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Relationship between PPARα activation and NO on proximal tubular Na^+ transport in the rat

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Abstract

Background: Nitric oxide (NO) regulates renal proximal tubular (PT) Na^+ handling through modulation of Na^+-K^+ ATPase. Peroxisome Proliferator Activated Receptorα (PPARα), a nuclear transcription factor, is expressed in PTs and has been reported to influence NO generation/activity in renal tissues. This study tested the hypothesis that PPARα interacts with NO and thereby affects renal tubular Na^+ transport. Urinary excretion of nitrite (UNO_XV) and Na^+ ($U_{Na}V$) and PT Na^+ transport (Na^+-K^+ ATPase activity) were determined in rats treated with clofibrate (250 mg/kg i.p) or WY14643 (45 mg/kg; i.p.), a PPARα ligand, 2% NaCl (orally), clofibrate/NaCl, L-NAME, an inhibitor of NO production (100 mg/kg; orally), L-NAME/Clofibrate.

Results: Clofibrate or WY14643 increased PPARα expression by 106 ± 7% ($p < 0.05$) and 113 ± 8% ($p < 0.05$), respectively. Similarly, clofibrate and WY14643 increased expression of MCAD, a downstream target protein of PPARα by 123 ± 8% ($p < 0.05$) and 143 ± 8% ($p < 0.05$), respectively. L-NAME attenuated clofibrate-induced increase in PPARα expression by 27 ± 2% ($p < 0.05$) but did not affect MCAD expression. UNO_XV excretion increased 3–4 fold in rats treated with clofibrate, WY14643 or NaCl from 44 ± 7 to 170 ± 15, 144 ± 18 or 132 ± 11 nmol/24 hr, respectively ($p < 0.05$). Similarly, clofibrate, WY14643 or NaCl elicited a 2–5 fold increase in $U_{Na}V$. L-NAME significantly reduced basal UNO_XV and $U_{Na}V$ and abolished the clofibrate-induced increase. Clofibrate, WY14643, NaCl or clofibrate + NaCl treatment reduced Na^+-K^+-ATPase activity in the PT by 89 ± 23, 62 ± 10, 43 ± 9 and 82 ± 15% ($p < 0.05$), respectively. On the contrary, L-NAME or ODQ, inhibitor of sGC, abolished the inhibition of Na^+-K^+-ATPase activity by clofibrate ($p < 0.05$). Clofibrate either alone or with NaCl elicited ~2-fold increase in the expression of the α1 subunit of Na^+-K^+ ATPase in the PT while L-NAME abolished clofibrate-induced increase in Na^+-K^+ ATPase expression.

Conclusion: These data suggest that PPARα activation, through increased NO generation promotes renal excretion of Na^+ through reduced Na^+-K^+ ATPase activity in the PT probably via post translational modification of Na^+-K^+-ATPase.

Background

The proximal tubule (PT) is a major player in the maintenance of salt and water hemostasis as it accounts for the absorption of approximately 70% Na^+ and water-filtered load [1]. The PT epithelial cells are highly enriched in Na^+-K^+ ATPase that is located at the basolateral domain of

epithelial cells and provides the driving force for active sodium and potassium translocation and for the secondary active transport of other solutes across the renal tubules. It consists of α (α_1-α_4), β (β_1-β_3) and γ (γ_1 & γ_2) subunits of which the α1 isoform represents the major Na^+-transporting system [2]. In different species and tissues, the renal Na^+-K^+ ATPase activity is regulated tightly by complex multi hormonal mechanisms and intracellular second messengers to maintain plasma electrolytes during dietary and pathological fluctuation of electrolytes and pH [1,3]. Such regulation involves the activation of distinct hormone- and tissue-specific intracellular signaling molecules such as protein kinase C (PKC) and protein kinase A (PKA) [4,5].

Nitric oxide (NO) is a key paracrine and autocrine regulator of physiological function in a number of organs including the kidney and NO has been reported to inhibit the α1 isoform of Na^+-K^+ ATPase in the PT probably via activation of PKC, or by inhibition of PKA [6-8]. The three isoforms of NO synthase (NOS) enzyme are differentially expressed throughout the kidney with the iNOS isoform expressed in the PT accounting for NO production in this segment [9-11].

