Effects of fumarate on renal vascular reactivity and the modulation of blood pressure in normotensive rats: Possible contribution of the nitric oxide synthase-nitric oxide system

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Abstract

Introduction: Fumarate, the tricarboxylic acid (TCA) cycle intermediary, has been linked to nitric oxide (NO) production. NO plays a prominent role in the physiological regulation of blood pressure and renal hemodynamics. This study is aimed to investigate any contribution of fumarate to blood pressure and renal hemodynamics in normotensive rats with a possible link to the nitricergic system.

Materials and methods: Fumarate (1, 3 and 10 µmol) was injected into isolated perfused kidneys, pre-constricted with epinephrine (30 µM). The fumarase inhibitor, pyromellitic acid (PMA) (1, 3 and 10 µM), was used to perfuse the isolated kidney and perfusate was collected for nitric oxide and fumarate assays. An acute blood pressure study involved the injection of bolus doses of fumarate (0.1, 0.3 and 1 µg/kg, iv) or PMA (1, 3 and 10 µg/kg, iv) to normotensive rats in the presence of N(ω)-nitro-L-arginine methyl ester (L-NAME) (10 mg/kg, iv) or PMA (1, 3 and 10 µg/kg).

Results and discussion: Fumarate reduced perfusion pressure and elicited a peak reduction at the highest dose. Perfusing the kidney with PMA caused a paradoxical increase in perfusion pressure (70%, p<0.05), compared to baseline. Bolus doses of fumarate reduced blood pressure (-29.3±6.2 mmHg, p<0.05), cortical blood flow (CBF) and increased medullary blood flow (MBF). L-NAME did not abolish the vasodilatory effect of fumarate, but reduced the magnitude of response (50%, p<0.05). PMA did not significantly affect the vasodilatory effect of fumarate (p>0.05).

Conclusion: These data suggest that fumarate exerts a vasodilatory effect on renal and systemic hemodynamics that may partly involve the nitric oxide signaling.
Graphical abstract:

Keywords

Tricarboxylic acid cycle, fumarate, blood pressure, nitric oxide, kidney.

Introduction

Mitochondrial bioenergetics is necessary for cell survival (Bhargava and Schnellmann 2017). Biochemical networks in the mitochondrion provides energy needed to drive the regulatory functions in the body. The recent evidence that these networks underlie the pathophysiology of certain diseases has shifted focus into the role of these biochemical frameworks (Eng et al. 2003; Che et al. 2014). Notable amongst this is the tricarboxylic acid (TCA) cycle. Downstream or upstream products of fumarate in the TCA cycle have been implicated in the pathogenesis of malignancies, neurological disorders and, recently, hypertension (Remes et al. 2004; Tian et al. 2009; Basile et al. 2012). It was discovered that a deficiency in fumarase hydratase (FH), an enzyme in the TCA cycle that catalyzes the conversion of fumarate to malate, underlined the increase in blood pressure in salt-sensitive (SS) rats (Tian et al. 2008; Tian et al. 2009), a genetic model of hypertension. Further reports stated that these intermediaries had a connection with nitric oxide (NO) synthesis and their deficiency led to a decrease in NO production (Hou et al. 2017). This same relationship between downstream products of fumarate and NO has been extended to deoxycorticosterone acetate (DOCA)-salt hypertension, a non-genetic model of hypertension (Edosuyi et al. 2021), thus suggesting a role of mitochondrial metabolism in both genetic and non-genetic hypertension (Liang 2011). Aside its connection to NO production, fumarate has been shown to interact with a host of mediators, such as hypoxia inducible factor (HIF-1), endothelial growth factor, and hydrogen peroxide which impact the cardiovascular system (Isaacs et al. 2005; Ashrafian et al. 2012; Czibik et al. 2014). Fumarate has also been shown to improve the renal function and particularly to protect the renal medulla. This improvement in the renal system function is instructive, as it directly impacts blood pressure in deoxycorticosterone acetate (DOCA)-salt hypertension (Edosuyi et al. 2021). Hence, there is a possibility that fumarate could contribute to the homeostatic regulation of blood pressure and renal hemodynamics. This study evaluates for any relationship between fumarate, the substrate for fumarase, in regulation of blood pressure and renal hemodynamics with a view to assessing the possible connection to nitric oxide production in normotensive rats.

