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Modulation of the Redox State and L-arginine Metabolism by Fumarate in Mitochondrial Fractions of the Rat Kidney

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ABSTRACT

Fumarate, a natural product, has been reported to modify the redox state and enhance nitric oxide (NO) synthesis in hypertensive rats. Considering the vital link between redox chemistry, L-arginine metabolism, and renal physiology, this study investigated fumarate for any effect on these parameters in normotensive rats. Kidneys were excised from anaesthetized (ketamine + xylazine) (100 mg/kg, i.p) Sprague Dawley rats. The renal cortex/medulla was homogenized to isolate the respective mitochondria fractions. The fractions were stimulated with angiotensin II (AII) (3, 10, 30 and 100 nM) to induce hydrogen peroxide (H_2O_2) production and incubated with fumarate (0.3,1, 3 and 10 µM) for 1 hour. Biochemical analyses were performed on these fractions. Fumarate (3 & 10 µM) exerted significant reductions in AII-induced H_2O_2 production in the cortex ($p < 0.05$). Fumarate reduced H_2O_2 in the medulla at 3 µM and exerted a time-related effect at 10 µM, compared to baseline ($p < 0.05$). Similarly, 3µM of fumarate significantly increased SOD activity in the medulla ($p < 0.05$). Catalase activity in the cortex peaked above baseline at 3 µM of fumarate only (2363.5 ± 332.2 vs 802.8 ± 189.1 units/ng, $p < 0.01$). However, fumarate increased catalase activity at all concentrations in the medulla ($p < 0.05$). Fumarate evoked a peak increase in NO and arginase activity at 3 µM in the cortex ($p < 0.05$). The data shows that fumarate improved the redox state via upregulation of SOD and CAT activities and may exert renoprotective action via an increase in NO.

Keywords: Fumarate, Mitochondrial metabolism, Hydrogen peroxide, Redox state, Nitric oxide

Introduction

The renal redox state is central to renal function.[1] An imbalance in the redox state of the kidneys has been reported to underlie a myriad of renal pathological disorders.[2,3] For instance, imbalances in the redox state can lead to the generation of radicals, notably, Hydrogen peroxide (H_2O_2). H_2O_2 is a radical of interest in renal physiology because of its vital signaling actions. Initially thought to be a harmless by-product of mitochondrial metabolism, the role of H_2O_2 evolved from a radical to a paracrine endogenous mediator capable of regulating internal mechanisms with far-reaching pathological consequences.[4] These properties of H_2O_2 are linked to its unique physical properties. Unlike most radicals, H_2O_2 is stable due to its paired electrons and has the exquisite ability to permeate into tissues to exert its biological effects. Similarly, H_2O_2 can interact with endogenous ligands to produce reactive radicals which possess deleterious properties. For instance, H_2O_2 can react with iron to produce a very unstable and reactive hydroxyl radical (OH^-).[5] Hence, aside from having its direct action on biological function, H_2O_2 can also exert indirect effects via its reactive by-products.

H_2O_2 has been documented to exert paracrine effects in the cardio-renal system.[6] Reports have shown that it exerts pro-hypertensive effects via increased vasoconstriction, organ remodelling and reduced renal natriuretic activity.[7]

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Recently, the increase in blood pressure in salt-sensitive rats due to insufficient catalytic activity of the tricarboxylic acid cycle intermediary, fumarase, was mechanistically linked to H_2O_2 and nitric oxide (NO).[8–10] The inability of fumarase to hydrolyze fumarate to malate (Figure 1), led to an excess of fumarate which increased H_2O_2 production and reduced NO production.[11] This established a link between renal mitochondrial metabolism and the pro-hypertensive action of H_2O_2 on blood pressure in genetic hypertension. [12] Due to the intricate link between renal H_2O_2, NO and blood pressure regulation, this present study investigated the effect of fumarate on H_2O_2 production, L-arginine metabolism, and the possible role of redox enzymes in mitochondrial fractions of the normotensive rat kidney.