Peroxisome Proliferator Activated Receptors (PPARs) constitute a subfamily of the nuclear receptor super-family which are activated by natural ligands such as fatty aids and eicosanoids [12-14]. PPARs regulate gene expression through binding with retinoic acid receptor (RXR) to specific DNA sequence elements termed PPAR response elements (PPREs) or by interfering with other transcription factor pathways in a DNA binding-independent manner [15,16]. Of the three PPARs, namely PPARα, β and γ, only the PPARα stimulates the β-oxidative degradation of fatty acids, resulting in high yield of ATP production and playing an essential role in supporting kidney reabsorptive functions [17,18]. Fatty acid β-oxidation occurs in the kidney peroxisomes which are found exclusively in the PT of the nephron and ranks among the largest peroxisomes known in different cell types [19,20]. PPARα is highly expressed in the rat kidney PT as well as in other nephron segments but its functional role remains unclear [21,22]. Based on the co-expression of PPARα and iNOS in the PT and the fact that PPARα activation modulates the production of NO in non renal cells, we tested the hypothesis that PPARα activators regulate renal tubular Na^+ transport by modulating PT Na^+-K^+-ATPase activity via NO production [23].

In this study, we employed NaCl loading as a stimulant for NO production [24] and investigated the effect of clofibrate and WY14643, PPARα activators, on renal NO production and Na^+-K^+ ATPase activity in the freshly isolated renal proximal tubular cells of the rat.

Results

Fifty five age and weight-matched male Sprague-Dawley rats were used in this study. Initial body weights of the animals were 194 ± 6, 191 ± 3, 198 ± 4, 195 ± 3, 201 ± 3, 199 ± 4, and 199 ± 6 g for the control, clofibrate, NaCl, clofibrate/NaCl, L-NAME, Clofibrate/L-NAME and WY14643 groups, respectively. After the treatment, body weights were unchanged except in the control group where the weight slightly increased by 12 ± 1 % ($p < 0.05$) to 218 ± 6 g.

PPARα and MCAD protein expression

The effects of treatment with different agents on PPARα and MCAD protein expression are illustrated in Fig. 1. Fig. 1a shows that, treatment with clofibrate or WY14643 for 7 days increased PPARα expression by 106 ± 7% ($p < 0.05$) or 113 ± 8% ($p < 0.05$), respectively. L-NAME did not affect basal expression of PPARα but it attenuated clofibrate-induced increase in PPARα expression (27 ± 2%; $p < 0.05$). However, PPARα expression in groups treated with NaCl alone or in combination with clofibrate was not significantly altered (data not shown). Fig. 1b shows that clofibrate or WY14643 increased MCAD protein expression by 123 ± 8% ($p < 0.05$) or 143 ± 8% ($p < 0.05$), respectively. Unlike the data obtained with PPARα, L-NAME was without effect on basal or clofibrate-induced MCAD expression.

Urinary excretion of Nitrite and Na^+

Compared to the control rats, treatment with clofibrate, WY14643 or NaCl elicited a 3–4 fold increase ($p < 0.01$) in 24 hr urinary nitrite excretion rats (Fig. 2a). Combined administration of clofibrate and NaCl produced an increase in nitrite excretion that was not greater than that produced by clofibrate alone. On the other hand, L-NAME reduced basal nitrite excretion by 46 ± 7% ($p < 0.05$) and abolished clofibrate-induced increase in nitrite excretion ($p < 0.05$). Accompanying these changes was a 2–5-fold increase in urinary Na^+ excretion (Fig. 2b) following treatment with WY14643, clofibrate or NaCl. L-NAME reduced 24 hr Na^+ excretion by 80 ± 14% ($p < 0.05$) and abolished clofibrate-induced increase in Na^+ excretion ($p < 0.05$).

Na^+-K^+ATPase activity and the expression of Na^+-K^+ATPase αI protein

The effects of these treatments on Na^+-K^+ATPase activity and expression are illustrated in Fig. 3. Basal Na^+-K^+ATPase activity in these experiments was 226 ± 28 nmol/mg protein/min. Clofibrate, NaCl or WY14643 caused a significant reduction in Na^+-K^+ ATPase activity in the PT by 89 ± 33% ($p < 0.05$), 43 ± 4% ($p < 0.05$) or 63 ± 23% ($p < 0.05$), respectively (Fig. 3a). Combined administration of clofibrate and NaCl elicited a greater reduction in Na^+-K^+-ATPase activity (81 ± 14%, $p < 0.05$), that was higher than that produced by NaCl alone but not