Materials and methods

Chemicals

Ketamine + Xylazine, Ketamine, Krebs solution (NaCl 1.18, KCl 0.046, CaCl 0.022, KH2PO4 0.012, MgSO4 0.25 NaHCO3, and glucose 0.0115 M), fumarate sodium, pyromellitic acid (PMA), L-arginine methyl ester (L-NAME), phenylephrine, sodium nitroprusside, thiobutabarbital, heparin, sulphamethazine, phosphoric acid, distilled water, N-(-1-naphthyl) ethylenediamine (NEDD), sodium nitrite (NaNO2), and fumarate assay kit. All the chemicals/drugs were purchased from Millipore Sigma (MA, USA).
Experimental animals

The studies were carried out using male Sprague Dawley rats (200–350 g; Harlan Sprague Dawley, Houston, TX) inbred in the animal facility of Texas Southern University, Houston, Texas. The animals were housed under 12-hour controlled lightening conditions, placed on standard rat feed (Purina Chow; Purina, St Louis MO, USA) and water ad libitum, in standard cages. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Texas Southern University, Houston, Texas, (Protocol #9004/Rats/1034) and the guidelines for the Care and Use of Laboratory Animals guidelines of the National Institutes of Health were strictly adhered to in this study.

Isolated perfused kidney (IPK) procedure

Male Sprague Dawley normotensive rats (250 g) were anaesthetized with ketamine + Xylazine (100 mg/kg, ip). A mid-line abdominal laparotomy was done to expose the right kidney. The right renal artery and abdominal aorta were carefully isolated, and a tie was placed above and below the abdominal aorta while another tie was placed on the right renal artery. An incision was made on the abdominal artery, and a 25G needle was carefully passed through the incision to the right renal artery, and the kidney was immediately perfused with Krebs solution. The tie above the abdominal aorta was quickly fastened; the kidney was excised and immediately transferred to a thermostatically controlled surface (Harvard Apparatus, Cambridge, MA, USA) to maintain body temperature (37 °C). A tracheostomy was performed, and a cannula (PE 250) was inserted to aid respiration. A cannula (PE 50) flushed with heparin (200 I.U./mL) was inserted into the left carotid artery for mean arterial pressure (MABP) measurements. The left jugular vein was cannulated (PE 50) for bolus administration of drugs. A left sided laparotomy was performed to expose the kidney, and renal blood flow was measured simultaneously by a laser-Doppler flowmeter (version 1.20; PeriFlux, Perimed AB, Stockholm, Sweden) through a surface probe (model PF 407) placed on the cortex to record corticomedullary blood flow (CBF) and using an optical fiber (model PF 402) inserted 6 mm below the cortex to measure medullary blood flow (MBF). The animal was allowed a 45-minute equilibration before the commencement of drug administrations. Blood pressure returned to baseline in-between drug administrations (Igbe et al. 2012). The drugs were administered as follows: fumarate (0.1, 0.3, and 1 µg/kg), sodium nitroprusside (10, 30, and 100 µg/kg) or PMA (1, 3, and 10 µg/kg).

In a second experiment to assess the role of FH, pyromellitic acid (fumarase inhibitor) was administered, followed by the same doses of fumarate (0.1, 0.3, and 1 µg/kg).

In a third experiment, there was slow bolus administration of L-arginine methyl ester (L-NAME) (10 mg/kg, iv) to normotensive rats, followed by repeated doses of fumarate (0.1, 0.3 and 1 µg/kg). L-NAME elevated MABP by 28±1.7 mmHg, reduced CBF and MBF by -14±23.6 and -6.8±19.3 PU, respectively. Blood pressure readings were recorded after each drug administration.

