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Original Article

Activation of the Na/K-ATPase oxidant amplification loop by uremic toxins drives adipocyte dysfunction *in vitro*

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Abstract

Oxidative stress is a major contributor to chronic kidney disease (CKD) progression. In experimental CKD, circulating uremic toxins (UTs) increase reactive oxygen species (ROS) production, triggering the Na/K-ATPase-ROS amplification loop (NKAL). Dysfunctional adipocytes, as seen in obesity, are a source of ROS and inflammatory cytokines, further exacerbating oxidative stress. We hypothesized that UT exposure activates this signaling pathway in adipocytes, leading to redox imbalance and phenotypic changes, and that the Na/K-ATPase antagonist pNaKtide can mitigate these effects. Murine 3T3-L1 preadipocytes were treated for 5 days with indoxyl sulfate (IS) (50, 100, 250 µM) or p-cresol (50, 100, 200 µM), with or without pNaKtide (0.7 µM), in adipogenic media. Adipogenesis was assessed by Oil Red O staining, superoxide levels by dihydroethidium fluorescence, and gene expression of adipogenic, inflammatory, and apoptotic markers by RT-PCR. In parallel, visceral fat from lean West Virginian donors was used to isolate mesenchymal stem cells (MSCs), which were differentiated into adipocytes and treated for 14 days with IS (25, 50, 100 µM) with or without pNaKtide (1 µM) for morphological and molecular analyses. UT treatment reduced adipogenesis and increased apoptotic and inflammatory markers in both 3T3-L1 and MSC-derived adipocytes, consistent with NKAL activation. Treatment with pNaKtide restores redox balance and improves cellular phenotype, both in *in vitro* models, though its effects on inflammatory and oxidative markers. Our data showed for the first time UT-induced activation of the NKAL as a driver of adipocyte dysfunction *in vitro*. Targeting this pathway with pNaKtide may represent a novel therapeutic approach to reduce oxidative stress-mediated metabolic disturbances in CKD.

Keywords: Uremic toxins, Adipocytes, Indoxyl sulfate, p-cresol, Oxidative stress, Na/K-ATPase signaling, CKD

1. Introduction

Chronic kidney disease (CKD) is a progressive condition that leads to a gradual decline in renal function, ultimately increasing susceptibility to kidney damage [1]. In the United States, it affects an estimated 14% of the population, with the highest mortality rates occurring in individuals at stage 5 CKD, also known as end-stage renal disease (ESRD) [2]. The clinical course of CKD is frequently worsened by coexisting disorders such as diabetes mellitus, obesity, and cardiovascular disease [1]. Although the complete molecular pathways remain incompletely understood, impaired renal clearance results in the accumulation of uremic toxins (UTs), which can accelerate both the onset and progression of CKD [3].

The accumulation of UTs in CKD is associated with excessive generation of reactive oxygen species (ROS) and oxidative stress, both of which are well-documented in ESRD and earlier stages of CKD [4-8]. UTs in the serum of CKD patients promote ROS overproduction and pro-inflammatory cytokine release, leading to disease progression [9]. Oxidative stress is not only a hallmark but also a pathogenic factor in CKD context, having macrophage activation, the Ras and nuclear factor-kappa B (NF-κB) pathways, and increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity as the major source [10, 11]. Increased levels of pro-inflammatory cytokines [interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1)] are correlated with disease severity [12].

Given the association between CKD, metabolic dysfunction, and systemic inflammation, adipose tissue has emerged as an important contributor to CKD-related oxidative stress and inflammation [13, 14]. Adipocytes are active endocrine regulators that release adipokines and inflammatory mediators capable of influencing renal and cardiovascular homeostasis [14, 15]. It has been shown

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that adipocytes are significant contributors to oxidative stress in obesity and metabolic syndrome, where changes in their phenotype lead to metabolic imbalance [16]. Therefore, understanding how UTs affect adipocyte function may help explain how CKD exacerbates systemic inflammation and metabolic impairment. In 3T3-L1 adipocytes, the UT indoxyl sulfate (IS) increases ROS production, linking adipose tissue dysregulation to an inflammatory state [2, 17].