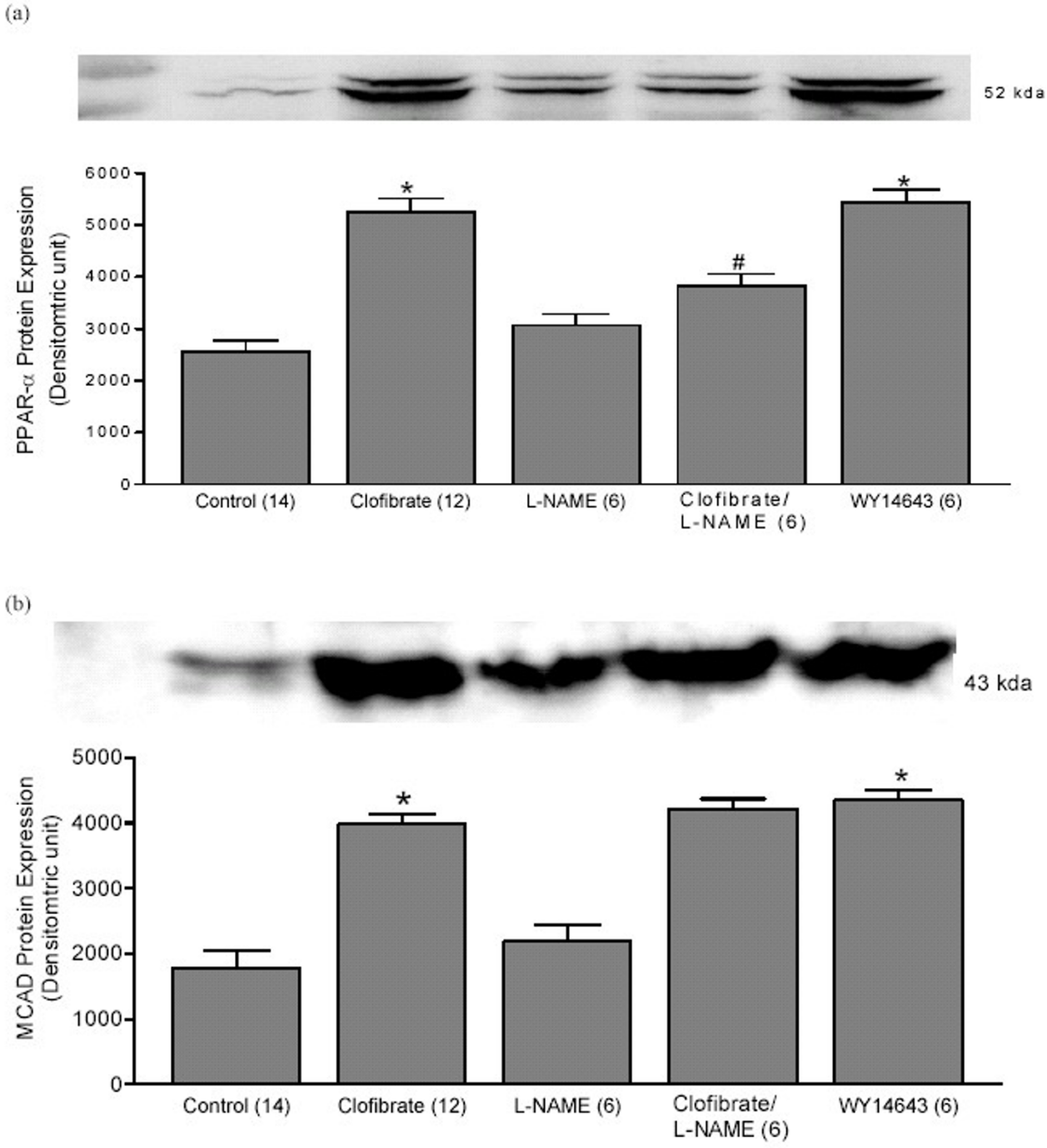

Figure 1
Changes in PPARα and MCAD protein expression. Expression of PPARα (a) and MCAD (b) proteins in vehicle-treated (Control) rats, or rats treated for 7 days with clofibrate (Clofibrate; 250 mg/kg; i.p.), L-NAME (L-NAME; 100 mg/kg; p.o.), WY14643 (WY14643; 45 mg/kg; i.p.), or clofibrate and L-NAME (Clofibrate/L-NAME). The upper tracing in each figure shows a typical immunoblot for PPARα (a) or MCAD (b) proteins. Values are mean ± SEM. Values in parentheses are the number of experiments. * $p < 0.05$ versus Control. #-$p < 0.05$ versus Clofibrate.

