Received: 13 July 2022 | Accepted: 19 September 2022

DOI: 10.1111/dme.14963

DIABETIC Medicine

RESEARCH ARTICLE

Blocking connexin 43 hemichannel-mediated ATP release reduces communication within and between tubular epithelial cells and medullary fibroblasts in a model of diabetic nephropathy

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Funding information
Diabetes UK

Abstract

Introduction: Fibrosis of renal tubules is the final common pathway in diabetic nephropathy and develops in the face of tubular injury and fibroblast activation. Aberrant connexin 43 (Cx43) hemichannel activity has been linked to this damage under euglycaemic conditions, however, its role in glycaemic injury is unknown. This study investigated the effect of a Cx43 blocker (Tonabersat) on hemichannel activity and cell–cell interactions within and between tubular epithelial cells and fibroblasts in an in vitro model of diabetic nephropathy.

Methods: Human kidney (HK2) proximal tubule epithelial cells and medullary fibroblasts (TK173) were treated in low (5 mM) or high (25 mM) glucose ± transforming growth factor beta-1 (TGFβ1) ± Tonabersat in high glucose. Carboxyfluorescein dye uptake and ATPlite luminescence assessed changes in hemichannel-mediated ATP release, while immunoblotting determined protein expression. Co-incubation with the ATP-diphosphohydrolase apyrase or a P2X7R inhibitor (A438079) assessed ATP-P2X7R signalling. Indirect co-culture with conditioned media from the alternate cell type evaluated paracrine-mediated heterotypic interactions.

Results: Tonabersat partially negated glucose/TGFβ1-induced increases in Cx43 hemichannel-mediated ATP release and downstream changes in adherens junction and extracellular matrix (ECM) protein expression in HK2 and TK173 cells. Apyrase and A438079 highlighted the role for ATP-P2X7R in driving changes in protein expression in TK173 fibroblasts. Indirect co-culture studies suggest that epithelial cell secretome increases Tonabersat-sensitive hemichannel-mediated dye uptake in fibroblasts and downstream protein expression.

Conclusion: Tonabersat-sensitive hemichannel-mediated ATP release enhances TGFβ1-driven heterotypic cell–cell interaction and favours myofibroblast

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activation. The data supports the potential benefit of Cx43 inhibition in reducing tubulointerstitial fibrosis in late-stage diabetic nephropathy.

KEYWORDS

adenosine triphosphate, connexin 43, diabetic nephropathy, epithelial cells, fibroblasts, fibrosis, Tonabersat

1 | INTRODUCTION

Worldwide, diabetic kidney disease develops in around 40% of those with diabetes and is the leading cause of chronic kidney disease (CKD).[1] In 2017, 1.2 million people died from CKD while 697.5 million cases were recorded globally.[2] In the United Kingdom, 30% of those entering maintenance renal replacement therapy have diabetic nephropathy as their primary renal disease.[3] Currently, there are no curative therapeutic options to prevent transition to end-stage kidney disease and innovative approaches are urgently required.

As the main contributor to end-stage renal failure, understanding mechanisms by which glucose alters renal function is critical to identifying future strategies for prevention and/or arrest of diabetic nephropathy (DN). Early damage in DN is characterised by persistent inflammation and fibrosis of the tubulointerstitium, where fibrosis develops in response to various morphological and phenotypic changes, including partial epithelial-to-mesenchymal transition (pEMT), and extracellular matrix (ECM) remodelling.[4] In the face of sustained glycaemic injury, soluble chemokines, adhesion molecules and cytokines, for example, transforming growth factor beta-1 (TGFβ1), recruit and activate infiltrating immune cells and resident fibroblasts to mediate inflammation and fibrosis, severity of which dictates disease progression.[5]

Work by Abed et al., reported that heterozygous connexin (Cx)43$^{+/-}$ mice exhibited decreased tubular macrophage infiltration and reduced numbers of active fibroblasts when surgically induced to exhibit interstitial inflammation and fibrosis via unilateral ureteral obstruction (UUO).[6] Moreover, our laboratory demonstrated that these mice exhibit reduced disassembly of adherens junction and tight junction proteins, the former of which is considered an initiating trigger for pEMT in the injured tubules.[7]