Several sources contribute to ROS generation, among them, evidence suggests the role of Na/K-ATPase (NKA) signaling in amplifying oxidative stress [18-20]. We have shown that the α1 subunit of NKA functions as a ROS receptor and feed-forward amplifier, activating a Src-mediated signaling cascade that promotes further ROS production and pro-inflammatory cytokine release [20, 21]. In pathological states, ROS-induced carbonylation of the α1 subunit facilitates α1/Src complex formation, triggering Src phosphorylation and downstream signaling that increases ROS and impairs antioxidant defenses. This Na/K-ATPase oxidant amplification loop (NKAL) has been shown to contribute to obesity, metabolic syndrome, and related disorders [18, 22].

To further explore this signaling mechanism, we employed pNaKtide, a cell-permeable peptide antagonist derived from the NaKtide sequence within the α1 subunit of the NKA fused to a TAT transduction domain [23]. pNaKtide selectively binds to the Src kinase associated with the NKA-Src complex, thereby disrupting their interaction and preventing Src phosphorylation and downstream ERK activation [20, 23, 24], without affecting the ion-pumping activity of the enzyme [23]. Previous studies have demonstrated that pNaKtide effectively inhibits NKAL and attenuates oxidative stress, inflammation, and tissue injury in multiple models, including uremic cardiomyopathy, renal fibrosis, vascular function, neurovascular function, myocardial ischemia injury, ischemic retinopathy, and obesity [17, 18, 25-30]. We also demonstrated that pNaKtide significantly attenuated oxidative stress and lipid accumulation in murine pre-adipocytes in a dose-dependent manner and improved redox homeostasis by reducing adipogenesis and inflammation. Structural evidence that α1-NKA forms a signaling complex with c-Src and caveolin under native conditions further supports this mechanism [31]. In this study, pNaKtide was used as a pharmacological tool to determine whether the effects of uremic toxins in adipocytes are mediated through NKA signaling.

This study investigates *in vitro* models to assess whether exposure of adipocytes to UTs activates the NKAL, leading to redox imbalance and phenotypic changes. We also report, for the first time, the effects of UTs on mesenchymal stem cell (MSC)-derived adipocytes obtained from visceral fat of the West Virginian population. These changes are mitigated by pNaKtide, which improves associated pathophysiological outcomes and highlights a potential therapeutic approach for CKD.

2. Material and Methods

2.1. Experimental design for in vitro studies

2.1.1. 3T3-L1 Cell Culture, Differentiation and Treatments

Frozen murine preadipocytes (3T3-L1; ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. Cells were plated at 1–5 × 10^6 per 25 cm^2 dish and maintained at 37°C with 5% CO_2. Medium was changed every 48 h and then every 3-4 days. Upon confluence (80%), cells were trypsinized and replated in 6- or 24-well plates at 10,000 cells/cm^2. Differentiation was induced using adipogenic medium containing high-glucose DMEM, 10% fetal bovine serum (FBS), insulin (10 µg/mL), dexamethasone (0.5 mM), isobutylmethylxanthine (0.5 mM), and indomethacin (0.1 mM) for 5 days. Cells were treated with indoxyl sulfate (IS; Sigma-Aldrich, St. Louis, MO, USA; Cat. I3875) at 50, 100, or 250 µM, or p-cresol (Sigma-Aldrich; Cat. C85751) at 50, 100, or 200 µM, with or without pNaKtide 0.7 µM; (obtained from Bioscience, China), and analyzed for morphological and molecular changes.

2.1.2. Human MSC Isolation, Differentiation, and Treatments

Visceral adipose tissue was collected from lean donors (BMI <30 kg/m^2) undergoing surgery at Cabell Huntington Hospital, with the Ethics Committee of Marshall University approval and informed consent. Exclusion criteria included cancer history, trauma surgery, and age <18 or >70 years. Tissue samples were processed by iXCell Biotechnology for MSC isolation. Purity was confirmed by positive expression of CD90, CD44, and CD105, and absence of CD14, CD31, and CD45. Upon confluence (80%), cells were trypsinized and replated in 6- or 24-well plates at 10,000 cells/cm^2 and grown in minimal essential medium eagle-alpha (α-MEM) until confluence, then differentiated for 14 days in the same adipogenic medium described above and the treatments included IS (25, 50, 100 µM) with or without pNaKtide (1 µM) and at the end of the experiment, studies were done for morphological and molecular changes.

