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ORIGINAL ARTICLE

Kidney

Simultaneous angiotensin receptor blockade and glucagon-like peptide-1 receptor activation ameliorate albuminuria in obese insulin-resistant rats

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Abstract

Insulin resistance increases renal oxidant production by upregulating NADPH oxidase 4 (Nox4) expression contributing to oxidative damage and ultimately albuminuria. Inhibition of the renin-angiotensin system (RAS) and activation of glucagon-like peptide-1 (GLP-1) receptor signalling may reverse this effect. However, whether angiotensin receptor type 1 (AT1) blockade and GLP-1 receptor activation improve oxidative damage and albuminuria through different mechanisms is not known. Using insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats, we tested the hypothesis that simultaneous blockade of AT1 and activation of GLP-1r additively decrease oxidative damage and urinary albumin excretion ($U_{alb}V$) in the following groups: (a) untreated, lean LETO (n = 7), (b) untreated, obese OLETF (n = 9), (c) OLETF + angiotensin receptor blocker (ARB; 10 mg olmesartan/kg/d; n = 9), (d) OLETF + GLP-1 mimetic (EXE; 10 µg exenatide/kg/d; n = 7) and (e) OLETF + ARB +exenatide (Combo; n = 6). Mean kidney Nox4 protein expression and nitrotyrosine (NT) levels were 30% and 46% greater, respectively, in OLETF compared with LETO. Conversely, Nox4 protein expression and NT were reduced to LETO levels in ARB and EXE, and Combo reduced Nox4, NT and 4-hydroxy-2-nonenal levels by 21%, 27% and 27%, respectively. At baseline, $U_{alb}V$ was nearly double in OLETF compared with LETO and increased to nearly 10-fold greater levels by the end of the study. Whereas ARB (45%) and EXE (55%) individually reduced $U_{alb}V$, the combination completely ameliorated the albuminuria. Collectively, these data suggest that AT1 blockade and GLP-1 receptor activation reduce renal oxidative damage similarly during insulin resistance, whereas targeting both signalling pathways provides added benefit in restoring and/or further ameliorating albuminuria in a model of diet-induced obesity.

KEYWORDS

chronic kidney disease, diabetes, obesity, oxidative stress, renin-angiotensin system

Rodriguez and Escobedo shared first authorship.

1 | INTRODUCTION

The prevalence of chronic kidney disease (CKD) in the US adult population was 14.8% from 2011 to 2014.[1] Many factors are associated with the development of CKD including older age, hypertension, cardiovascular disease, obesity and diabetes mellitus (DM), with the latter co-existing in 30%-40% of individuals with CKD.[2] Although DM is a primary factor for the development of CKD, the deterioration of renal function precedes the development of overt DM. Therefore, factors that precede the development of DM such as obesity, the metabolic syndrome and insulin resistance are major risk factors for the development of CKD.[3,4] Obesity and its comorbidities are major risk factors because they inappropriately activate the renin-angiotensin system (RAS),[5] increase production of oxidants and/or decrease the expression of antioxidant enzymes leading to oxidative stress.[6,7] In patients with CKD further deterioration of renal function is associated with higher urinary angiotensinogen excretion ($U_{Agt}V$), an index of intrarenal angiotensin II activity.[8,9] Additionally, CKD patients present with higher plasma 8-isoprostane levels (a marker of oxidant stress status in vivo),[10] which were inversely correlated with lower estimated glomerular filtration rates (eGFR).[11] However, the mechanisms by which inappropriately activate RAS and oxidative stress compromise renal health, and ultimately, function remain unclear in insulin-resistant conditions.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that acts through the GLP-1 receptor (GLP-1r), which is expressed in many tissues including the kidneys.[12-14] GLP-1 increases insulin secretion in a glucose-dependent manner,[15] suggesting that it may be a useful therapeutic agent for targeting the maladies associated with the metabolic syndrome and insulin resistance. GLP-1r agonists and dipeptidyl peptidase inhibitors may also serve as novel antihypertensive agents by increasing Na^+ excretion[16-18] and may have protective effects against diabetic nephropathy by downregulating NADPH oxidase (Nox) enzymes.[19-21] Nox enzymes are major producers of oxidants in the kidney under high glucose or high angiotensin II (Ang II) conditions leading to oxidative damage.[21,22] Of the seven Nox isoforms, Nox1, Nox2 and Nox4 are expressed in the rodent kidney,[23] with Nox4 being the most prominent in the renal cortex.[24] Collectively, these relationships suggest that increased renal Nox4 mediates oxidative renal injury during insulin resistance and that GLP-1 based therapies may be beneficial in preventing the development of CKD.

Monotherapy of either angiotensin receptor blockade (ARB) or GLP-1 receptor agonist decreased urinary albuminuria excretion in patients with DM, suggesting that these two signalling pathways may be involved in the manifestation of renal injury.[25,26] While the blockade of AT1 and the activation of the GLP-1r ameliorate renal oxidative stress, their combined effects on oxidant/antioxidant signalling, and subsequently, on renal injury during insulin resistance remain unexamined. Using insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of diet-induced obesity, insulin resistance and elevated intrarenal RAS,[27-30] we tested the hypothesis that simultaneous blockade of AT1 and activation of GLP-1r additively decrease oxidative damage and albuminuria.