Connexins oligomerise to form hexameric structures, before being transported to the cell membrane where they form hemichannels which allow for the local release of various danger-associated molecular patterns (DAMPs), for example, adenosine triphosphate (ATP), in response to pathological triggers, including hyperglycaemia[8] and inflammation.[9] Excess ATP within the intercellular environment activates P2X7 purinergic receptors[10] to trigger inflammatory and fibrotic events in both resident tubule epithelial cells[11] and other associated cell types local to the proximal kidney, for example, macrophages.[10] We previously demonstrated that pharmacologically inhibiting Cx43-mediated hemichannel activity using Cx43 hemichannel blocker Peptide 5, protects against early tubular injury in an in vitro model of experimental chronic kidney disease under euglycaemic conditions.[12] Similar to Peptide 5, Tonabersat; a benzopyran derivative originally reported to inhibit gap junction communication,[13] has recently been shown to be equivalent to Peptide 5 in its capacity to act as a blocker of Cx43 hemichannels.[14–16] Consequently,

Novelty statement

What is already known?

- Cx43 hemichannels release ATP in TGFβ1-treated tubular epithelial cells.

What this study found?

- TGFβ1 increases Cx43 hemichannel-mediated ATP release in TK173 renal fibroblasts and HK2 tubular epithelial cells, an effect amplified by high glucose.
- Aberrant ATP release initiates phenotypic changes via P2X7 receptors.
- Co-culture of TK173 cells with high glucose conditioned HK2 media, increased Cx43 hemichannel-mediated dye uptake and ECM protein expression.
- Characteristic of late-stage kidney damage, effects were partly blocked with Tonabersat.

Implications?

- Tonabersat blocks Cx43 hemichannel-mediated communication within and between tubular epithelial cells (HK2) and medullary renal fibroblasts (TK173), protecting against phenotypic changes characteristic of diabetic nephropathy.

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Tonabersat (Xiflam) has since been reported to confer protection in both in vivo models of age-related macular degeneration[14] and diabetic retinopathy.[14,17] Currently entering Phase2b clinical trials for diabetic macular oedema and retinopathy, its ability to target hemichannels in the diabetic kidney and confer anti-inflammatory protection are yet to be reported.

In this study, we tested whether blocking Cx43-mediated hemichannel ATP release decreased P2X7R activation within and between tubule epithelial cells and renal fibroblasts in conditions designed to mimic diabetic nephropathy. We posit that a heterotypic pro-inflammatory relationship exists between tubular epithelial cells and resident fibroblasts, and that blocking Cx43-mediated ATP release can dampen this interaction. The data demonstrate the importance of Cx43 hemichannel-mediated paracrine signalling in kidney damage, and suggest the potential benefit of using Cx43 hemichannel blockers in managing tubulointerstitial fibrosis in late-stage DN.

2 | METHODS

2.1 | Materials

Human kidney (HK2) proximal tubule epithelial cells were purchased from American Type Culture Collection (ATCC) (LGC Standards; Manassas, United States). Medullary renal fibroblasts (TK173) were derived from normal human kidneys and gifted from Dr. Isak Demirel (Örebro University, Sweden). Tissue culture plastic and supplies were from Sarstedt Inc (Leicester, UK), while tissue culture media and fetal calf serum (FCS) were from Fisher Scientific (Loughborough, UK). Penicillin/Streptomycin was from Sigma-Aldrich (Dorset, UK), while epithelial growth factor (EGF) was purchased from ProSpec (Rotherham, UK). Glass-bottomed fluorodishes for carboxyfluorescein dye uptake studies were from Thistle Scientific (Glasgow, UK). For western blotting, all membranes, buffers and equipment were obtained from Licor Biosciences (Lincoln, USA). Recombinant TGFβ1 (PHG9214) was from Fisher Scientific (Loughborough, UK), while Tonabersat was purchased from Cambridge Bioscience (Cambridge, UK). ATPlite luminescence assay was from PerkinElmer (Llantrisant, UK). Unless otherwise stated, all chemicals were from Sigma-Aldrich (Dorset, UK).