2.2. Oil Red O Staining

Lipid accumulation was assessed by Oil Red O staining as previously described. 3T3-L1 adipocytes and MSC-derived adipocytes were fixed in 10% formaldehyde, stained with 0.21% Oil Red O in 100% isopropanol for 10 min, rinsed with 60% isopropanol, and eluted with 100% isopropanol for 10 min. Absorbance was measured at 490 nm after 5 days of differentiation for 3T3-L1 adipocytes and after 14 days of differentiation for MSC-derived adipocytes.

2.3. DHE staining for superoxide measurement

Superoxide levels were measured by dihydroethidium (DHE). Following treatment with IS or p-cresol (100 µM), with or without pNaKtide, cells were incubated with 10 µM DHE for 30 min at 37°C in 5% CO_2. Fluorescence was measured using a Spectramax i3x spectrometer with excitation/emission settings of 530/620 nm.

2.4. MTT Assay

To identify the optimal UT concentrations that induce phenotypic changes without leading to cytotoxicity, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. 3T3-L1 adipocytes and MSC-derived adipocytes were seeded in 24-well plates and treated with IS or p-cresol, with or without pNaKtide. Cell viability was assessed using the Vybrant MTT Cell

Proliferation Assay Kit (Invitrogen) following the manufacturer's instructions.

2.5. Cytokine measurement

IL-6 levels in conditioned media were measured using an ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

2.6. RNA extraction and RT-PCR analysis

Total RNA from 3T3-L1 adipocytes and MSC-derived adipocytes was isolated using the Qiagen RNeasy Kit (Qiagen) following the manufacturer's instructions. RNA purity and concentration were determined by 260/280 nm absorbance using a NanoDrop Analyzer (Thermo Scientific). 1µg of RNA was reverse-transcribed into cDNA with the GeneAmp Kit (Applied Biosystems) and diluted 1:40 in RNase-free water. Gene expression was assessed for Caspase 3, BCL2-associated X protein (Bax), peroxisome proliferator-activated receptor gamma (PPARγ), TNF-α, and MCP-1 using specific primers (IDT Technologies) and two technical replicates per sample on a 7500 Fast Real-Time PCR System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene [32]. Gene expression was analyzed using the $2^{-}\Delta\Delta Ct$ method. Ct values were normalized to the housekeeping gene GAPDH, and data are presented as expression relative to the mean ΔCt of the control group.

2.7. Estimation of Src phosphorylation by Western Blot analysis

The cell lysates from 3T3-L1 adipocytes and MSC-derived adipocytes were prepared using NP-40 buffer, and c-Src activation was assessed as previously described [33].

2.8. Statistical analysis

Data were analyzed using two-way ANOVA, followed by Tukey's post hoc test for all pairwise group comparisons. Tukey's test was done as it is a standard method for controlling type I error in multiple comparisons. Statistical significance was set at $p < 0.01$ and $p<0.05$. Data are presented as mean ± SEM, and individual data points were included in all graphs to illustrate the distribution of each dataset.

2. Results

3.1. pNaKtide Attenuates Indoxyl Sulfate-Induced Dysfunction in 3T3-L1 Adipocytes

3T3-L1 murine pre-adipocytes were treated with IS at 50, 100, or 250 µM, with or without pNaKtide (0.7 µM), for five days under adipogenic conditions. Previously, we demonstrated through concentration-response analysis that pNaKtide at 0.7 µM effectively suppresses adipogenesis in 3T3-L1 cells [18]. Oil Red O staining showed that pNaKtide alone reduced lipid accumulation compared to control (Figure 1A). IS at 50 µM had minimal effect on lipid accumulation, whereas 100 and 250 µM significantly reduced adipogenesis. Co-treatment with pNaKtide (0.7 µM) attenuated these inhibitory effects. Similarly, pNaKtide alone decreased IL-6 secretion compared to control (Figure 1B). IS at 100 and 250 µM also induced a significantly increased IL-6 secretion, while 50 µM caused no substantial change; pNaKtide reduced IL-6 levels in adipocytes that were exposed to IS. MTT assays indicated no cytotoxicity of pNaKtide, as well as at 50 or 100 µM IS, whereas 250 µM IS caused a significant decrease in cell viability (Figure 1C).