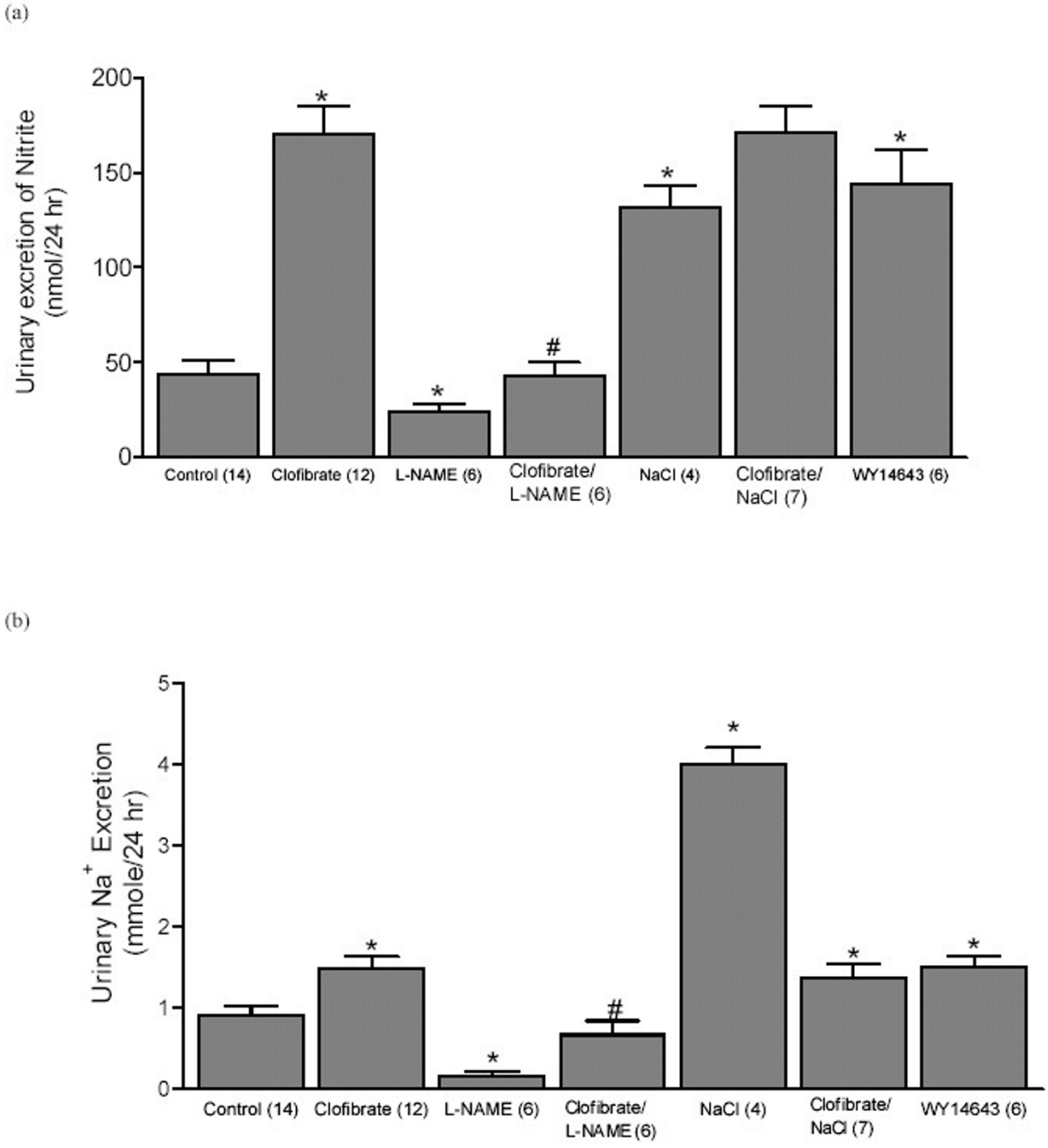

Figure 2
Urinary excretion of nitrite and Na^+. 24 hr urinary excretion of nitrite (a) and Na^+ (b) after 7 days of treatment with vehicle (Control), clofibrate (Clofibrate; 250 mg/kg; i.p.), L-NAME (L-NAME; 100 mg/kg; p.o.), 2 % NaCl in drinking water (NaCl), WY14643 (WY14643; 45 mg/kg; i.p.), clofibrate and NaCl (Clofibrate/NaCl) or clofibrate and L-NAME (Clofibrate/L-NAME). Values in parentheses are the number of animals. Values are mean ± SEM. *-$p < 0.05$ versus Control. #-$p < 0.05$ versus Clofibrate.