2 | RESULTS

2.1 | OLETF rats are characterized by increased arterial pressure, insulin resistance and urinary angiotensinogen excretion

Body mass (BM), absolute and relative fat masses, absolute kidney mass and $U_{Agt}V$ were measured to assess whether AT1 blockade and activated GLP-1r signalling blunted the development of obesity, insulin resistance and elevated intrarenal RAS. Data on BM, the homeostasis model assessment of insulin resistance (HOMA-IR), and $U_{Agt}V$ have been previously published,[31] but are briefly included here for completeness. On day 41, mean arterial pressure (MAP) was 22% higher in OLETF than in LETO and EXE was not significantly different from OLETF. Notwithstanding, MAP was 22% and 27% lower in ARB and Combo, respectively, compared with OLETF (Table 1).[31] Additionally, BM was 34% greater in OLETF compared with LETO, whereas ARB and EXE had no significant effect on BM compared with OLETF. On the other hand, BM was 10% lower in Combo compared with OLETF (Table 1). Mean absolute and relative fat masses were greater in OLETF compared with LETO. However, there were no significant changes among the treatment groups and OLETF (Table 1). Mean absolute kidney mass was 45% greater in OLETF compared with LETO. Nevertheless, there were no significant changes among the treatment groups and OLETF (Table 1). Fasting plasma glucose (FPG) and fasting plasma insulin (FPI) were 32% and 128% higher, respectively, in OLETF than in LETO. FPI was not significantly different in ARB, EXE and Combo compared with OLETF. Yet, FPG was 9% lower in ARB compared with OLETF, and EXE and Combo were not significantly different from OLETF (Table 2).[31] Mean plasma leptin was nearly 4-fold greater ($P < .001$) in OLETF compared with LETO, and ARB and Combo reduced ($P < .05$) values 48% and 52%, respectively, but still almost double of LETO ($P < .05$) (Table 2). EXE had no detectable effect on plasma leptin with values similar to OLETF (Table 2). HOMA-IR was 240% greater in OLETF compared with LETO; however, there were no significant changes among ARB, EXE and Combo compared with OLETF (Table 2).[31] On day 40, mean $U_{Agt}V$ was greater in OLETF compared with LETO (748 ± 66 vs 135 ± 10 ng/day; $P < .05$) and was lower in ARB (332 ± 43 ng/d; $P < .05$), EXE (287 ± 25 ng/d; $P < .05$) and Combo (313 ± 26 ng/d; $P < .05$) compared with OLETF.[31] Collectively, these results demonstrate that OLETF rats are characterized by obesity, insulin resistance and elevated intrarenal RAS.

2.2 | AT1 blockade and GLP-1r activation reduce Nox4 protein expression in the kidney

Kidney Nox4 protein expression, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities were measured to assess whether AT1 blockade and activated GLP-1r signalling improved renal oxidant potential and antioxidant balance. Mean Nox4 protein expression was 30% greater in OLETF compared with LETO and was 20%, 15% and 21% lower in ARB, EXE and Combo, respectively (Figure 1). Mean SOD activity was 50% lower in OLETF compared with LETO,

TABLE 1 Mean body mass, absolute and relative fat masses and absolute kidney mass in LETO, OLETF, OLETF + olmesartan (ARB), OLETF + exenatide (EXE) and OLETF + combination (Combo) group of rats at 16 wk of age

	LETO (n = 7)	OLETF (n = 9)	ARB (n = 9)	EXE (n = 7)	Combo (n = 6)
MAP (mm/Hg)	94 ± 2	115 ± 3*	90 ± 3**	110 ± 1*,***	84 ± 2*,**,****
Body mass (g)	425 ± 14	568 ± 13*	530 ± 6*	547 ± 11*	509 ± 19*,**
Retrofat (g)	6.0 ± 0.4	22.5 ± 1.9*	19.5 ± 1.4*	19.3 ± 1.5*	16.1 ± 1.1*
Relative retrofat (g/ 100 g BM)	1.6 ± 0.1	4.2 ± 0.2*	3.9 ± 0.2*	3.9 ± 0.2*	3.5 ± 0.1*
Epi-fat (g)	6.0 ± 0.2	11.3 ± 1.0*	11.3 ± 0.8*	10.4 ± 0.7*	8.6 ± 0.7*
Relative epi-fat (g/100 g BM)	1.6 ± 0.1	2.1 ± 0.1*	2.3 ± 0.1*	2.1 ± 0.1*	1.9 ± 0.1
Total fat (g)	12 ± 0.6	33.8 ± 2.6*	30.9 ± 2.2*	29.6 ± 2.1*	24.8 ± 1.6*
Relative total fat (g/100 g BM)	3.1 ± 0.1	6.3 ± 0.3*	6.2 ± 0.4*	5.9 ± 0.3*	5.4 ± 0.2*
Kidney mass (g)	1.1 ± 0.03	1.6 ± 0.07*	1.5 ± 0.03*	1.6 ± 0.04*	1.6 ± 0.04*

Abbreviations: epi, epididymal; Retro, retroperitoneal.

*P < .05, vs LETO.

**P < .05 vs OLETF.

***P < .05 vs ARB.

****P < .05 vs EXE.

TABLE 2 Mean (± SE) fasting plasma biochemical and hormone measurements, and calculated HOMA-IR in LETO, OLETF, OLETF + olmesartan (ARB), OLETF + exenatide (EXE) and OLETF + combination (Combo) group of rats at 16 wk of age

	LETO (n = 7)	OLETF (n = 9)	ARB (n = 9)	EXE (n = 7)	Combo (n = 6)
Glucose (mmol/dL)	5.6 ± 0.2	7.4 ± 0.2*	6.7 ± 0.1*,**	7.1 ± 0.2*	7.0 ± 0.1*
Insulin (mmol/L)	130 ± 18	296 ± 31*	229 ± 28	325 ± 45*	300 ± 27*
HOMA-IR (relative units)	0.8 ± 0.1	2.7 ± 0.4*	1.6 ± 0.2	2.5 ± 0.4*	2.3 ± 0.2
Leptin (ng/mL)	2.33 ± 0.21	9.08 ± 0.83*	4.76 ± 0.67*,**	6.84 ± 0.97*	4.35 ± 0.36*,**
Creatinine (mg/dL)	0.71 ± 0.06	0.35 ± 0.04*	0.38 ± 0.04*	0.40 ± 0.05*	0.52 ± 0.07*

Abbreviation: HOMA-IR, homeostasis model assessment of insulin resistance.