3.2. pNaKtide Attenuates Indoxyl Sulfate-Induced Oxidative Stress, Adipogenesis, Apoptosis, and Inflammation in 3T3-L1 Adipocytes

IS at 100 µM was identified as the optimal concentration for reducing lipid accumulation and increasing IL-6 production compared to the control group. Therefore, this concentration was used for subsequent studies. DHE staining showed that pNaKtide alone reduced superoxide levels compared to control (Figure 2A). IS significantly increased superoxide levels, while the treatment with pNaKtide reduced superoxide levels. Additionally, pNaKtide alone downregulated PPARγ expression compared to control (Figure 2B), a key regulator of adipocyte differentiation. IS downregulated PPARγ expression compared to control; pNaKtide showed a significant upregulation of PPARγ expression induced by IS. Western blot analysis showed that pNaKtide alone decreased Src phosphorylation (pSrc) compared to control (Figure 2C). IS leads to an increase in pSrc expression; this increase in pSrc expression was inhibited by pNaKtide treatment. Furthermore, RT-PCR demonstrated that pNaKtide alone downregulated TNF-α and MCP-1 expression compared to control (Figure 2D). IS upregulated TNF-α and MCP-1 expression; pNaKtide showed a significant downregulation of

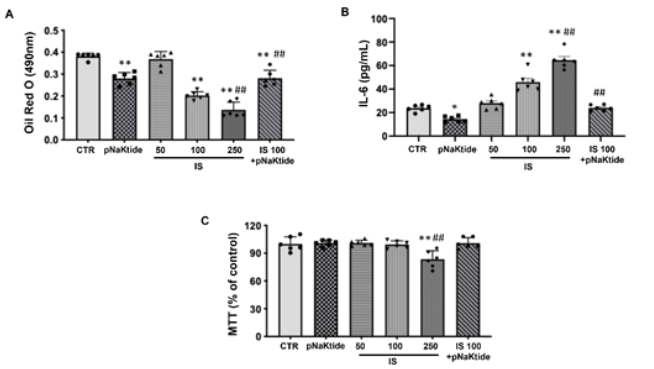

Fig. 1. Dose-dependent concentration effect of IS on 3T3-L1 adipocytes with or without pNaKtide. (A) Lipid accumulation by Oil Red O staining (N=6). (B) IL-6 quantification (N=6). (C) MTT assay, expressed as % of control (N=6). Data represent mean ± SEM. *p<0.05 vs. CTR; **p<0.01 vs. CTR; ##p<0.01 vs. IS 100 µM.

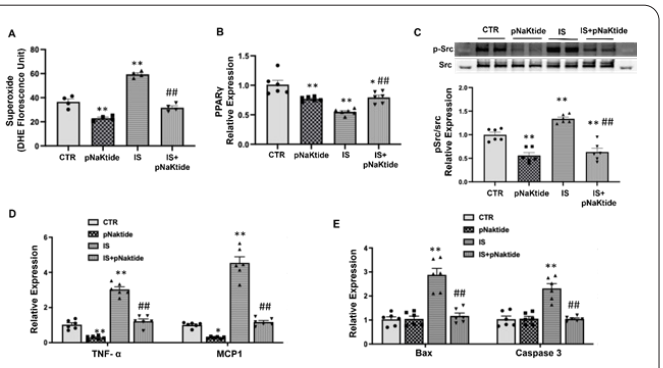

Fig. 2. Effect of IS (100 µM) on 3T3-L1 adipocytes with or without pNaKtide. (A) Superoxide levels by DHE staining (N=4). (B) PPARγ mRNA expression (N=6). (C) Western blot for pSrc levels (N=6). (D) TNF-α and MCP-1 mRNA expression (N=6). (E) Bax and Caspase 3 mRNA expression (N=6). Data represent mean ± SEM. *p<0.05 vs. CTR; **p<0.01 vs. CTR; ##p<0.01 vs. IS.

TNF-α and MCP-1 expression induced by IS. In addition, pNaKtide alone did not change Bax and Caspase 3 expression compared to control (Figure 2E). IS significantly increased both Bax and Caspase 3 expression, and these increases were attenuated by pNaKtide treatment.

