 0.75$ ,  $P < 0.001$ ) and between the ROS generation and the insulin level ( $r = 0.67$ ,  $P < 0.01$ ).

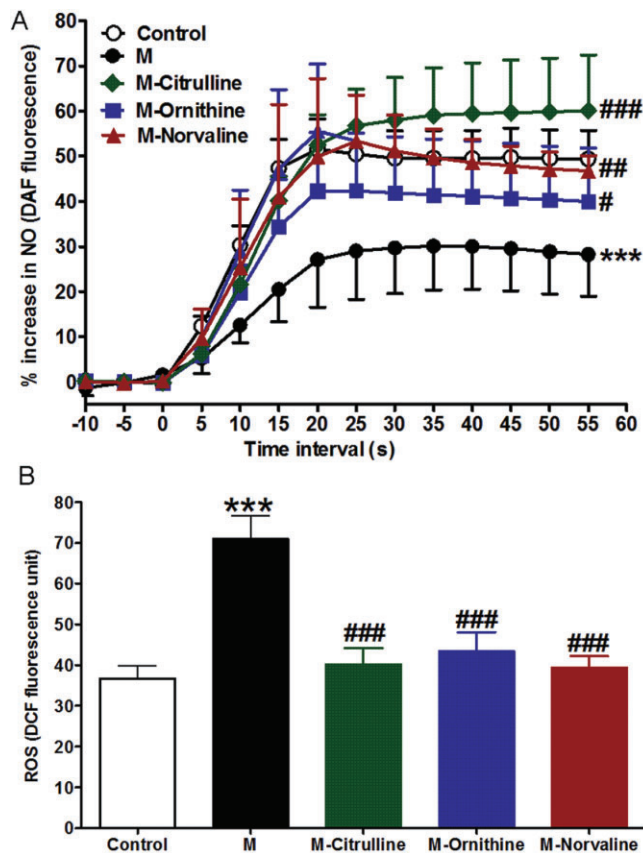
### Control experiments

None of the used arginase inhibitors citrulline, norvaline or ornithine affected the previously mentioned parameters compared to control when given to normal animals for the same periods of time (data not shown).

## Discussion and conclusions

The current study is the first to report on the potential protective effect of arginase inhibition against the hypertension associated with metabolic syndrome. The arginase inhibitors citrulline, ornithine or norvaline virtually inhibited the development of hypertension in fructose-induced metabolic syndrome in rats. The following findings can explain the ability of arginase inhibition to counteract hypertension accompanying metabolic syndrome: (i) arginase inhibition ameliorated the impaired ACh-induced relaxations associated with metabolic syndrome; (ii) the impaired NO and increased ROS generation in metabolic syndrome were abrogated by arginase inhibition; and (iii) arginase inhibition prevented the development of insulin resistance and hypertriglyceridaemia.





**Figure 6**

Effect of fructose-induced metabolic syndrome (M, 10% in drinking water, for 12 weeks) and chronic daily oral administration (last 6 weeks) of citrulline ( $50 \text{ mg}\cdot\text{kg}^{-1}$ ), norvaline ( $50 \text{ mg}\cdot\text{kg}^{-1}$ ) or ornithine ( $200 \text{ mg}\cdot\text{kg}^{-1}$ ) on ACh-stimulated NO generation (A) and basal ROS formation (B). Symbols indicate mean  $\pm$  SEM for  $n = 6-8$  animals; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the corresponding control group values; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared with the corresponding M group values; by one-way ANOVA and Newman-Keuls *post hoc* test.

mia in metabolic syndrome. These findings provide convincing evidence that arginase offsets the hypertension accompanying the metabolic syndrome through endothelial-dependent relaxation NO signalling protection.

We reasoned that arginase inhibition would mitigate or at least minimize the hypertension components of the metabolic syndrome. The validity of this assumption was ascertained in this work because all the arginase inhibitors used significantly inhibited the elevations in diastolic and systolic BP seen in animals with metabolic syndrome. The elevated systolic (after load) BP in diabetic animals could be due to cardiac complication and/or aortic stiffness while the elevated diastolic (pre-load) BP is attributed to the impaired vascular reactivity observed in diabetic animals. Aortic stiffness has been reported to be responsible for augmented pressure in late systole (O'Rourke and Nichols, 2005).

We focused on the impairment in vascular reactivity because of its importance in the development of hypertension (Mansour *et al.*, 2011) and in metabolic syndrome-

evoked hypertension in particular (Schinzari *et al.*, 2010). The findings of the present study highlight a pivotal role for vascular reactivity in the amelioration of metabolic syndrome-evoked hypertension by arginase inhibition. We have found increased contraction of isolated aorta to PE and KCl and decreased relaxation to ACh in animals with metabolic syndrome, while arginase inhibitors prevented the impaired relaxation to ACh in aorta isolated from animals with metabolic syndrome without affecting the response to PE or KCl. These findings are supported by previous reports from us and other laboratories, which showed increased response to different vasoconstrictors (El-Bassossy *et al.*, 2011a; Mahmoud *et al.*, 2012) and impaired endothelium-dependent dilatation (Verma *et al.*, 1996; Takagawa *et al.*, 2001) due to insulin resistance. In addition, we have previously shown that arginase inhibition alleviates hypertension and impaired endothelial-dependent relaxation associated with insulin deficiency (El-Bassossy *et al.*, 2012b).