*P < .05, vs LETO.

**P < .05 vs OLETF.

whereas activity was 81% and 100% higher in ARB and Combo, respectively, compared with OLETF (Figure 2A). Although SOD activity was 61% greater in EXE compared with OLETF, it was not statistically significant (P = .065) (Figure 2A). Mean catalase and GPx activities were similar among all groups (Figure 2B,C). Collectively, these results suggest that blockade of AT1 and activation of GLP-1r-mediated signalling share a common pathway to improve Nox4 protein expression in the kidney. Furthermore, these results suggest that the degree of insulin resistance-associated renal dysfunction in OLETF rats is sufficient to impair the superoxide radical quenching capacity (SOD), but not sufficient to impair the antioxidant capacity of the hydrogen peroxide quenching enzymes (catalase and GPx).

2.3 | AT1 blockade and GLP-1R activation improve renal injury

Urinary 8-isoprostane excretion ($U_{8\text{-iso}}V$), renal nitrotyrosine (NT) and 4-hydroxy-2-nonenal (4-HNE) were measured to determine whether AT1 blockade and activated GLP-1r signalling reduced the obesity-associated systemic and renal oxidative damage. Mean $U_{8\text{-iso}}V$ was 102% higher in OLETF compared with LETO (Figure 3A) but was not different among the treatment groups and OLETF (Figure 3A). Mean renal NT was 46% greater in OLETF compared with LETO, whereas levels were reduced by 25%, 22% and 27% in ARB, EXE and Combo, respectively, compared with OLETF (Figure 3B). Mean renal 4-HNE levels were not different among LETO, OLETF, ARB and EXE; however, they were 27% lower in Combo compared with OLETF (Figure 3C). Collectively, these results suggest that the insulin resistance associated with OLETF rats is characterized by systemic oxidative stress that is not reversed by either AT1 blockade or activation of GLP-1r signalling. However, renal protein nitration is reversed by AT1 blockade and activation of GLP-1r signalling in a non-additive fashion.

2.4 | AT1 blockade in conjunction with GLP-1r activation ameliorates albuminuria in OLETF rats

Urinary albumin excretion ($U_{alb}V$), urinary albumin:creatinine ratio (ACR) and the PAS-positive area in glomeruli were assessed to

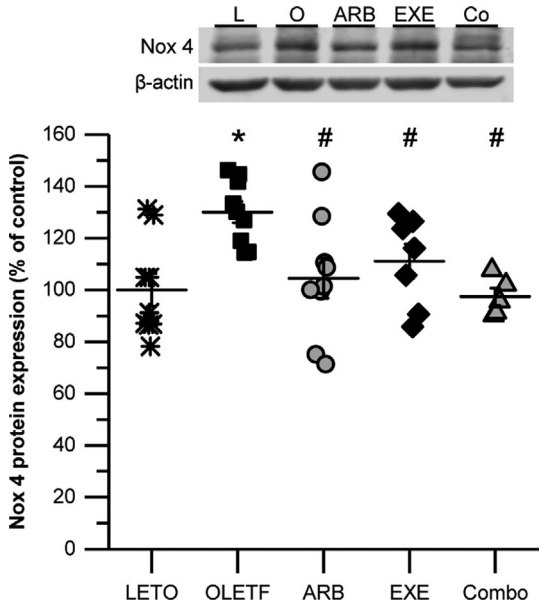

FIGURE 1 AT1 blockade in conjunction with GLP-1r activation reduced kidney Nox 4 protein expression. Mean (±SE) kidney Nox 4 (% change from LETO), and the representative western blot bands of Long-Evans Tokushima Otsuka (LETO; n = 7), Otsuka Long-Evans Tokushima Fatty (OLETF; n = 9), OLETF + ARB (ARB; n = 8), OLETF + EXE (EXE; n = 7) and OLETF + ARB+EXE (Combo; n = 6). * $P < .05$ vs LETO; # $P < .05$ vs OLETF

determine the potential benefits of AT1 blockade and activated GLP-1r signalling to the insulin resistance-associated nephropathy. At baseline, mean $U_{alb}V$ was almost double in OLETF compared with LETO, and levels increased nearly 10-fold in OLETF over the next 6 wks while they remained unchanged in LETO (Figure 4). Mean $U_{alb}V$ decreased nearly 45% and 55% in ARB and EXE, respectively, compared with OLETF, and COMBO completely ameliorated the increase (Figure 4). At baseline, mean ACR was 54% greater in OLETF compared with LETO. On day 40, mean ACR was 12.4-fold greater in OLETF compared with LETO and increased 2.4-fold compared with its baseline levels. When corrected for creatinine excretion, the changes in ARB and EXE were not significantly reduced compared with OLETF, suggesting that subtle, non-significant changes in glomerular filtration were just sufficient enough to abolish the statistical difference. However, mean ACR was reduced 83% in Combo compared with OLETF, suggesting that the improvements in albuminuria were more profound and not a function of alternations in filtration alone. Periodic acid-Schiff (PAS)-positive area in glomeruli among the groups detected no significant changes that would reveal overt renal damage or protective benefits of the treatments at this stage of the insulin resistance-associated renal injury (Figure 5). Collectively, these results suggest that the protective effects of AT1 blockade and GLP-1r activation against the insulin resistance-associated albuminuria in OLETF rats are additive. Furthermore, the increased ACR is not associated with overt glomerular injury at this stage of the insulin resistance. The changes in $U_{crt}V$ were not statistically different among the groups at the end of the study.