3.3. pNaKtide Attenuates p-cresol-Induced Dysfunction in 3T3-L1 Adipocytes

To validate our data with another uremic toxin, 3T3-L1 adipocytes cells were exposed to p-cresol at 50, 100, or 200 μM, with or without pNaKtide. Oil Red O staining showed that pNaKtide alone reduced lipid accumulation compared to control (Figure 3A). P-cresol at 100 and 200 μM significantly decreased lipid accumulation, while 50 μM had minimal effect. Treatment with pNaKtide attenuated the decrease in adipogenesis caused by 100 μM p-cresol. Similarly, pNaKtide alone decreased IL-6 secretion compared to control (Figure 3B). Our results showed that p-cresol at 100 and 200 μM induced a significantly increased IL-6 secretion, while 50 μM caused no substantial change; pNaKtide treatment reduced IL-6 levels. MTT assays showed no cytotoxicity of pNaKtide, as well as at 50 or 100 μM p-cresol, whereas 200 μM caused a decrease in cell viability (Figure 3C).

3.4. pNaKtide Attenuates p-cresol-Induced Oxidative Stress, Adipogenesis, Apoptosis, and Inflammation in 3T3-L1 Adipocytes

P-cresol at 100 μM was effective in inducing adipocyte dysregulation. This concentration was used for subsequent studies. DHE staining showed that pNaKtide alone reduced superoxide levels compared to control (Figure 4A). P-cresol significantly increased superoxide levels; these increases were attenuated by pNaKtide treatment. Additionally, pNaKtide alone downregulated PPARγ expression compared to control (Figure 4B). P-cresol downregulated PPARγ expression compared to control; pNaKtide showed a significant upregulation of PPARγ expression induced by p-cresol. Western blot analysis showed that pNaKtide alone had no effect on pSrc compared to control (Figure 4C); while p-cresol increased pSrc, an effect inhibited by pNaKtide treatment. Additionally, RT-PCR demonstrated that pNaKtide alone has no effect on TNF-α but significantly downregulates MCP-1 expression compared to control (Figure 4D). P-cresol upregulated TNF-α and MCP-1 expression, while pNaKtide showed a significant reduction of TNF-α and MCP-1 expression induced by p-cresol. Furthermore, pNaKtide alone did not change Bax and Caspase 3 expression compared to control (Figure 4E). P-cresol significantly increased both Bax and Caspase 3 expression, and these increases were decreased by pNaKtide treatment.

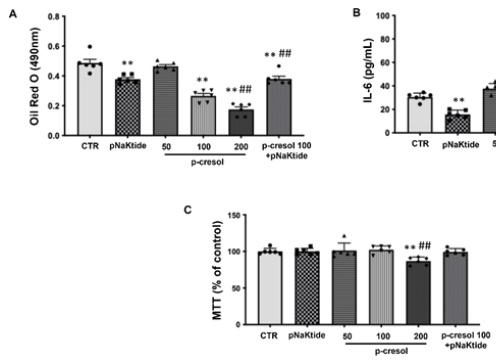

Fig. 3. Dose-dependent concentration curve of p-cresol on 3T3-L1 adipocytes with or without pNaKtide. (A) Lipid accumulation by Oil Red O staining (N=6). (B) IL-6 quantification (N=6). (C) MTT assay, expressed as % of control (N=6). Data represent mean ± SEM. **p<0.01 vs. CTR; ##p<0.01 vs. p-cresol 100 μM.

3.5. pNaKtide Attenuates Indoxyl Sulfate-Induced Dysfunction in MSC-derived adipocytes

To study the role of NKAL in adipocytes in a human system, MSC-derived adipocytes from visceral fat of the West Virginian population were treated with IS at 25, 50, or 100 μM, with or without pNaKtide. Oil Red O staining showed that pNaKtide alone did not affect lipid accumulation compared to control (Figure 5A). IS at 25 μM caused no change in lipid accumulation, whereas 50 and 100 μM reduced adipogenesis. Our results showed that treatment with pNaKtide attenuated the inhibitory effects of IS on lipid accumulation. Similarly, pNaKtide alone decreased IL-6 secretion compared to control (Figure 5B). IS at 25, 50, and 100 μM increased IL-6 secretion; pNaKtide reduced IL-6 levels in IS-treated adipocytes.