Estimation of nitric oxide in kidney perfusate

The colorimetric method previously described (Green et al. 1982) was carried by preparation of Griess reagent, which involved mixing Solution A (1 g sulphanilamide in 5 mL of phosphoric acid and 95 mL of distilled water) and solution B (100 mg of N-(1-naphthyl) ethylenediamine (NEDD) in 100 mL of distilled water) in a 1:1 ratio. This was followed by the addition of 0.5 mL of kidney perfusate to 0.5 mL of Griess reagent. Standard concentrations of sodium nitrite (NaNO₂) (1, 2, 5, 10 and 20 µM) were prepared and subjected to the same procedure. The resulting reaction mixtures were immediately read at 540 nm against a reagent blank.
Estimation of fumarate in kidney perfusate

The assay was performed as described in the fumarate assay kit (Millipore-Sigma, St Louis, MO, USA). Fumarate enzyme mixture was reconstituted with 0.22 mL of fumarate assay buffer. Fumarate developer solution was prepared with 0.9 mL of distilled water. Standard preparations of fumarate were prepared (5, 10, 15, 20, and 25 nM) with 0.99 mL of assay buffer. 0.01 mL of the provided master reaction mix (MRM) containing 0.09 mL fumarate assay buffer, 0.08 mL of fumarate developer and 0.02 mL of enzyme mix was added to the microplate containing 0.05 mL of standard preparations of fumarate and kidney perfusate. The microplate was incubated at room temperature for 30 minutes with mild agitation. The absorbance was read at 450 nm, using a microplate reader (spectra max M5, Molecular Devices, CA, USA).

Statistical analyses

Data are expressed as mean±SEM. Data from the isolated perfused kidney experiment and the acute blood pressure study are expressed as changes from a recorded baseline perfusion/blood pressure. One-way analysis of variance (ANOVA) followed by Dunnet post-hoc test for multiple comparisons was carried out on all data. (Graphpad Prism 6, San Diego, USA).

Results and discussion

Fumarate reduced perfusion pressure in the pre-constricted isolated kidney

As Fig. 1A illustrates, fumarate reduced perfusion pressure in a dose-related manner. Fumarate tended to reduce perfusion pressure at all doses but produced a peak effect at 3 µmol (-19.3±17.3 mmHg, p>0.05) and elicited a significant reduction at 10 µmol (79.3±1.7 to 70.0±6.7 mmHg, p<0.05), when compared to basal pressure (n=4). Sodium nitroprusside (Fig. 1B), a nitric oxide donor, which acts as a vasodilator, reduced perfusion pressure at all doses (n=4).

Inhibition of fumarase evoked an increase in perfusion pressure

FH, an enzyme that metabolizes fumarate in the TCA cycle, was inhibited using PMA. As shown in Fig. 2A, perfusing the kidney with PMA elicited a significant increase at all doses in perfusion pressure, compared to baseline pressure (p<0.05, n=4). PMA evoked a peak response (70%, p<0.05) at 1 µmol. Fig. 2B shows the typical dose response relationship of a vasoconstrictor, phenylephrine.

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Figure 1. Effect of fumarate (A) and sodium nitroprusside (SNP) (B) on perfusion pressure in the isolated kidney perfused with Krebs-Henseleit and pre-constricted with 30 µM phenylephrine. Note: * – p<0.05 compared to basal perfusion pressure

Figure 2. The elevation in perfusion pressure due to the inhibition of fumarase (FH) with pyromellitic acid (PMA) (A) and dose-response relationship of phenylephrine (B) in the isolated perfused kidney. Note: * – p<0.05 compared to basal perfusion pressure
Inhibition of fumarase increased fumarate levels and nitric oxide production in the isolated kidneys

The diagram (Fig. 3A, B) shows that inhibition of fumarase evoked a time-related, concentration-dependent increase in nitric oxide and fumarate levels in the perfusate of the isolated kidney, with the most significant effect occurring at 10 µM (0.7±0.1 and 93.9±22.9 µM/mL, p<0.05), respectively.