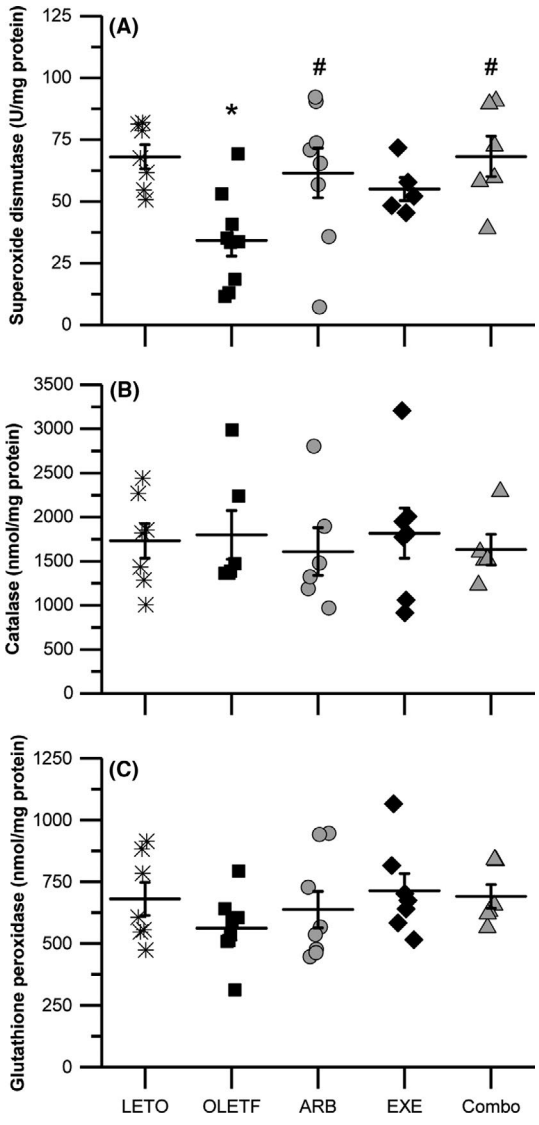

FIGURE 2 AT1 blockade normalizes kidney superoxide dismutase enzyme activity. Mean (±SE) activity of kidney (A) superoxide dismutase, (B) catalase and (C) glutathione peroxidase of Long-Evans Tokushima Otsuka (LETO; n = 7), Otsuka Long-Evans Tokushima Fatty (OLETF; n = 6-9), OLETF + ARB (ARB; n = 6-8), OLETF + EXE (EXE; n = 5-7) and OLETF + ARB+EXE (Combo; n = 5-6). * $P < .05$ vs LETO; # $P < .05$ vs OLETF

3 | DISCUSSION

The prevalence of CKD in the adult US population was 14.8% from 2011 to 2014.[1] Although many factors are associated with the development of CKD, DM is a primary risk factor and accounts for the majority of cases of end-stage renal disease in the United States.[32] Furthermore, DM with the early development of kidney disease reduces lifespan by 16 years, whereas DM and early kidney disease on their own reduce lifespan by 10 and 6 years, respectively.[33] Therefore, renal function should be monitored in high-risk individuals such as those with obesity, insulin resistance and hypertension. Currently, RAS inhibitors are recommended as first-line treatments