3.6. pNaKtide Attenuates Indoxyl Sulfate-Induced Oxidative Stress, Adipogenesis, Apoptosis, and Inflammation in MSC-derived adipocytes

IS at 50 μM concentration was effective in inducing adipocyte dysregulation. This concentration was used for subsequent studies. DHE staining showed that pNaKtide alone reduced superoxide levels compared to control (Figure 6A). IS at 50 μM increased superoxide levels, and

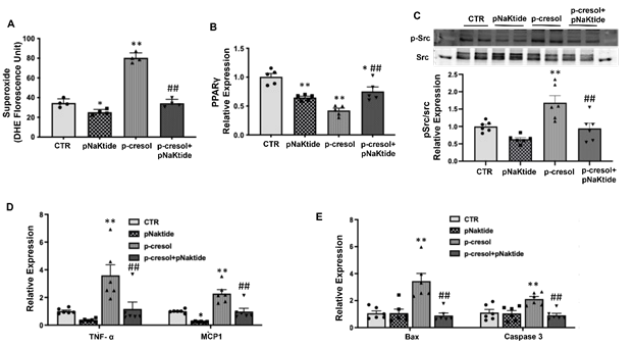

Fig. 4. Effect of p-cresol (100 μM) on 3T3-L1 adipocytes cells with or without pNaKtide. (A) Superoxide levels by DHE staining (N=4). (B) PPARγ mRNA expression (N=5). (C) Western blot for pSrc levels (N=6). (D) TNF-α and MCP-1 mRNA expression (N=6). (E) Bax and Caspase 3 mRNA expression (N=6). Data represent mean ± SEM. *p<0.05 vs. CTR; **p<0.01 vs. CTR; ##p<0.01 vs. p-cresol.

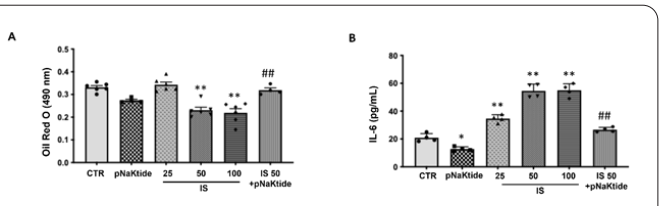

Fig. 5. Effect of IS (25, 50, 100 μM) on MSC-derived adipocytes with or without pNaKtide. (A) Adipogenesis by Oil Red O staining (N=4–6). (B) IL-6 quantification (N=4). Data represent mean ± SEM. *p<0.05 vs. CTR; **p<0.01 vs. CTR; ##p<0.01 vs. IS 50 μM.

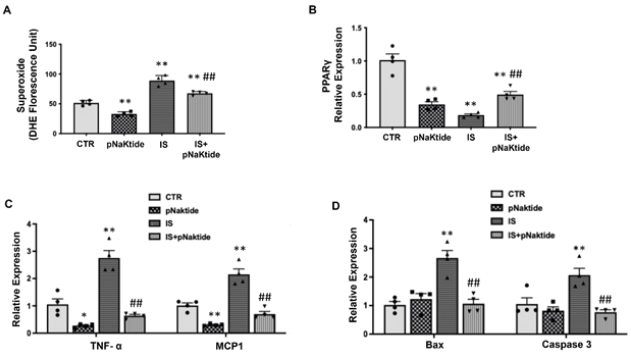

Fig. 6. Effect of IS (50 µM) on MSC-derived adipocytes with or without pNaKtide. (A) Superoxide levels by DHE staining (N=4). (B) PPARγ mRNA expression (N=4). (C) TNF-α and MCP-1 mRNA expression (N=4). (D) Bax and Caspase 3 mRNA expression (N=4). Data represent mean ± SEM. *p<0.05 vs. CTR; **p<0.01 vs. CTR; ##p<0.01 vs. IS.

treatment with pNaKtide reduced these levels compared to IS alone. Similarly, pNaKtide alone downregulated PPARγ expression compared to control (Figure 6B). IS downregulated PPARγ expression compared to control; pNaKtide showed a significant upregulation of PPARγ expression induced by IS. RT-PCR analysis demonstrated that pNaKtide alone reduced TNF-α and MCP-1 expression compared to control (Figure 6C). IS increased TNF-α and MCP-1 expression, whereas pNaKtide treatment attenuated IS-induced upregulation. For apoptotic markers, pNaKtide alone did not affect Bax and Caspase 3 expression compared to control (Figure 6D). These apoptotic markers were significantly increased by IS treatment, and these increases were attenuated by pNaKtide treatment.