Materials and Methods

Animals

Sprague Dawley rats 180 – 250 g; (Harlan Sprague Dawley, Houston, TX) of both sexes were inbred in standard cages, under-regulated lightening conditions kept on standard rat food (Purina Chow; Purina, St Louis MO, USA) and water *ad libitum* in the animal facility of Texas Southern University, Houston, Texas. Protocols for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Southern University, ethical approval number: #9014. All experiments were carried out in accordance with the Care and Use of Laboratory Animals guidelines of the National Institutes of Health.

Experimental procedures

Animal surgery

A midline abdominal incision was made on an anaesthetized rat (ketamine + xylazine, 100 mg/kg, i.p). Visceral tissues were cleared to expose the kidneys which were excised and immediately transferred to an ice pack. The kidneys were carefully separated into the cortex

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(200mg) and medulla (200 mg) using a sterile scalpel. Both sections of the kidneys were separately placed in a homogenization buffer and homogenized with a homogenizer (PRO Scientific, Oxford, CT, USA). After homogenization, mitochondrial fractions of the cortex and medulla were obtained using the modified method of Williamson et al.[13]

Tissue homogenization
The isolated renal cortex/medulla were homogenized using the protocols described in the Thermo Scientific® NE-PER nuclear and cytoplasmic extraction reagents kit. Briefly, 200 mg of the renal cortex/medulla was suspended in 1 mL of phosphate-buffered saline (PBS, pH 7.4). The resulting mixture was then centrifuged for 5 minutes at 500 g and the supernatant was discarded. Homogenization mixture (3 mL of cytoplasmic extraction reagent I (CER I), 0.16 mL of cytoplasmic extraction reagent II (CER II) and 0.15 mL of nuclear extraction reagent (NER) was then added to the residue and the resulting mixture was homogenized.

Protein concentration assay
Thermo Scientific® micro-BCA protein assay kit was used to quantify the protein content of the renal cortex or medulla fractions. The BCA working reagent was prepared by mixing 25 parts MA + 24 parts MB + 0.50 parts MC. Briefly, 0.03 mL of the renal cortex/medulla was added to 0.50 mL of BCA working reagents and 0.49 mL of distilled water. The mixture was then incubated at 60 ℃ and read at 562 nm against a blank solution (0.50 mL of BCA working reagent and 0.49 of distilled water) using a spectrophotometer. Standard albumin concentrations (4, 8, 10, 12, 16 and 20 µg/mL) were subjected to the same procedure.

Isolation of mitochondrial fractions
The homogenised mixtures of the renal cortex/medulla were centrifuged at 800 g for 10 minutes at 4 ^{0}C. The resulting supernatant was aspirated into a clean tube and centrifuged at 15,000 g for 10 minutes at 4 ^{0}C The supernatant was discarded, and the pellets were re-suspended in phosphate-buffered saline (PBS) to give the mitochondria fractions.[13]

Detection of H_2O_2 production in vitro
Hydrogen peroxide production was assayed with the aid of a free radical analyser® (Apollo 4000, WPI, Sarasota, FL, USA).[14] The free radical analyzer® consists of a detecting electrode which was submerged in a beaker containing 5 mL of phosphate-buffered saline (PBS) and 40 µg of the renal cortex/medulla mitochondrial fraction. A baseline of 5 minutes was allowed before the addition of angiotensin II (AII) (3, 10, 30, and 100 nM) to stimulate hydrogen peroxide production. After the baseline of 5 minutes, observation continued at 5, 15, 30 and 60 minutes after each addition of the various concentrations of AII and changes in hydrogen peroxide production was recorded. The same procedure was repeated with the addition of 3, 10, 30 and 100 µM of fumarate.

In vitro determination of superoxide dismutase (SOD), catalase (CAT), arginase activity, and nitric oxide (NO) production
The renal cortex/medulla (40 µg) was suspended in 1 mL of phosphate-buffered saline. Fumarate (3, 10, 30 and 100 µM) was added to the resulting mixture and the reaction mixture was incubated at 37 ^{0}C for 1 hour. After 1 hour, the reaction mixture was subjected to biochemical analyses stated as follows:

Nitric oxide assay
Briefly, 0.5 mL of cortex/medulla homogenate to 0.5 mL of Griess reagent (1 g of sulphanilamide + 100 mg of N-(-1-naphthyl) ethylenediamine (NEDD). The same procedure was repeated for standard concentrations of sodium nitrite (NaNO2) (1, 2, 5, 10 and 20 µM). The reaction mixtures were immediately read at 540 nm against a reagent blank (0.5 mL of distilled water + 0.5 mL of Griess reagent),[15] using a spectrophotometer.