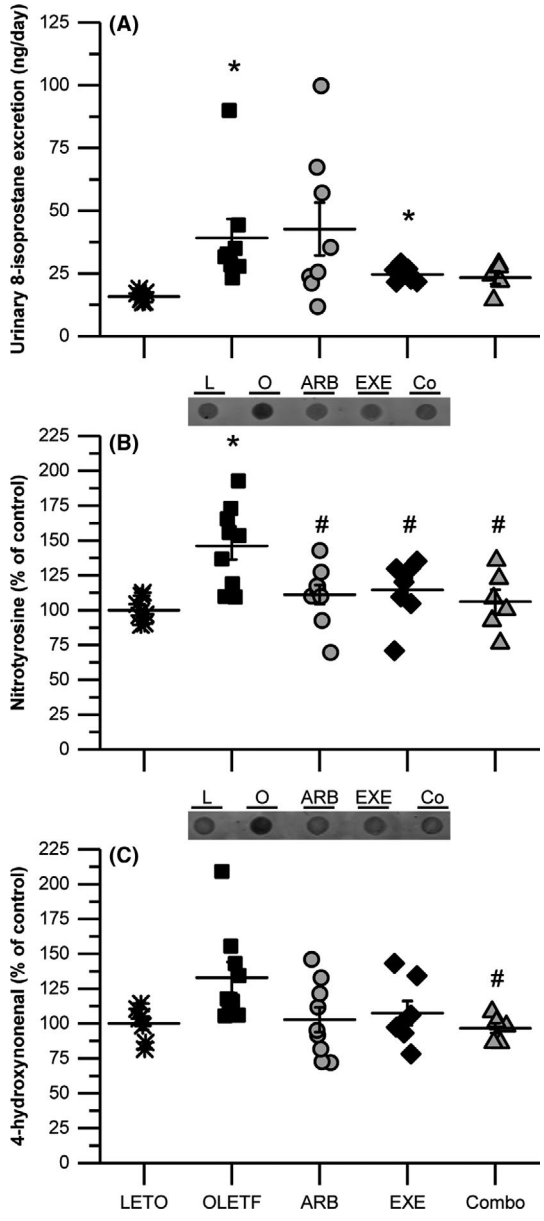

FIGURE 3 Effects of AT1 blockade alone and in combination with GLP-1r activation on oxidative damage. Mean (±SE) urinary (A) 8-isoprostane excretion, (B) kidney nitrotyrosine and (**C**) 4-hydroxynonenal, and the representative dot blots of Long-Evans Tokushima Otsuka (LETO; n = 7), Otsuka Long-Evans Tokushima Fatty (OLETF; n = 7-9), OLETF + ARB (ARB; n = 7-9), OLETF + EXE (EXE; n = 7) and OLETF + ARB+EXE (Combo; n = 5-6). * $P < .05$ vs LETO; # $P < .05$ vs OLETF

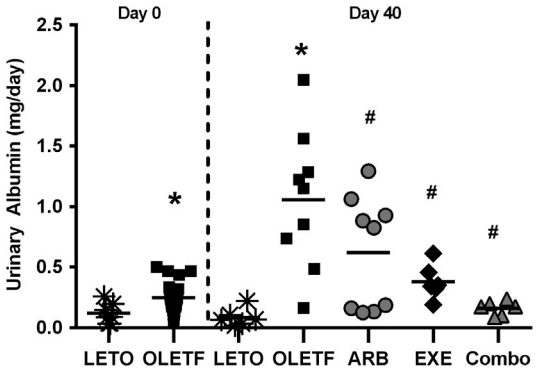

FIGURE 4 AT1 blockade and GLP-1r activation ameliorate albuminuria. Mean (±SE) urinary albumin excretion of Long-Evans Tokushima Otsuka (LETO; n = 7), Otsuka Long-Evans Tokushima Fatty (OLETF; n = 9), OLETF + ARB (ARB; n = 9), OLETF + EXE (EXE; n = 7) and OLETF + ARB+EXE (Combo; n = 6). * $P < .05$ vs LETO; # $P < .05$ vs. OLETF; a $P < .05$ vs baseline for OLETF

for individuals with concomitant DM, hypertension and elevated ACR.[34] Nevertheless, in individuals with DM and high cardiovascular risk, liraglutide (a GLP-1r agonist) added to standard diabetes treatment (83% of participants were on RAS inhibitors) demonstrated that GLP-1r activation lowered the rates of the development and progression of kidney disease,[25] suggesting that simultaneous RAS inhibition and GLP-1r activation may be an appropriate therapeutic option for kidney disease in high-risk individuals. Experimental studies have demonstrated that both RAS inhibitors and GLP-1r agonists improve renal function in diabetic animals by decreasing oxidative damage.[19,21,22,35] Nevertheless, it remains unknown whether simultaneous AT1 blockade and GLP-1r activation improve renal oxidant balance and albuminuria in an insulin-resistant setting. Therefore, the objectives of this study were to evaluate (a) the potential of the added benefits of targeting two signalling pathways (AT1 and GLP-1) that contribute to renal oxidant balance and (b) whether these benefits in renal oxidant balance translated into amelioration of albuminuria. To this end, we found that AT1 blockade and GLP-1r activation reduced renal Nox4 protein expression and NT levels and that combined administration did not have consistent additive effects. However, simultaneous AT1 blockade and GLP-1r activation reduced the $U_{alb}V$ more than monotherapy alone, suggesting that this is a critical early marker for evaluating the renal dysfunction associated with the onset of insulin resistance.

Oxidative damage is a result of increased oxidant production and/or reduced antioxidant capacity.[36] Nox enzymes are the primary oxidant generators in the kidney,[37] with Nox4 being the most prominent in the renal cortex.[23,38] Nox4 is constitutively active[39] meaning that its higher expression in the renal cortex makes glomeruli and proximal tubules susceptible to Nox4-mediated oxidative injury. In rodent models of type 1 diabetes (T1D), global or podocyte deletion/knockdown of Nox4 reduced ACR and attenuated glomerular structural changes.[40-42] Furthermore, the deletion of Nox4 in Dahl salt-sensitive rats blunted the effects of a high Na^+ diet on systolic blood pressure, albuminuria and glomerular injury.[38] Collectively, these studies suggest that Nox4-mediated oxidant overproduction is the primary mediator of renal oxidative damage during impaired metabolic conditions. In the present study, renal Nox4 protein expression was increased, and renal SOD activity was decreased in OLETF rats indicating that impaired renal oxidant-antioxidant balance is a consequence of the early onset of insulin resistance. Furthermore, the hydrogen peroxide quenching enzymes (catalase and GPx) were not elevated in response to the increase in Nox4, which predominantly generates hydrogen peroxide, suggesting that this oxidative