2.2 | Cell culture and treatment

HK2 cells (passages 9–21) were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium, while TK173 (passages 4–10) were maintained in DMEM(1x) + pyruvate. HK2 media contained penicillin/streptomycin (2%) and EGF (5 ng/ml). TK173 media were supplemented with minimum essential medium non-essential amino acids (MEM NEAA). All culture media contained FCS (10%). Cells were cultured at 37°C in a humidified environment with 5% CO_2.

For HK2 treatments, cells were cultured in low (5 mM) glucose DMEM/F12 (5 mmoL/L) for 48 h, followed by serum starvation overnight prior to treatment with TGFβ1 (10 ng/ml) ± Tonabersat (100 μM) at either 5 mM or 25 mM D-glucose. For TK173 treatments, cells were seeded in culture medium for 24 h, followed by overnight serum starvation before treatment in DMEM(1x), containing 1% FCS with TGFβ1 (10 ng/ml) ± Tonabersat (10 μM) ± apyrase (100 IU/ml) ± P2X7R inhibitor A438079 (50 μM). For co-culture experiments, cells were treated for 48 h in 5 mM or 25 mM D-glucose ± Tonabersat (10 μM), before harvesting the media and freeze-thawing 10x. Conditioned media were then used for treatment of the alternative cell type for 48 h. For this, both cell types were grown in DMEM (1x).

2.3 | Carboxyfluorescein dye uptake

Cells were cultured on glass-bottomed fluorodishes before exposure to Ca^{2+}-free balanced salt solution (BSS) + carboxyfluorescein (200 μM) for 10 min and Ca^{2+}-containing BSS + carboxyfluorescein (200 μM) for 5 min. Cells were washed with Ca^{2+}-containing BSS before imaging with a Cool Snap HQ CCD camera (Roper Scientific, Gottingen, Germany) and Metamorph software (v7.75, Universal Imaging Corp., Marlow, UK). Approximately 10–12 images were taken per dish, and regions of interest (ROI) drawn around individual cells (~15 cell/image). Two separate dishes were imaged per condition for each N number. The integrated density was measured using Fiji software (v2.1.0, ImageJ, LOCI, Wisconsin, USA), as described in detail previously.[18] Percentage dye uptake was calculated using these values, where treatment was set to 100%.

2.4 | ATP luminescence assay

The ATP luminescence assay from PerkinElmer is a quantitative assay which measures the amount of ATP in the cell supernatant. Cells were seeded at 1×10^4 following the treatment protocol before being incubated with the substrate solution as provided in the kit. The foil-covered plate was placed on a shaker for 5 min, before being dark-adapted for 10 min. Luminescence was measured using a Chameleon plate reader and provided a direct measure of hemichannel-mediated ATP release.

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2.5 | Immunocytochemistry

HK2 and TK173 cells were stimulated with TGFβ1 (10 ng/ml) ± Tonabersat at 100 μM and 10 μM respectively for 48 h prior to fixing with paraformaldehyde (4%), and subsequent blocking with goat serum (10%) for 1 h at room temperature (RT). Antibodies against fibronectin (Santa Cruz sc-271098), N-cadherin (Abcam ab18203), Cx43 (Abcam ab11370), and vimentin (Cell Signaling Technology D21H3), (all 1:200) were used for incubation overnight at 4°C. After washing, cells were incubated with nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI) (1 mmoL/L) for 3 min. Cells were incubated with Alexa-Fluor 488 for 1 h at RT before visualisation using a Leica TC SP8 confocal microscope (Wetzlar, Germany).