Catalase assay
Briefly, 40 µg of the renal cortex/ medulla was added to 0.48 mL of Phosphate buffer saline and 0.50 mL of 20 mM H2O2. The absorbance of the mixture was immediately read spectrophotometrically at 240 nm, every 30 s for 210 s, against a blank (0.50 mL of 50 mM of phosphate buffer saline + 0.50 mL of 20 mM H2O2).[16]

Superoxide dismutase assay
Briefly, 40 µg of cortex/medulla was mixed with to 0.50 mL of 0.05M carbonate buffer (pH 10.2) and 0.50 mL of 0.3 mM adrenalin. The resulting mixture was read every 30 s for 180 s at 480 nm against a blank (0.50 mL of carbonate buffer + 0.50 mL 0.3 mM adrenalin).[17]

Arginase assay
This assay procedure involved the mixture of 40 µg of renal cortex/medulla with 1 M of magnesium chloride (MgCl2) at 37^{0}C for 30 minutes. The mixture was further mixed with 0.5 mL of L-arginine buffered solution and allowed to stand for 1 hour at 37^{0}C. After incubation, a developer solution (0.05 mL of Ninhydrin + 0.45 mL of acetic acid + 0.05 mL of phosphoric acid solution) was added, and the mixture was incubated at 95^{0}C for 1 hour. The cooled reaction mixture was read at 530 nm against a blank solution (0.01 mL of 1 M magnesium chloride + 0.49 mL of L-Arginine buffer + 0.50 mL of developer solution).[18]

Statistical analysis
All experimental data are presented as mean ± SEM and one-way ANOVA was used for statistical analysis, followed by Dunnet post hoc test. GraphPad prism® 6.0 software was used to analyze the data and a value of $p < 0.05$ was considered significant. Only fumarate concentrations that gave significant effects are displayed in the hydrogen peroxide assay.

Results and Discussion

As illustrated in Figures 2A&B, AII exerted a time-dependent increase in basal H_2O_2 production in the renal cortex and medulla ($p < 0.05$). Fumarate (3 µM) did not have any significant effect on H_2O_2 production in the cortex (Figure 1A). However, fumarate (10 µM) caused a 2-fold reduction in H_2O_2 production in the cortex at 60 minutes only, compared to baseline ($p < 0.05$) (Figure 2A). At 10 nm of angiotensin II (AII), fumarate (3 µM) reduced H_2O_2 production at 30 (0.3 + 0.0 vs 0.1 + 0.0) and 60 minutes (0.4 + 0.0 vs 0.1 + 0.0), when compared to baseline ($p < 0.05$, n=5) (Figure 2B). Also, fumarate (10 µM) significantly reduced 3 nM AII-induced increase in H_2O_2 production at 30 minutes ($p < 0.05$, n=5) in the cortex (Figure 2B). H_2O_2 production in the medulla was significantly attenuated by a 3 µM concentration of fumarate at 3nM AII within the observed times (Figure 3A). Similarly, 10 µM of fumarate significantly reduced H_2O_2 at 30 minutes only ($p < 0.05$, n=5) (Figure 2A). At 10 nM of AII, H_2O_2 was significantly reduced at 3 µM of fumarate only (Figure 3B). Figure 4A illustrates that in the cortex, fumarate did not have any significant effect on SOD activity, when compared to baseline ($p > 0.05$, n=5). In the medulla (Figure 4B), 0.3 µM of fumarate elicited peak effect at 30 and 180 s, (40 and 55%) respectively, compared to baseline ($p < 0.05$, n=5).

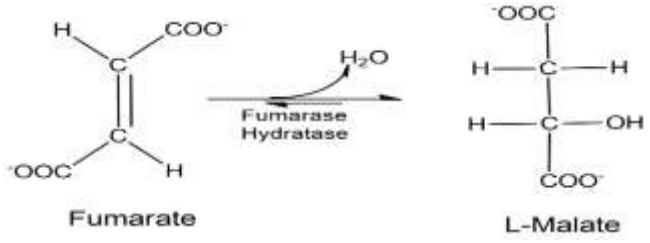

Figure 1: Catalytic conversion of fumarate to malate by the enzyme, fumarase hydratase.