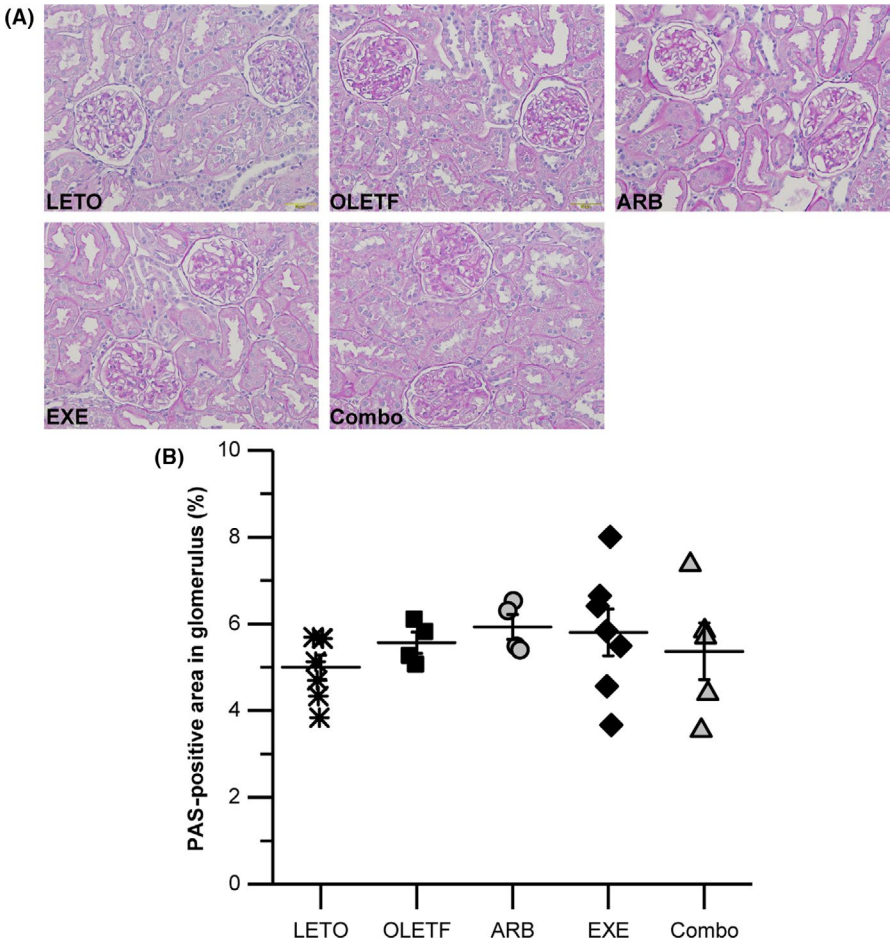

FIGURE 5 Effects of AT1 blockade alone and in combination with GLP-1r activation on glomerulosclerosis. Mean (±SE) PAS-positive area in glomerulus of Long-Evans Tokushima Otsuka (LETO; n = 7), Otsuka Long-Evans Tokushima Fatty (OLETF; n = 4), OLETF + ARB (ARB; n = 4), OLETF + EXE (EXE; n = 6) and OLETF + ARB+EXE (Combo; n = 5)

environment was likely driven by elevated cellular oxidant production. Nevertheless, AT1 blockade or GLP-1r activation reduced Nox4 protein expression, and their combination had no additional benefits, suggesting that these two signalling pathways may share a common mediator of Nox4 regulation. The suppression of Nox4 expression in the treatment groups likely reduced the necessity to increase catalase and GPx, and thus potentially explains the lack of a change in their activities. While Nox2 is also expressed in the kidney, albeit to a much lesser extent than Nox4,[43] we performed additional measurements of phox47 translocation as previously described in the heart of Ang II-infused rats[44] and did not observe a significant group effect (data not shown), suggesting that the oxidative damage observed was likely the primary result of Nox4-generated oxidants and not from Nox2.

Nox4 is induced by high glucose or Ang II,[45-48] which are both elevated in obesity and insulin-resistant conditions.[5,27] In this study, OLETF rats are characterized by fasting hyperglycaemia and increased $U_{Agt}V$, but only AT1 blockade reduced the glucose levels[31] suggesting that overactivation of RAS contributes significantly to the early-onset hyperglycaemia. Conversely, $U_{Agt}V$ was reduced equally by AT1 blockade, GLP-1r activation and their combination, suggesting that these two signalling pathways may reduce Nox4 protein expression through their regulation of intrarenal Ang II levels. The decrease in Ang II-induced cellular damage in renal mesangial cells with GLP-1r agonism[49] corroborates this previous contention.

Nitrotyrosine formation is a result of peroxynitrite production, which is a product formed by the reaction between nitric oxide and superoxide.[50] Although Nox4 mainly generates hydrogen peroxide, it has been suggested that it may also generate superoxide,[47,51,52] which may then interact with nitric oxide to form peroxynitrite. The increase in Nox4 expression and NT levels, in the presence of reduced SOD, suggests that renal Nox4 may have generated sufficient superoxide, which was not sufficiently dismutased because of the suppressed levels of SOD, to promote nitrosative stress in the kidney. The blockade of AT1, activation of GLP-1r and their combination effectively reduced Nox4 expression and ameliorated the suppression of SOD, which resulted in the equivalent reconciliation of the nitrotyrosine levels corroborating the Nox4 mediation of nitrosative stress. In mesangial cells, incubation with high glucose or Ang II increased Nox4 expression, superoxide generation and peroxynitrite formation leading to the uncoupling of endothelial nitric-oxide synthase and fibronectin formation.[47,52] Furthermore, these data suggest that AT1 blockade and activated GLP-1r signalling contribute equivalently to the reduction in oxidants in the kidney during insulin resistance. Although GLP-1r activation did not significantly alter SOD activity here, in streptozotocin-induced diabetic Wistar rats, liraglutide treatment lowered the expressions of Nox4, and the subunits of Nox2, gp91phox and p47phox,[21] suggesting that the protective effects come from decreasing oxidant production rather than oxidant detoxification.