3. Discussion

This study demonstrates that UTs exert distinct effects on different *in vitro* models of CKD, with NKA signaling playing a central role in mediating these responses. Our data showed that pNaKtide alone was sufficient to modulate key experimental markers in adipogenic conditions, showing its independent mechanistic action. Both mouse preadipocytes and human MSC-derived adipocytes treated with IS or p-cresol demonstrated increased ROS production, upregulation of inflammatory and apoptotic markers, as well as reduced adipogenesis and lipid accumulation. In 3T3-L1 cells, pNaKtide attenuated IS-induced changes in adipogenesis, IL-6, PPARγ, Bax, and Caspase 3, consistent with inhibition of the NKAL. Similarly, with p-cresol, pNaKtide improved adipogenesis, reduced superoxide, and restored PPARγ, Bax, and Caspase 3 levels. Furthermore, in MSC-derived adipocytes, pNaKtide attenuated IS-induced reductions in adipogenesis and increases in IL-6, Bax, and Caspase 3. Our data suggest that NKA signaling has a central role in UT-induced adipocyte dysfunction.

In CKD, and particularly in ESRD, the increase of UTs in circulation contributes to oxidative stress and pro-inflammatory cytokine production, with elevated IL-6, TNF-α, and MCP-1 levels correlating with disease severity [9, 12]. It has been shown that adipose tissue has a central role in systemic imbalance, leading to oxidative stress condition. Obesity, a common comorbidity in CKD, is associated with dysfunctional adipose tissue, altered adipokine secretion, chronic inflammation, and reduced antioxidant defenses [16, 34, 35]. Both human and murine studies have linked fat accumulation to increased systemic oxidative stress through increased ROS and cytokine production [16]. Thus, UT-induced adipose dysfunction may exacerbate CKD progression by amplifying oxidative stress and inflammation. A study in 3T3-L1 cells treated with physiological concentrations of IS showed that IS is absorbed in a dose-dependent manner, stimulating ROS generation mainly through NADPH oxidase, along with increased secretion of TNF-α and IL-6 [17]. Similarly, the exposure of 3T3-L1 cells to p-cresol reduces proliferation, increases apoptosis, decreases lipid accumulation, and downregulates PPARγ expression in a dose-dependent manner [36]. Our findings align with these reports, demonstrating that both IS and p-cresol modulate adipogenesis, increase ROS, trigger inflammatory cytokine release, and promote apoptosis in adipocytes. Importantly, pNaKtide alone reduced ROS, inflammatory markers, and restored PPARγ expression compared to adipogenic controls.

Obesity can be associated with CKD, and a significant proportion of patients exhibit adipose tissue loss and metabolic waste in the setting of chronic inflammation. Studies have shown that uremic solutes suppress adipogenic differentiation, reduce lipid accumulation, and promote a metabolically impaired and pro-inflammatory adipocyte phenotype [14, 36, 37]. These effects are consistent with published studies showing that CKD is accompanied by adipose tissue dysfunction, which can manifest either as impaired lipid storage or as adipose wasting, depending on disease severity and inflammatory status [14, 38-40].

UTs are known to disrupt cellular homeostasis by inducing oxidative stress and altering redox-sensitive signaling pathways [41]. A study demonstrated that in MSCs, from healthy donors, exposure to p-cresol, IS, and hippuric acid increased ROS under both normoxic and hypoxic conditions, reduced antioxidant enzyme expression, and suppressed hypoxia-inducible factor 1 subunit alpha (HIF-1α), along with its downstream targets vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1(SDF-1), impairing wound healing in experimental models [37]. These effects were linked to persistent activation of prolyl hydroxylase domain 2 (PHD2), a negative regulator of HIF-1α, likely sustained by elevated ROS. Our study extends these observations to MSC-derived adipocytes for the first time, using visceral fat from the West Virginian population. In adipocytes from lean donors, IS did not significantly affect lipid accumulation but induced increases in pro-inflammatory cytokines, oxidative stress, and apoptosis. These results suggest that UTs can reprogram adipocytes toward a pro-inflammatory and pro-apoptotic phenotype, contributing to CKD-related metabolic dysfunction even in the absence of increased lipid storage.