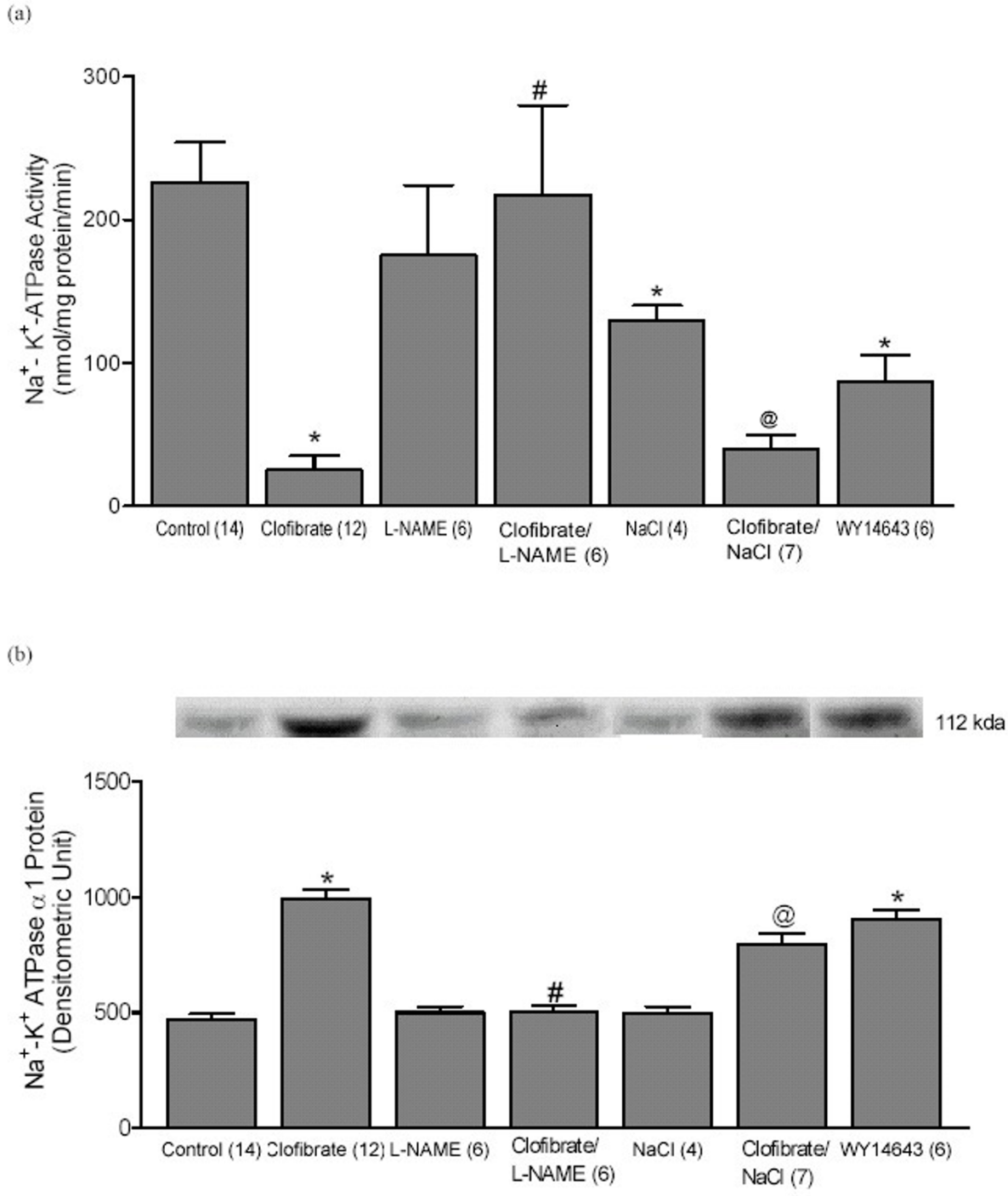

Figure 3
Na^+-K^+-ATPase activity and Expression of Na^+-K^+-ATPase α1 protein. Na^+-K^+-ATPase activity (a) and expression of Na^+-K^+-ATPase α1 protein (b) in the renal proximal tubules of different experimental groups: Control (vehicle-treated), clofibrate (clofibrate 250 mg/kg; i.p.), L-NAME (L-NAME; 100 mg/kg; p.o.), Clofibrate/L-NAME (Clofibrate and L-NAME), NaCl (2% NaCl in drinking water), Clofibrate/NaCl (clofibrate and NaCl) or WY14643 (WY14643; 45 mg/kg; i.p.). Values are mean ± SEM. Values in parentheses are the number of animals. *-$p < 0.05$ versus Ccontrol. #-$p < 0.05$ versus Clofibrate. @-$p < 0.05$ versus NaCl.

greater than that produced by clofibrate alone. On the other hand, L-NAME did not affect basal Na^+-K^+-ATPase activity but abolished clofibrate-induced reduction in Na^+-K^+ATPase activity. The role of cGMP in clofibrate-induced reduction in Na^+-K^+-ATPase activity was evaluated by measuring the Na^+-K^+-ATPase activity in the presence of ODQ (10^{-6}M), an inhibitor of sGC. ODQ reversed clofibrate-induced reduction in Na^+-K^+-ATPase activity from 25 ± 10 to 58 ± 7 nmol/mg protein/min (data not shown).