Increased renal Ang II levels are associated with the pathophysiology of renal disease.[8] In the present study, $U_{alb}V$ and ACR were increased in insulin-resistant OLETF rats at baseline, suggesting that the dysmetabolic condition in the model is sufficient to promote early-onset renal injury, which progresses rapidly over the following 6 weeks of age. The progression of the albuminuria is most likely attributed to Ang II-associated renal dysfunction in this model.[53] While ARB and EXE individually provided modest improvements in $U_{alb}V$ suggesting that AT1 and GLP-1 signalling are contributing elements to the progression of the model-associated albuminuria, the combination of these treatments completely ameliorated $U_{alb}V$ and ACR, suggesting that the benefits of AT1 blockade and GLP-1r activation are additive. Additionally, while correction of the $U_{alb}V$ by creatinine excretion removed the statistical difference in $U_{alb}V$ in the ARB and EXE suggesting that subtle, non-significant changes in glomerular filtration partially contributed to the reduction in $U_{alb}V$, the reduction in $U_{alb}V$ remained significant even after correcting for $U_{crt}V$, suggesting that the benefits of co-therapy were not significantly influenced by changes in filtration. While this was one of the few additive effects detected, it is highly biologically significant because a profound improvement in $U_{alb}V$ was observed despite the lack of consistent parallel improvements in oxidant/antioxidant balance and oxidative/nitrosative damage, suggesting that the mechanisms driving proteinuria during the early stages of insulin resistance are not primarily mediated by impaired redox balance signalling and the accompanying injury. Thus, ACR may serve as a critical marker for early detection before the kidney becomes irreversibly compromised with the progression of the insulin resistance into frank type 2 diabetes. This finding is in agreement with that previously reported in which 11 weeks of co-therapy with telmisartan (ARB) and linagliptin (dipeptidyl peptidase 4 inhibitor) in mice induced with T1D normalized albuminuria, whereas the reductions with monotherapy were not statistically significant.[54] Additionally, our findings support the results from the LEADER trial, which found that liraglutide added to standard diabetes treatment (83% of participants were on RAS inhibitors) lowered the rates of the development and progression of kidney disease, with these benefits being a result of a reduction in new onset of persistent macroalbuminuria.[25] Interestingly, the reductions in $U_{alb}V$ and ACR here were independent of improvements in glomerulosclerosis (PAS staining), suggesting that at this stage of the disease the improvements in $U_{alb}V$ and ACR may be a result of altered albumin reabsorption[55] rather than overt glomerular injury.

While the improvement in fasting plasma glucose with ARB was not accompanied by statistically significant reductions in plasma insulin and calculated HOMA-IR values, the reductions in these variables trended ($P < .10$) in the appropriate directions. Furthermore, these analyses in the present study were based on static plasma analyses, whereas we have demonstrated that the same dosage of ARB potently improves glucose tolerance and insulin resistance index calculations using the more robust glucose tolerance test in this model.[27] Interestingly, exenatide and the combination therapies did not translate into static improvements in either fasting plasma glucose or insulin, and thus calculated HOMA-IR. While the present study was not designed to examine the mechanisms of peripheral glucose regulation, we speculate that the improvements in fasting glucose and glucose tolerance in this model[27] stem from the maintenance of pancreatic health and potential amelioration of leptin resistance. This is based on our previous study[27] demonstrating that ARB partially normalized the hyperleptinaemia, which is characteristic of the OLETF. The progression of the diabetes in this model is associated with the development of leptin resistance.[56,57] Chronic blockade of AT1 with telmisartan improved glucose tolerance and other symptoms of the metabolic syndrome via improved leptin signalling and sensitivity associated with amelioration of the hyperleptinaemia,[58,59] suggesting that the improvements in fasting plasma glucose in the present study were derived from similar mechanisms. In the STZ-induced diabetic mouse, exogenous leptin restored the insulinotropic effect of exenatide,[60] suggesting that elevated leptin facilitates GLP-1-mediated effects on insulin signalling. In the present study, OLETF presented with the characteristic hyperleptinaemia, which is partially ameliorated with ARB and combination treatments. Consistent with clinical trials in humans,[61] exenatide did not reduce plasma leptin and the effects of exenatide on circulating leptin must have been sufficient to mask the benefits of ARB because the combination group also did not translate to a significant improvement in fasting plasma glucose despite an equivalent reduction in plasma leptin. While telmisartan[59] and an exenatide analogue[62] have been shown to reduce body mass independently, body mass was only reduced in the combination group, suggesting that the lack of improvements in fasting plasma glucose and HOMA-IR was independent of decreases in body mass. The interactions of these treatments on leptin receptor signalling and glucose metabolism warrant further investigation.

4 | CONCLUSION

In summary, the present study demonstrates that combined GLP-1r activation and AT1 blockade produce an additive improvement in albuminuria, which is independent of any overt glomerular injury. Furthermore, we demonstrated that AT1 blockade, GLP-1r activation and their combination equally reduced $U_{Agt}V$, renal Nox 4 protein expression and nitrotyrosine levels in the kidney. Interestingly, these beneficial effects in combination were independent of improvements in insulin resistance.