Acute bolus doses of fumarate reduced blood pressure and cortical blood flow (CBF) but increased medullary blood flow (MBF) in normotensive rats

As shown in Fig. 4A, fumarate elicited a non-dose dependent decrease in MABP and CBF, accompanied by an increase in MBF, when compared to baseline (p<0.05, n=4). Fumarate produced a peak effect on MABP at 0.1 µg/kg (-29.3±6.2 mmHg, p<0.05), CBF at 1 µg/kg (-34±6.3 PU, p<0.05) and MBF at 0.3 µg/kg (-71.3±44.3 PU, p>0.05). SNP, a nitric oxide donor, elicited significant dose-dependent reductions in MABP (p<0.05, n=4) and CBF (p>0.05, n=4), when compared to baseline (n=4) (Fig. 4B). However, MBF was significantly increased at the highest dose only when compared to baseline (p<0.05, n=4).

Effect of the inhibition of fumarase on fumarate-induced changes on MABP, CBF and MBF

The effect of fumarate in normotensive rats pre-treated with a fumarase inhibitor, PMA, was evaluated on renal and systemic haemodynamics. As shown in Fig. 6A, fumarate elicited a non-dose related but significant reduction in blood pressure (white bars). There were dose related decreases in CBF (Fig. 6B) and increases in MBF (Fig. 6C) (white bars). Contrary to

Acute bolus injection of a fumarase inhibitor, PMA, altered renal and systemic hemodynamics in normotensive rats

The contribution of fumarase to MABP, CBF and MBF was evaluated via its inhibition with PMA. As illustrated in Fig. 5, pyromellitic acid reduced blood pressure at lower doses (1 and 3 µg/kg), but elicited an increase at the highest dose, 1 µg/kg (8.7±2.6 mmHg, p<0.05, n=4). PMA elicited non-dose dependent effects on CBF and MBF, causing peak effects at 1 µg/kg (-22.3±5.9 PU, p<0.05) and (14.3±2.7 PU, p<0.05), on CBF and MBF, respectively.
expectation, infusion of fumarate to PMA-treated rats tended to reduce, but not reverse, the magnitude of fumarate-induced reduction in blood pressure at 0.1 µg/kg (27%, p>0.05, n=4) and 0.3 µg/kg (54.5%, p>0.05, n=4) (hatched bars) (Fig. 6A). There were no significant changes in fumarate-induced reduction in CBF (Fig. 6B); however, there was 4-fold, p<0.05, reduction and 3-fold, p>0.05, reduction at 0.1 µg/kg and 0.3 µg/kg, respectively, in MBF (Fig. 6C) evoked by fumarate in PMA-treated rats.

**Effect of N(ω)-nitro-L-arginine methyl ester (L-NAME) on fumarate-induced changes in MABP, CBF and MBF**

Any connection between fumarate and nitric oxide synthase downstream was assessed by pre-treating normotensive rats with L-NAME, a nitric oxide synthase (NOS) inhibitor. As illustrated in Fig. 7A, fumarate caused a non-dose related decreases in blood pressure and CBF (Fig. 7B), but increased in MBF (Fig. 7C), when compared to baseline (p<0.05, n=4) (white bars). L-NAME did not reverse the fumarate-induced decrease in blood pressure, but there was a 50% reduction at 0.1 µg/kg, when compared to fumarate-treated rats (p<0.05, n=4) (white bars). L-NAME also evoked a 49% reduction at 0.3 µg/kg. (Fig. 7A).