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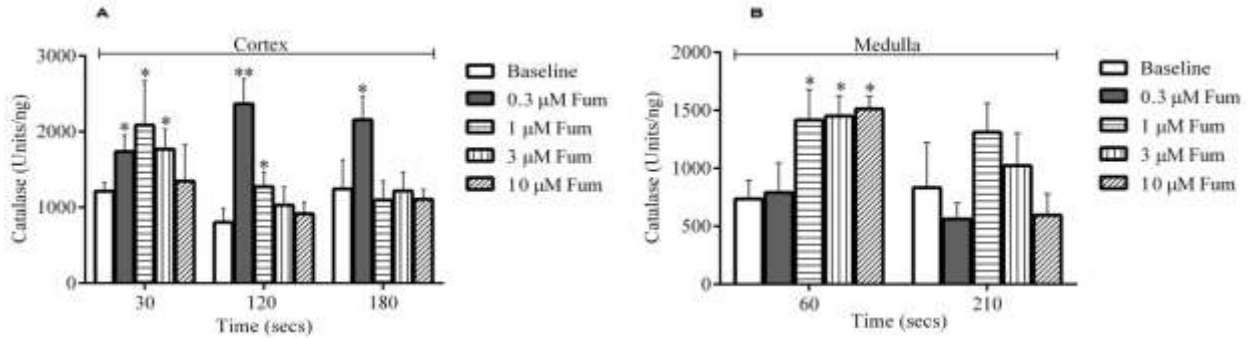

Figure 2: Effect of fumarate (3 and 10 µM) on hydrogen peroxide (H_2O_2) production in mitochondrial fractions of the renal cortex stimulated with angiotensin II (3 and 10 nm) *in vitro*. *p < 0.05, compared to baseline (n=5). Key: Fum (fumarate), AII=angiotensin II. Only concentrations of fumarate with significant activity are shown.

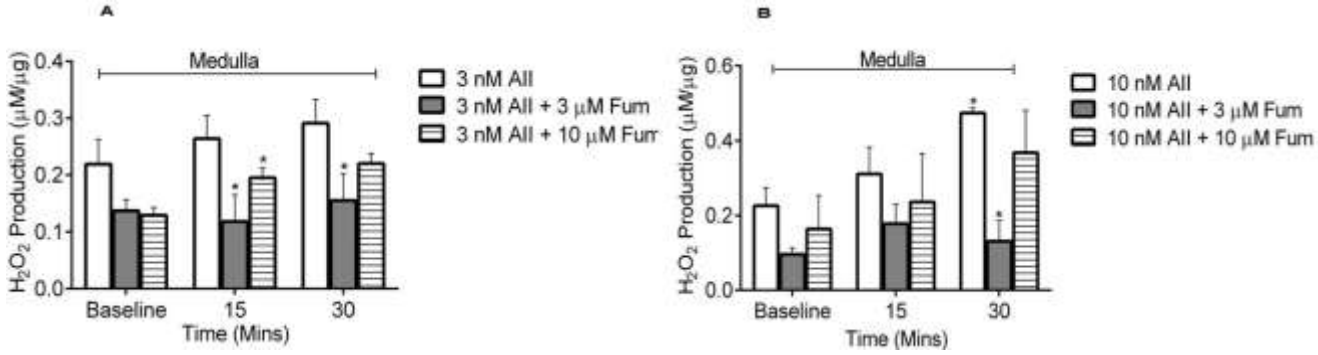

Figure 3: Hydrogen peroxide (H_2O_2) production in mitochondrial fractions of the renal medulla stimulated with angiotensin II (AII) and treated with fumarate (3 and 10 µM) *in vitro*. *p < 0.05 vs baseline (n=5). Key: Fum (fumarate). Only concentrations of fumarate with significant activity are shown.