In this study, pNaKtide inhibited the effects of UTs in both murine and human adipocytes. While adipogenesis and apoptosis were preserved, they had their influence on ROS, inflammatory responses, and related phenotypic changes. By preventing NKA-Src complex formation, pNaKtide inhibits the feed-forward oxidant-amplification loop [23], supporting the concept that UTs trigger ROS generation, which in turn drives downstream phenotypic alterations. Although cardiotonic steroids such as ouabain are well-documented activators of NKA signaling, this is the first evidence that UTs can initiate the same NKAL me-

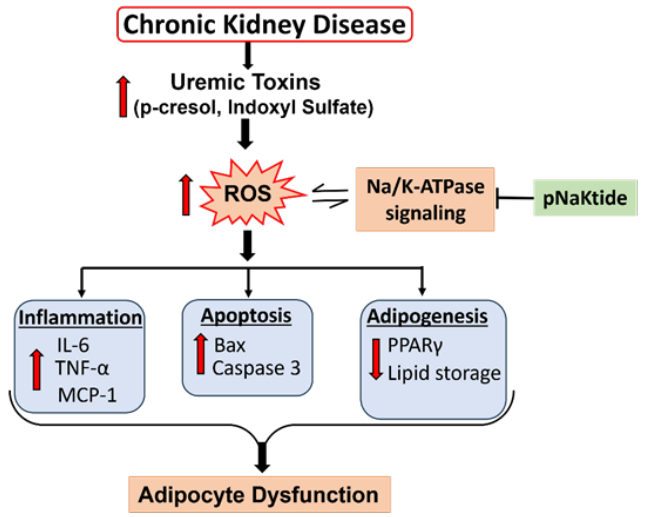

Fig. 7. Proposed model of Na/K-ATPase oxidant-amplification loop–mediated adipocyte dysfunction induced by UTs in 3T3-L1 and MSC-derived adipocytes.

chanism. Given that oxidative stress appears central to the observed cellular changes, UT-induced activation likely involves Src phosphorylation and epidermal growth factor receptor (EGFR) transactivation, as described for cardiotonic steroids [42]. The contribution of other sources, such as NADPH oxidase, remains to be clarified, which justifies further investigation of the mechanisms involved.

We acknowledge a few limitations. First, CKD is a complex, multifactorial condition involving diverse inflammatory and apoptotic pathways that contribute to disease progression. Second, heterozygous α1 knockout mice and PNx models should be incorporated to understand the mechanistic role of the NKAL in adipocytes. These approaches would extend our findings and provide evidence for UT-mediated activation of NKA signaling in adipocytes as a contributor to CKD development and progression.

4. Conclusion

Our findings demonstrate that uremic toxins distinctly activate the NKAL in both murine 3T3-L1 adipocytes and human MSC-derived adipocytes, leading to increased ROS production and phenotypic alterations consistent with adipocyte dysfunction (Figure 7). These changes could promote chronic oxidative stress in CKD, potentially accelerating disease progression. The confirmation of these effects in MSCs derived from human adipose tissues underscores their clinical relevance and supports the oxidant-amplification loop as a potential therapeutic target. The NKA signaling antagonist pNaKtide attenuated UT-induced alterations in adipogenesis and apoptosis, partially restoring a healthier phenotype. Our future studies will focus on validating the molecular mechanisms by which UTs trigger this signaling cascade, confirming these observations *in vivo*, and exploring the therapeutic potential of targeting NKA signaling in CKD.

Conflicts of Interest

The authors declare no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate:

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Marshall University (protocol # 715). And conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Declaration regarding previous retraction

The authors acknowledge that a previous version of this manuscript was retracted by the International Journal of Molecular Sciences due to concerns regarding image integrity. The current manuscript represents a thoroughly revised and independently validated version that addresses all prior issues. Raw data and repeated experimental results are available upon request. This submission adheres to the highest standards of scientific integrity and transparency to ensure the reliability and reproducibility of the findings.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Hari V. Lakhani: Perform the experiments and write the original draft; Muhammad A. Chaudhry, Tilak Khanal, D. Blaine Nease; Perform the experiments; Joseph I. Shapiro and Komal Sodhi: Supervision, write, review and edit.

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