5 | METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of Kagawa Medical University, Japan, and the University of California, Merced. The present manuscript complements our previous study using the same animals.[31]

5.1 | Animals

Male, eight-week-old male, Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats were studied

(Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan). Rats were divided into the following groups: (a) untreated LETO (n = 7) + vehicle (0.5% methylcellulose by oral gavage once daily), (b) untreated OLETF (n = 9) + vehicle, (c) OLETF + ARB (ARB; 10 mg olmesartan/kg/d by oral gavage once daily for 42 days; n = 9), (d) OLETF + GLP-1 receptor agonist (EXE; 10 μg exenatide/kg/d by osmotic mini-pumps for 42 days; n = 7) and (e) OLETF + ARB +EXE (Combo; n = 6). Initially, animals in the OLETF groups were assigned so that the initial mean BM of the groups was within 5% of each other. Animals were housed in individual metabolic cages, and urine was collected at baseline and 40 days following the start of the treatments. After collection, urine samples were centrifuged (3000 *g* x 15 minutes at 4°C), and an aliquot transferred to a cryovial and immediately stored at −20°C for later analyses. All animals were housed in a specific pathogen-free facility under temperature (23°C) and humidity-controlled (55%) conditions on a 12-h light-dark cycle. All animals were given free access to water and standard laboratory chow (Teklad Diets, Madison, WI). At 16 weeks of age, all tissues were harvested from animals.

5.2 | Body mass and food intake

BM was measured daily to calculate the appropriate ARB dose.[31]

5.3 | Tissue collection

At 16 weeks of age (following 42 days of treatment), animals were fasted for 12 hours, and tissues were collected the following morning. After BM measurements had been obtained, animals were decapitated, and trunk blood was collected into chilled vials containing 50 mmol/L EDTA and protease inhibitor cocktail (PIC; Sigma-Aldrich). Immediately following, retroperitoneal and epididymal fat masses, and the left kidney were removed, their mass was recorded, and the kidney was snap-frozen in liquid nitrogen and stored at −80°C until analysed. Additionally, the right kidney was perfused with PBS, removed and a mid-transverse section was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Blood samples were centrifuged (3000 *g* x 15 minutes at 4°C), and plasma was transferred to cryovials and stored at −80°C for later analyses.

5.4 | Western blot

Renal protein expression of Nox4 was determined as previously described.[63] Blots were incubated with Nox4 (1:500; Abcam) and β-actin (1:500; Santa Cruz Biotechnology) antibodies. Membranes were washed, incubated for 1 hour with specific secondary antibodies (IRDye: LI-COR Biosciences) in TBS-T + 5% non-fat milk + 0.01% SDS, rewashed and scanned in an Odyssey infrared imager (LI-COR Biosciences). In addition to consistently loading the same amount of total protein per well, densitometry values were further normalized by correcting for the densitometry values of β-actin.

5.5 | Renal oxidative injury and antioxidant enzyme activities

A piece of frozen kidney cortex was homogenized in 50 mmol/L potassium phosphate buffer containing EDTA, PIC (Thermo) and PMSF (EMD Millipore). Subsequent supernatants were used to measure renal NT and 4-HNE levels as markers of oxidative renal injury as previously described.[44] Membranes were incubated with NT (1:1000; Cell Signaling, Danvers, MA) and 4-HNE (1:1000; EMD Millipore) antibodies and developed similarly to western blots (described above). Uniformed protein loading was confirmed by Ponceau S staining. Renal SOD, catalase and GPx activities were measured using commercially available kits (Cayman Chemical, Ann Arbor, MI) as previously described.[63,64] Total protein content was measured with the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) and used to normalize enzyme activities.[63,64]

5.6 | Biochemical analyses and homeostasis model assessment of insulin resistance

Fasting plasma glucose was measured using an Analox GM7 analyzer (Analox Instruments). Fasting plasma creatinine (Sigma-Aldrich), insulin (EMD Millipore) and leptin (R&D Systems) were measured using commercially available kits. All samples were analysed in duplicate and ran in a single assay with intra-assay coefficients of variability of <10%. HOMA-IR was calculated as follows: HOMA-IR = (fasting plasma glucose (mg/dL) x fasting plasma insulin (μU/mL))/2.430, as previously described in rats.[65]

5.7 | Urine analyses

Levels of urinary angiotensinogen (BL America), urinary albumin (Bethyl, Montgomery, TX), urinary 8-isoprostane (Cayman Chemical) and creatinine (Sigma-Aldrich) were measured using commercially available colorimetric kits. Urine albumin concentrations were measured using a Hitachi 7020 chemistry analyzer (Diamond Diagnostics). Urinary excretion of each variable was calculated as the product of daily urine volume and urinary concentration. Additionally, urinary albumin excretion ($U_{alb}V$) was further corrected for urinary creatinine excretion to account for potential effects of changes in glomerular filtration. All samples were analysed in duplicate and run in a single assay with intra-assay, per cent coefficients of variability of <10% for all assays.

5.8 | Histology

Histological evaluation of the glomeruli by periodic acid-Schiff staining (PAS) at 16 weeks of age was performed as described previously.[66] The ratio of the affected lesions to each glomerulus was calculated using the ImageJ software. For each glomerulus, the affected lesion

where the intensity was beyond a threshold calculated by the background signal was measured automatically by the software, and this affected area, in turn, was divided by the total area of the glomerulus. For each kidney sample, 15 glomeruli were examined, and the averaged percentages of the affected lesions were obtained.

5.9 | Statistics

Means (± standard error) were compared by one-way ANOVA followed by Newman-Keuls or Games-Howell post hoc test. For urinary albumin excretion ($U_{alb}V$), we used a two-factor repeated-measures ANOVA with time as a within-subjects factor and group as a between-subjects factor followed by pairwise comparisons using a Bonferroni correction. Means were considered significantly different at $P < .05$, and analyses were performed with SPSS version 24 (IBM).

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CONFLICT OF INTERESTS

At the time this work was performed, DG Parkes was an employee and stockholder at Amylin Pharmaceuticals Inc.

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