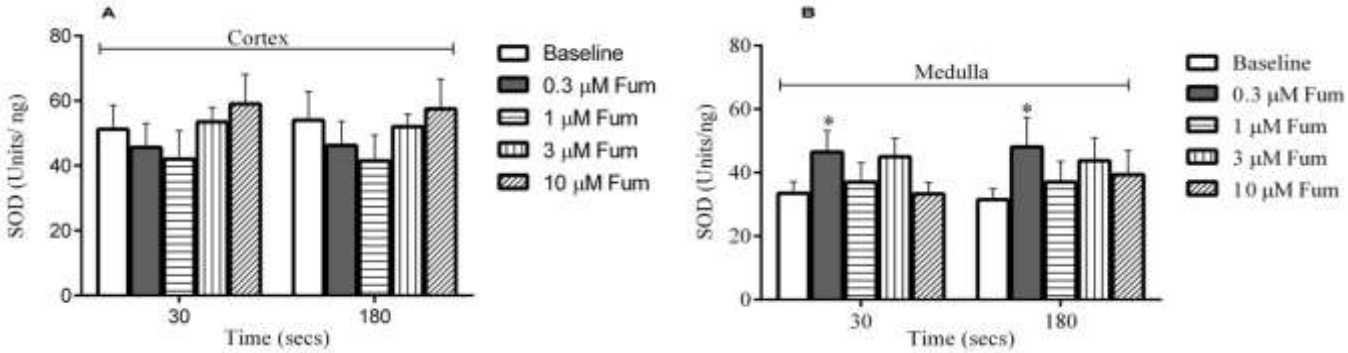

Figure 4: Effect of fumarate (0.3, 1, 3 and 10 µM) on superoxide dismutase (SOD) activity in mitochondrial fractions of the kidney *in vitro*. *p < 0.05, compared to baseline (n=5). Key: Fum (fumarate).

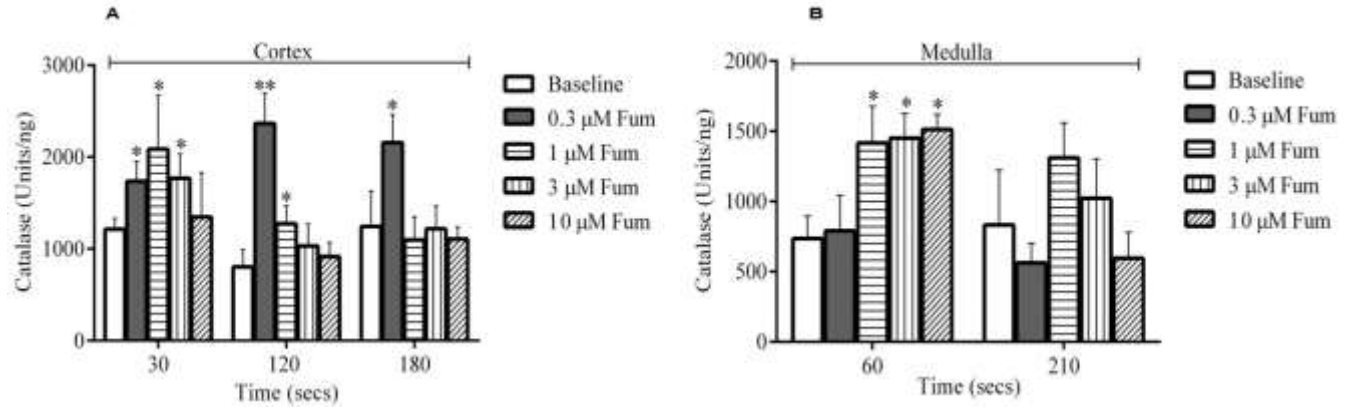

Figure 5: Effect of fumarate (0.3, 1, 3 and 10 µM) on catalase (CAT) activity in mitochondrial fractions of the kidney *in vitro*. *p < 0.05, **p < 0.01, compared to baseline (n=5). Key: Fum (fumarate).