L-NAME tended to exacerbate the fumarate-induced reduction in CBF (3-fold, p>0.05) at 0.1 µg/kg (Fig. 7B, but elicited no effect on fumarate-induced increase in MBF at 0.1 µg/kg and caused a 5-fold reduction at 0.3 µg/kg (Fig. 7C), when compared to rats treated with fumarate alone (p>0.05, n=4) (white bars).

The isolated perfused kidney offers unique possibilities to understand the renal function and unearth putative ligands that modulate renal vascular resistance and function, which ultimately impacts blood pressure.
In this present study, fumarate non-dose dependently relaxed renal vessels pre-constricted with epinephrine; thus, fumarate appeared to act as a vasodilator. Ideally, inhibiting FH should increase upstream availability of fumarate and hence exacerbate this putative vasodilatory response. However, perfusing the kidney with an FH inhibitor, PMA, caused an unexpected increase in perfusion pressure. It is likely that the constant perfusion of the kidney with the FH inhibitor increased fumarate availability above optimal levels causing the generation of free radicals, thus leading to increased perfusion pressure (Zheng et al. 2019). Zheng et al. (2019) showed that a genetic alteration in FH reduced its catalytic activity (similar to inhibition in this present study) and led to an increase in free radicals via fumarate-induced succination of glutathione. It is also possible that the fumarate-induced increase in free radicals due to PMA eclipsed the advantageous vasodilatory effect of NO by catalysing the NO-induced generation of peroxynitrite, which could increase perfusion pressure (McIntyre et al. 1999; Wilcox 2005). However, although it seems counter-intuitive, NO levels were elevated in the kidney perfusate treated with PMA and may have underlined the perceived vasodilatory effect of fumarate on renal vessels. The effect of TCA cycle products in the isolated perfused kidney showed that these products may interact independently with the renal system to impact blood pressure regulation. It also indicates that the renal system may be central to the perceived effects of fumarate in blood pressure regulation. This was reflected in the observed effects of these products on acute studies on blood pressure. Acute bolus administration of fumarate to normotensive rats reduced MABP, which was an effect that was marked by an initial, transient pressor response and quickly followed by a lasting, depressor response. The drop in blood pressure expectedly reduced blood flow to the kidneys resulting in reduced cortical blood flow (CBF). However, the reduction in CBF was accompanied by an increase in medullary blood flow (MBF), an adaptive mechanism to prevent hypoxia in the medulla via increased perfusion. Hence, TCA cycle products, downstream of fumarate, appear to exert a vasodilatory effect on systemic and renal haemodynamics. This is consistent with the earlier observed vasodilatory effect in the isolated kidney in this study. The fumarase inhibitor, PMA, increases upstream availability of fumarate; hence, this inhibition was expected to exaggerate the effect of fumarate. However, there were dual and dose-dependent effects on blood pressure and renal haemodynamics. Lower doses of PMA elicited effects consistent with that seen with fumarate on blood pressure; however, in consonance with the increase in perfusion pressure elicited by PMA on the renal vessels in the isolated kidney, the highest dose of PMA paradoxically increased blood

![Figure 7](image-url)