Figure 3b illustrates that treatment with WY14643 or clofibrate elicited a significant increase in the expression of the α1 subunit of the Na^+-K^+-ATPase protein, the major Na^+ transporting isoform and this remained elevated even during combined administration of NaCl and clofibrate. However, though NaCl its own inhibited Na^+-K^+ATPase activity, it did not affect the expression of Na^+-K^+-ATPase α1 protein. Similarly, L-NAME alone did not alter Na^+-K^+-ATPase α1 expression but abolished clofibrate-induced increase in Na^+-K^+-ATPase expression.

Discussion

The main highlight of this study is that activation of PPARα increased renal NO production and inhibited Na^+ transport in freshly isolated PT of the rat. These results therefore add to a growing list of actions of PPARα which had until recently being thought were limited to specific tissue types. The biologic function of PPARα was initially limited to lipid catabolism and peroxisome proliferation in the liver [17]. However, with the discovery that PPARα is present in a variety of different cell types and a growing list of their activities, their functions and biological effects have grown tremendously [25].

PPARα is highly expressed in the kidney especially in the PT cells. Indeed, PPARα-mediated β-oxidation of long chain fatty acids occurs predominantly in the S3 segment of the PT and represents an important mechanism for energy production in the kidney cortex. In addition, fatty acid β-oxidation enzymes which exist in the mitochondria, peroxisomes and microsomes of this nephron segment are very important in the preservation of renal function following damage to the kidney [26-29]. The importance of fatty acid β-oxidation and PPARα is also underscored by the recognition that the kidney is the only other organ in addition to the liver in which fatty acid β-oxidation occurs in the peroxisomes which are found exclusively in the PT of the nephron, and rank among the largest peroxisomes known in different cell types [20].

The association of PPARα with PT drew us to this nephron segment with a view to evaluating its effect on ion transport. PT cells possesses an abundance of Na^+-K^+ ATPase that regulates transepithelial Na^+ movement and a number of agents, endogenous and exogenous, and disease conditions can modulate the activity of the enzyme. Prominent amongst the endogenous regulators of Na^+-K^+ATPase activity in the PT is NO. However, the effects of NO on PT are controversial as increase as well as inhibition of Na^+ transport have been reported [6,30,31].

Following the demonstration in some recent reports that PPARα ligands modulate NO production in vascular tissues and macrophages, we wondered whether PPARα activators affect NO production in the kidney and if so, whether NO plays a role in the effect of PPARα on sodium excretion and renal Na^+ transport in the PT. We first established that clofibrate and WY14643 are PPARα ligands based on their capacity to increase PPARα expression and its downstream target protein MCAD. These effects were accompanied by increased nitrite excretion. However, L-NAME inhibited PPARα but not MCAD expression (Fig. 1). Using NaCl loading as a stimulus for NO production we observed that NaCl caused a 3–4 fold increase in urinary excretion of nitrite as did clofibrate and WY14643, prototype PPARα activators [24]. The increase in urinary nitrite excretion is indicative of the ability to stimulate renal NO production. In parallel to this, clofibrate, WY14643 and NaCl decreased Na^+-K^+-ATPase activity and the combined administration of clofibrate and NaCl further enhanced this reduction. In addition, ODQ, an inhibitor of sGC, or L-NAME attenuated the effects of clofibrate on Na^+-K^+ATPase activity. The decrease in Na^+-K^+-ATPase activity following sodium loading is in agreement with studies that demonstrated increased level of endogenous digitalis-like factor and is consistent with the known capacity of the PT Na^+-K^+-ATPase to participate in promoting Na^+ excretion [32]. Taken together, these data suggest that PPARα activation affects Na^+ transport and that this effect may involve NO production. This being the case, it is expected that Na^+ and water excretion should increase when NO production increased. This was indeed the case as we observed a marked increase in urinary Na^+ excretion following administration of WY14643, clofibrate or NaCl alone or in combination with each other. Though NO production was of similar magnitude in the groups treated with clofibrate, WY14643 or NaCl, the corresponding effect on $U_{Na}V$ and urine output is greatest for the NaCl group. We interpret these data to mean that salt loading promotes natriuretic and diuretic effects that are not exclusively linked to NO production in the PT. It thus appears that high salt concentration may increase the osmolar gradient in the medulla and countercurrent multiplier system therein and thereby elicit a strong natriuretic effect that involves additional mechanism apart from NO production. The lower effect on PT Na^+-K^+ATPase activity in NaCl-treated group despite the strong natriuresis suggests additional effects on other nephron segments possibly in the loop of Henle where inhibition of Na^+, K^+

or Cl^- transport can produce strong natriuretic effect as seen with the loop diuretics.