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As illustrated in Figure 5A, fumarate increased catalase activity in the cortex in a non-concentration-dependent manner and produced a peak effect on catalase activity at 0.3 µM (2-fold, $p < 0.01$, n=5), compared to baseline (n=5). In the medulla, fumarate increase catalase activity in a concentration and time-dependent fashion (Figure 5B), exerting significant increases in catalase activity at 1, 3 and 10 µm ($p<0.05$) (Figure 5B). As shown in Figure 6A, fumarate (3 µM) exerted a peak increase in arginase activity in the cortex, compared to baseline (9.6 ± 1.4 vs 23.7 ± 6.7 µM/µg, $p < 0.05$, n=5). Fumarate reduced arginase activity in a concentration-dependent manner with the 10 µM concentration eliciting peak reduction in the cortex (9.6 ± 1.4 vs 4.8 ± 1.5 µM/µg, $p < 0.05$, n=5). All concentrations of fumarate tended to reduce arginase activity in the medulla, eliciting a 50% reduction at 1 µM when compared to control ($p > 0.05$, n=5). Figure 6B shows fumarate significantly increased production of nitric oxide at 0.3 and 1 µM in the cortex, compared to baseline ($p < 0.05$, n=5). In the medulla, fumarate evoked a significant increase at 3 µM only, when compared to control (21.0 ± 6.9 vs 36.3 ± 0.7, $p < 0.05$, n=5).

This study investigated the effect of fumarate on AII-induced stimulation of H_2O_2 production in mitochondrial fractions of the renal cortex and medulla of normotensive rats. AII induces H_2O_2 via activation of nicotinamide adenine dinucleotide phosphate (NADPH) which is the major source of H_2O_2 in most cells.[19] The produced H_2O_2 was monitored via the aid of a free radical analyzer®.[20] The principle of the free radical analyser® is based on the oxidation of H2O2 through an electrode (microsensor) to create an electrical (redox) current (pA) which is proportional to the rate of change in H2O2 production occurring in the sample. The free radical analyzer thus measures the real-time production of H_2O_2 via microsensors submerged in physiological buffered solutions.[14] In this present study, the microsensors connected to the free radical analyzer were submerged in the mitochondria fraction of the renal cortex/medulla. Consistent with earlier reports,[8] AII caused a time-dependent increase in the production of H_2O_2 in the renal cortex and medulla. In the renal cortex, fumarate exerted concentration-related effects in that, it reversed H_2O_2 production at higher concentrations. However, as H_2O_2 increased, the lower concentration of fumarate exerted a consistent reduction in H_2O_2 production. Fumarate had a faster onset of action in the medulla and produced consistent effects at its lower concentrations. Fumarate thus reversed AII-induced production of H2O2 and thus appears to function as an antioxidant, especially at lower concentrations in the medulla. Generally, it was observed that fumarate exerted better control on H_2O_2 production at lower concentrations in both regions of the kidneys. This aligns with previous reports that have stated that an excess of fumarate can contribute to increasing redox signalling in the kidneys.[8,10,21] Similarly, we observed that at higher concentrations of AII, fumarate exerted a minimal effect on production and returned H_2O_2 production to baseline levels.