**Figure 7.** Effect of acute bolus dose of fumarate in the absence (white bar) or presence (hatched bars) of N(ω)-nitro-L-arginine methyl ester (L-NAME) (10 mg/kg, iv), a NOS inhibitor, on (A) mean arterial blood pressure (MABP), (B) cortical blood flow (CBF) and (C) medullary blood flow (MBF). L-NAME was administered 15 minutes before fumarate. Note: * – p<0.05 compared to baseline; # – p<0.05 compared to rats that received only fumarate (white bar); PU – perfusion units.
pressure in normotensive rats. This observation may be a protective (reflex) homeostatic response to prevent excessive reduction in blood pressure occasioned by the increased levels of fumarate. Hence, the system tends to initiate counter-regulatory mechanisms to increase blood pressure and restore systemic haemodynamics (Arendse et al. 2019). It is also likely that increased fumarate levels enhanced the bioavailability of NO, and excess NO has been documented to autoinhibit NOS via an interaction with the haem group of NOS (Griscavage et al. 1995). Furthermore, excess NO could interact with superoxide anion to form peroxynitrite and other NO-related radicals that subsequently impact NOS function and increase blood pressure (Miner et al. 2010). The above mechanisms may underlie the paradoxical increase in BP in the presence of excess fumarate, especially as fumarate excess can favour the generation of free radical (Zheng et al. 2019). Interestingly, PMA produced a similar effect as seen with fumarate on renal haemodynamics; while CBF was consistently decreased, MBF was increased. There is a somewhat correlation between the vasodilatory effect of these TCA cycle products in the isolated kidney and in vivo, in blood pressure regulation indicating that the mechanism of TCA cycle products downstream of fumarate may significantly depend on the kidneys. This becomes instructive when the intricate role of the kidney in blood pressure regulation is considered (Navar 1997; Crowley and Coffman 2014).

The inhibition of FH did not affect the depressor effect of exogenous fumarate on blood pressure but caused changes in its renal haemodynamic effects. Once again, the renal-specific effect of the TCA intermediaries is brought to the fore and was possibly due to the increased concentration of fumarate in the renal tissue, which increased its effect on the kidney. These observations are consistent with the inhibition of FH activity, which exacerbates upstream effects of fumarate while reducing its downstream effect. Earlier reports have shown a mechanistic link between fumarate and nitric oxide production (Hou et al. 2017). Hence, NOS was evaluated for any contribution it makes to the depressor effects of fumarate by the pre-administration of N(ω)-nitro-L-arginine methyl ester (L-NMMA), a NOS inhibitor, to normotensive rats. Fumarate reproduced its depressor effect on blood pressure, albeit with reduced magnitude in the presence of L-NMMA and maintained its decrease and increase in CBF and MBF, respectively. Also, while the action of fumarate appears linked to NOS, the inability of L-NMMA to abolish these effects indicates that other mechanisms may be involved in mediating the effect of fumarate as it is not uncommon to find endogenous ligands with multiple downstream targets; for instance, angiotensin II elicits vasoconstriction and vasodilation depending on its receptor subtype (Atlas 2007).

Experiments by Cowley (Cowley 1997) showed that ligands, such as norepinephrine, angiotensin II (AII) and inhibitors of NOS etc., which reduce MBF, also increase MABP, while those that stimulate or enhance the release of NO, such as Ach, which increase MBF, eventually reduce MABP. Data from this study presents a similar and classical observation for a TCA cycle enzyme, fumarase, and its substrate, fumarate, which reduced MABP and increased MBF. The increase in MBF confers a renoprotective effect, which improves a medulla function (Navar 1997) and attenuates adverse exacerbations in blood pressure (Cowley 1997). This may indicate that these TCA products have a homeostatic role in MABP regulation, which could be partly dependent on increased nitric oxide synthase activity in the kidneys. Taken together, this study has indicated that, aside its role in hypertension (Tian et al. 2009; Edosuyi et al. 2021), downstream products of fumarate in the tricarboxylic acid cycle may also be involved in the physiological regulation of blood pressure and this could open up new vistas as it relates to the mechanistic contribution of the mitochondrion to blood pressure regulation and give credence to the recently suggested dictum that the mitochondrial metabolism may contribute to the pathogenesis of hypertension (Liang 2011).

**Conclusion**

Our study has shown that fumarate appeared to act as a vasodilator in the kidney. This study has also reinforced the possible link between downstream products of fumarase in the TCA cycle and nitric oxide in the kidney. Hence, TCA cycle intermediaries downstream of fumarate appear to exert effects on MABP and renal haemodynamics, just like any other vasodilator. These acute effects of fumarate on blood pressure may be coupled to but not totally dependent on NOS-downstream signaling.

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**Conflict of interests**

The authors have declared that no conflict of interests exists.

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