Though there are many isoforms of Na^+-K^+-ATPase, the α-1 isoform represents the main sodium transporter in the basolateral membrane and is the target of many hormones that regulate PT sodium reabsorption [2,32]. In these experiments, it is note worthy that reduction in Na^+-K^+ATPase activity by clofibrate, clofibrate and NaCl, and WY14643 but not that by NaCl alone was accompanied by increased expression of α1 subunit of Na^+-K^+ATPase protein. Similarly, L-NAME abolished clofibrate-induced increase in Na^+-K^+-ATPase expression but not its activity. We interpret the incongruence in activity and expression of Na^+-K^+-ATPase to post translational modifications of the enzyme by NaCl and L-NAME. The reason for this is not clear but it is not surprising considering that dissociation has also been observed between PKC-inhibitable inhibition by dopamine of Na^+-K^+-ATPase activity and phosphorylation of the α subunit of the enzyme [33].

Conclusion

Data presented in this study suggest that PPARα activators increase NO generation and promote ion transport in the PT through a reduction in Na^+-K^+ ATPase activity. The effects produced by NaCl or L-NAME may involve a post translational modification of Na^+-K^+ ATPase.

Methods

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Southern University following the National Institutes of Health (NIH) guidelines for laboratory animal use.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Rabbit polyclonal antibody against Na^+-K^+ ATPase α1 and PPARα were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Antibody to MCAD (medium chain acyldehydrogenase) and 1H-[1,2,4] oxadiazolo [4,3-α] quinoxalin-1-one (ODQ) were obtained from Cayman (Cayman Chemicals, Ann Arbor, MI)

Adult male Sprague-Dawley rats (Harlan, Houston TX), 150–200 g were randomly allocated to 4 treatment groups as follows: Control (mineral oil, 50 μl i.p.; n = 8), clofibrate (250 mg/kg; i.p.; n = 6), NaCl (2% in drinking water; n = 4), and clofibrate/NaCl (n = 7). The NaCl group was included since salt loading is a known activator of NO production [24]. In another set of experiments 30 rats were randomly divided into five treatment groups (n = 6 in each group): control, clofibrate (250 mg/kg; i.p.), L-NAME (100 mg/kg; orally) clofibrate/L-NAME and WY14643 (45 mg/kg; i.p.) a synthetic PPARα ligand. Animals were maintained on the treatments for 7 days before they were sacrificed. They were kept in metabolic cages for collection of 24 hr urine output on days 1 and 7 of the study period. At the end of the treatment period, animals were anesthetized with pentobarbital sodium (50 mg/kg; i.p). and the kidneys were perfused with 20 ml of Krebs buffer containing iron oxide particles (100 μl/10 ml buffer).

Isolation of proximal tubule

PT from the rat kidney were isolated following the procedure described by Chibalin et al with little modification. Kidneys were isolated, cut longitudinally, and the cortex was separated from the medulla and the papilla [33]. Cortical tissues were minced and placed in scintillation vials containing 10 ml fresh ice-cold digestion solution (collagenase 230 u/ml, hyaluronidase 250 u/ml, trypsin inhibitor 10 μg/ml, sucrose 320 mM, and EDTA 0.1 mM). Using a plastic pipette, the samples were titurated for about 20 sec to loosen up tissue and to expose more tubules for digestion. These samples were then incubated in a shaking water bath for 15–20 min at 37°C while continuously bubbling with 95% O_2 and 5% CO_2. After a complete digestion, samples were placed in a 50 ml centrifuge tube which was placed on a BioMag magnetic separator (Polyscience Inc, PA). Vascular tissue and glomeruli containing iron were separated by magnetic attraction and tubular fraction carefully transferred to another tube. Proximal tubular fraction was further enriched by percoll density-gradient centrifugation in which samples were layered in between 5 ml of 35% percoll and 5 ml of 100% percoll solution and centrifuged at 13000 g for 15 min. Proximal tubular fraction was carefully collected. Viability of the tubules (95%) was confirmed by microscopy and trypan blue exclusion test.