SOD and CAT are the most extensively studied endogenous antioxidant enzyme systems and they act as the first-line defence against superoxide anion and H2O2, respectively.[22] SOD and CAT act synergistically to break down the superoxide anion and in situations of excess free radicals, they play vital roles in detoxification and thus serve as internal markers for redox activity.[23] Fumarate did not elicit any significant changes in SOD activity in the cortex, however, it exerted a peak effect at the lowest dose in the medulla. Hence, fumarate had no effect on superoxide generation in the cortex but appeared to selectively enhance the uncoupling of superoxide in the medulla. Catalase activity was significantly increased by fumarate at lower concentrations in both the cortex and medulla. Fumarate's effect on catalase activity was time-and concentration-dependent, especially in the renal medulla. Fumarate thus enhanced the activity of catalase in both regions of the kidneys. Ligands that modulate the effect of radicals such as H_2O_2 can either act directly through chemical inactivation or via upregulating the activities of the endogenous enzyme system.[24] There was a correlation between the effect of the lower concentrations of fumarate as regards the reduction in H_2O_2 production and the activities of SOD and CAT. This congruence in activity may well indicate that the upregulation of SOD and CAT activities partly underlies the mechanism involved in the reduction in H_2O_2 production by fumarate. L-arginine metabolism as represented by arginase activity and NO production is integral to optimal renal function.[25] Arginase metabolizes L-arginine to ornithine and urea while nitric oxide synthase converts L-arginine to NO and citrulline. Both enzymes have an inverse relationship and have their unique contribution to kidney function. Considering the novel link of fumarate to L-arginine metabolism, this study investigated to ascertain a possible link in the normotensive rat. The effect of fumarate on arginase and NO activity in the cortex was somewhat inconsistent. An increase in arginase activity would reduce L-arginine availability and should reduce NO production, however, fumarate increase both arginase activity and NO production at the same concentrations. This indicates that fumarate may also increase NO production via other mechanisms and rightly so, especially as fumarate has been reported to increase hypoxia inducible factor levels (HIF-1α) and HIF-1α also acts by increasing the production of NO.[26] The scenario in the medulla was quite consistent and fumarate increased NO production and simultaneously reduced arginase activity at the same concentrations, however, in contrast to the observation in the cortex, fumarate achieved its effect in the medulla at higher concentrations. Fumarate thus evoked differential effects on regional arginase activity in the kidney and consistently reduced arginase activity in the medulla. Although arginase reduces NO production by reducing L-arginine availability, the resultant increase in ornithine exerts beneficial effects on tissue repair.[27, 28]

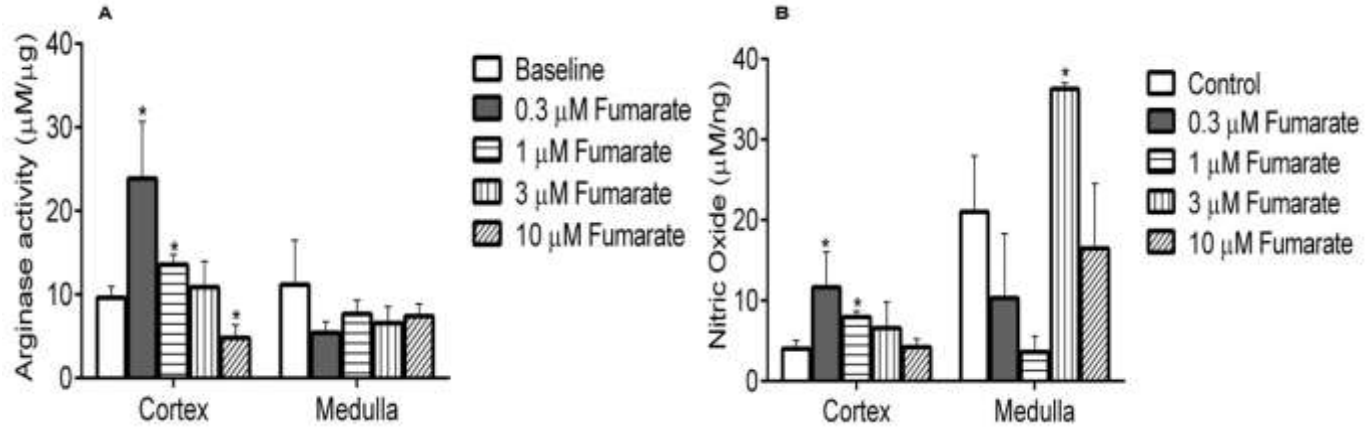

Figure 6: L-arginine metabolism in mitochondrial fractions of the kidney treated with fumarate (0.3, 1, 3 and 10 µM) *in vitro*. Arginase activity (A) and nitric oxide production (NO). *$p < 0.05$, compared to baseline (n = 5).

Conclusion

Observations from this study have shown that fumarate reduced renal mitochondrial production of radicals such as H_2O_2, thus improving the redox state of the kidney via mechanisms that partly involve upregulation of SOD and CAT activities. The relative increase in NO production also has direct effects on renal haemodynamics and although previous reports have highlighted these effects of fumarate in hypertensive rats, our study lends credence to these reports and postulates that fumarate also modulates renal function in normotensive rats. Considering the role of H_2O_2 and L-arginine metabolism in renal haemodynamics, future studies would be pursued to assess these probable effects of fumarate on blood pressure and renal blood flow.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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