We used three inhibitors that are known to inhibit arginase enzyme by different ways in order to confirm that the observed protection is most likely due to arginase enzyme inhibition and not due to a direct effect of the inhibitors. Ornithine inhibits arginase enzyme by competing with its substrate L-arginine on the active sites of the enzyme. Norvaline is a non-competitive inhibitor of the arginase enzyme (Hunter and Downs, 1945). L-citrulline is an allosteric inhibitor of arginase (Shearer *et al.*, 1997).

In the present work, we found increased arginase activity in animals with metabolic syndrome while all the inhibitors showed a significant inhibition of arginase activity at the doses and durations used. The protective effect of arginase inhibition on elevated diastolic BP in animals with metabolic syndrome seems to be through direct and indirect mechanisms. The direct protective effect is via improving relaxation. This is because acute *in vitro* arginase inhibition significantly increased endothelial-dependent relaxation in aorta isolated from animals with metabolic syndrome in this study. The indirect protective effect of arginase inhibition is through amelioration of the developed insulin resistance. In the present work, arginase inhibition was statistically correlated with the reduction in the developed insulin resistance. This points to the role of arginase enzyme activity in the metabolic syndrome. Hyperuricaemia is one of the important components of the metabolic syndrome and is a true risk factor for cardiovascular disease in metabolic syndrome. The proposed mechanism is through inhibiting endothelial-dependent relaxation and NO production (Heinig and Johnson, 2006). In this study, *in vitro* incubation with uric acid at the concentration seen in animals with metabolic syndrome significantly impairs endothelial-dependent relaxation in aorta isolated from normal animals. To test if the observed hyperuricaemia is responsible for arginase activation in metabolic syndrome, we found that *in vitro* incubation with uric acid did not significantly affect the arginase activity in isolated vessels. On the other hand, the observed uric acid-induced impairment in relaxation was prevented by co-incubation with any of the arginase inhibitors while none of them affected the serum level of uric acid in animals with metabolic syndrome. This suggests an indirect relationship between hyperuricaemia and arginase enzyme in metabolic syndrome.

The mechanism(s) by which arginase inhibition protects against the impairment in endothelium-dependent relaxation could be mediated, at least in part, by modulation of NO production. In this work, treatment with any of the arginase inhibitors prevented the impairment in ACh-stimulated NO generation in aorta isolated from animals with metabolic syndrome. Reducing arginase activity via dietary manganese deficiency has been previously reported to enhance endothelium-dependent vasorelaxation of rat aorta (Ensunsa *et al.*, 2004). The observed protection of ACh-stimulated NO generation due to arginase inhibition in metabolic syndrome could be due to inhibition of eNOS uncoupling and NO quenching by ROS. Arginase inhibition restores the availability of L-arginine for eNOS and limits NO inactivation by superoxide anion produced by eNOS as a result of arginine depletion (Santhanam *et al.*, 2008). In support of this, we have found a significant reduction in ROS generation in vessels isolated from animals with metabolic syndrome due to arginase inhibition. The decrease in ROS generation seems to be due to inhibition of eNOS uncoupling that follow arginase inhibition in this study. Modulation of eNOS co-factors like BH4 is another possibility of restored ACh-induced NO generation by arginase inhibition in metabolic syndrome as BH4 can be easily oxidized by ROS. In a previous report from our group, we found that external addition of BH4 blocks the attenuation in ACh-stimulated NO generation (El-Bassossy *et al.*, 2009).

The possibility that alterations in circulating lipid profile contributed to the metabolic syndrome–arginase hemodynamic interaction was also investigated. Dyslipidemia is associated with increased cardiovascular morbidity and mortality (Robins *et al.*, 2003). Our findings showed that the metabolic syndrome was characterized by hypertriglyceridaemia and hypercholesterolaemia. While arginase inhibition did not affect the developed hypercholesterolaemia, it completely prevented hypertriglyceridaemia associated with metabolic syndrome. In addition, serum triglyceride levels were strongly correlated with arginase activity in all groups. These observations strongly suggest a role of arginase enzyme in the hypertriglyceridaemia associated with metabolic syndrome. A possible explanation to this novel finding is that increased arginase activity limits L-arginine for eNOS and hence reduces NO production. NO plays an important role in reducing triglycerides level. It was found that diets rich in nitrite promote NO production and reduce triglycerides in humans (Zand *et al.*, 2011), while the NO synthase inhibitor L-N{omega}nitroarginine (L-NNA) elevates serum triglyceride (Goto *et al.*, 1999). The effect of NO on serum triglyceride levels might be due to reduced fatty acid oxidation as it has been shown that inhibiting NO synthase by L-NNA lowered the activity of hepatic carnitine palmitoyl transferase, the rate-limiting enzyme of fatty acid oxidation (Goto *et al.*, 1999).

In conclusion, arginase inhibition by citrulline, norvaline or ornithine alleviates hypertension associated with metabolic syndrome by direct and indirect protective mechanisms. The direct protective mechanism is through maintenance of endothelial-dependent relaxation and NO generation, while the indirect mechanism is through inhibition of insulin resistance and hypertriglyceridaemia.

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## Conflict of interest

The authors state no conflict of interest.

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