Na^+-K^+ ATPase activity assay

Na^+-K^+ ATPase activity was determined in the proximal tubular fraction as the rate of inorganic phosphate released in the presence or absence of ouabain [6,34]. Tubular suspension was washed three times with phosphate-free buffer (NaCl 2.36 M, $NaHCO_3$ 0.54 M, KCl 0.4 M, $MgCl_2$ 0.12 M) and the final proximal tubule suspension was adjusted to 1 mg protein/ml. Aliquots of tubular suspension (100 μl) were pipetted into 800 μl reaction mixture (NaCl 75 mM, KCl 5 mM, $MgCl_2$ 5 mM, NaN_2 6 mM, Na EGTA 1 mM, imidazole 37.5 mM, Tris HCl 75 mM and histidine 30 mM; pH 7.4) with or without ouabain (1 mM) in a final volume of 1 ml and pre-incubated for 5 min in a water bath at 37°C. Reaction was initiated by adding Tris ATP (4 mM) and was stopped after 15 min of incubation at 37°C by adding 50 μl of 50% TCA. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixture where ouabain was added. To quantify the amount of

phosphate produced, I ml of coloring reagent (10% ammonium molybdate in 10 N sulfuric acid + ferrous sulfate) was added to the reaction mixture, mixed thoroughly and centrifuged at 3000 g for 10 min. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm against a standard curve prepared from K_2HPO_4. Na^+-K^+ ATPase activity was estimated as the difference between total ATPase activity and the ouabain-insensitive ATPase activity and expressed as nmol phosphate produced per mg protein per min. In another set of experiments, proximal tubules isolated from clofibrate-treated rats were incubated with ODQ (10^{-6} M), an inhibitor of soluble guanylyl cyclase (sGC) for 30 mins before measurement of Na^+-K^+ ATPase activity.

Immunoblotting of Na^+-K^+ ATPase α1 protein

Solubilized protein collected from the PT of the rat kidney was separated in 10% SDS-PAGE gel and transferred to a PVDF membrane (Amersham Pharmacia, NJ). Blots were blocked with 5% non-fat dried milk (wt/vol) and 0.1% Tween 20 in TBS (50 mM Tris Base, 137 mM NaCl, 1 M HCl; pH 7.4) overnight at 4°C and incubated for 1 hr with isoform-specific anti α-1 mouse monoclonal antibody (1:500). After washing in TBST (TBS + 0.1% Tween 20), blots were incubated with corresponding peroxidase-conjugated affinity purified anti-mouse anti serum at 1:1000 dilution for 1 hr. Immunoreactivity was detected by enhanced chemiluminescence (Hyperfilm ECL, Amersham Pharmacia, NJ).

Immunoblotting of PPARα and MCAD protein

40 μg of protein from PT homogenate was electrophoresed on 12% polyacrylamide gels and transferred to PVDF membrane (Amersham Pharmacia, NJ). Blots were probed with rabbit polyclonal anti PPARα antibody and anti MCAD antibody at 1:1000 and 1: 500 dilutions, respectively, followed by addition of secondary antibody at 1:5000 dilution. Immunocomplexes were visualized using an enhanced chemiluminescence (ECL-Plus) detection system from Amersham Pharmacia, NJ.

The intensity of the bands was scanned and quantified using Personal Densitometer SI scanner and ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Biochemical Analysis

Protein concentration in the tissue preparation was determined by the BCA protein assay kit from Pierce (Rockford, IL). Urine Na^+ was determined by flame photometer (PFP7. Genway Ltd, Essex, UK) and urinary excretion of nitrite was determined colorimetrically by Griess Assay.

Statistical Analysis

Data were presented as mean ± SEM and compared between groups for significance using students 't' test. In all cases, $p < 0.05$ was considered as significant.

List of abbreviations

MCAD: Medium chain acyldehydrogenase

NO: Nitric Oxide

ODQ: 1H-[1,2,4]oxadiazolo [4,3-α]quinoxalin-1-one

PKA: Protein Kinase A

PKC: Protein kinase C

PPAR: Peroxisome Proliferator Activated Receptor

PT: Proximal Tubule

sGC: Soluble guanylyl cyclase

$U_{Na}V$: Urinary sodium

UV: Urine volume

UNO_XV: Urinary Nitrite

Authors' contributions

MAN carried out biochemical and molecular biology experiments, participated in the design of the study and drafted the manuscript. KR participated in western blot experiments. AOO conceived of the study, and participated in its design, redaction and coordination.

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