2.6 | Western blotting

Whole cell lysates were prepared and separated using SDS-PAGE gel electrophoresis at 125 volts (V) for 1.5 h, before transfer at 100 V for 1 h, as previously described.[19] Membranes were blocked before being probed overnight at 4°C with primary antibodies against Cx43, β-catenin (Cell Signaling Technology D10A8), N-cadherin, fibronectin, collagen I (Abcam ab34710), (all at 1:1000) and α-tubulin (Sigma-Aldrich T5168), at a dilution of 1:20,000, as a house-keeping protein. After washing, membranes were probed with secondary antibody (goat anti-rabbit 800 and/or goat anti-mouse) at 1:20,000 for 1 h at RT. Bands were visualised using an Odyssey Fc imaging unit before semi-quantification and analysis using ImageStudioLite software (5.2.5).

2.7 | Statistical analysis

All data are presented as mean value ± SEM. Statistical analysis was performed using ANOVA and Tukey post-test; $p \leq 0.05$ was considered statistically significant, with '*n*' denoting sample number.

3 | RESULTS

3.1 | Tonabersat blocks hemichannel-mediated ATP release in tubular epithelial cells

Previous studies link increased Cx43 expression to inflammation and fibrosis in a model of renal disease,[6,20] while findings from our laboratory report that aberrant Cx43 hemichannel-mediated ATP release from TGFβ1-treated tubular epithelial cells evoked phenotypic and functional changes, characteristic of tubular damage.[12,21,22] Consequently, the current study determined if this effect was accentuated in high glucose and if the response could be decreased using Tonabersat, an efficient Cx43 hemichannel blocker. Human kidney (HK2) cells were cultured at low (5 mM) and high (25 mM) glucose with TGFβ1 (10 ng/ml) ± Tonabersat (100 μM) at high glucose for 48 h. Glucose (25 mM) increased dye uptake (25.3 ± 8.8%, $p < 0.05$), compared to low glucose (5 mM) control (Figure 1b,c). The response was amplified by 16.4% in the presence of TGFβ1 ($p < 0.001$). The combined effect of high glucose and TGFβ1 was reduced by 42.3% ± 5.3% when cells were preincubated with Tonabersat (Figure 1c; $p < 0.001$). In Figure 1d, TGFβ1 increased ATP release at low (32.3 ± 10.2%; $p < 0.05$) and high (55.7%, $p < 0.001$) glucose, with the response at 25 mM glucose significantly decreased by Tonabersat (36.9 ± 12.3%, $p < 0.05$).

3.2 | Blocking Cx43 hemichannels inhibits TGFβ1/glucose-induced increases in expression of Cx43 and markers of tubular damage

We previously reported that UUO causes disassembly of the adherens junction complex[12] and that this is accompanied by increased interstitial fibrosis.[6] In the absence of glycaemic injury, this effect was partly restored in the $Cx43^{+/-}$ mouse model of UUO. Consequently, we evaluated the effect of TGFβ1 on Cx43, adherens junction (N-cadherin and β-catenin) and ECM (fibronectin and collagen I) protein expression in high glucose (Figure 2). Human kidney (HK2) cells were treated for 48 h in either low (5 mM) or high (25 mM) glucose with TGFβ1 (10 ng/ml) ± Tonabersat (100 μM) to block ATP release (Figure 2a). Compared to low glucose control, the cytokine increased expression of total Cx43 by 76.4 ± 10.2% (Figure 2b; $p < 0.001$), N-cadherin by 39.8 ± 7.8% (Figure 2d; $p < 0.01$), fibronectin by 52.1 ± 6.7% (Figure 2e; $p < 0.001$) and collagen I by 28.8 ± 8.6% (Figure 2f; $p < 0.05$). This effect was amplified in the presence of high (25 mM) glucose, where TGFβ1 increased N-cadherin (51.3%, $p < 0.001$), fibronectin (67.7%, $p < 0.001$) and collagen I (88.5%, $p < 0.001$) expression. The effect of TGFβ1 on Cx43 was not accentuated under high (25 mM) glucose conditions (64.4%, $p < 0.001$), and there were no effects of glucose or TGFβ1 on β-catenin expression (Figure 2c).

Having determined that Tonabersat blocks TGFβ1-induced changes in hemichannel activity (Figure 1), we assessed if TGFβ1-evoked changes in protein expression under conditions of high glucose were hemichannel

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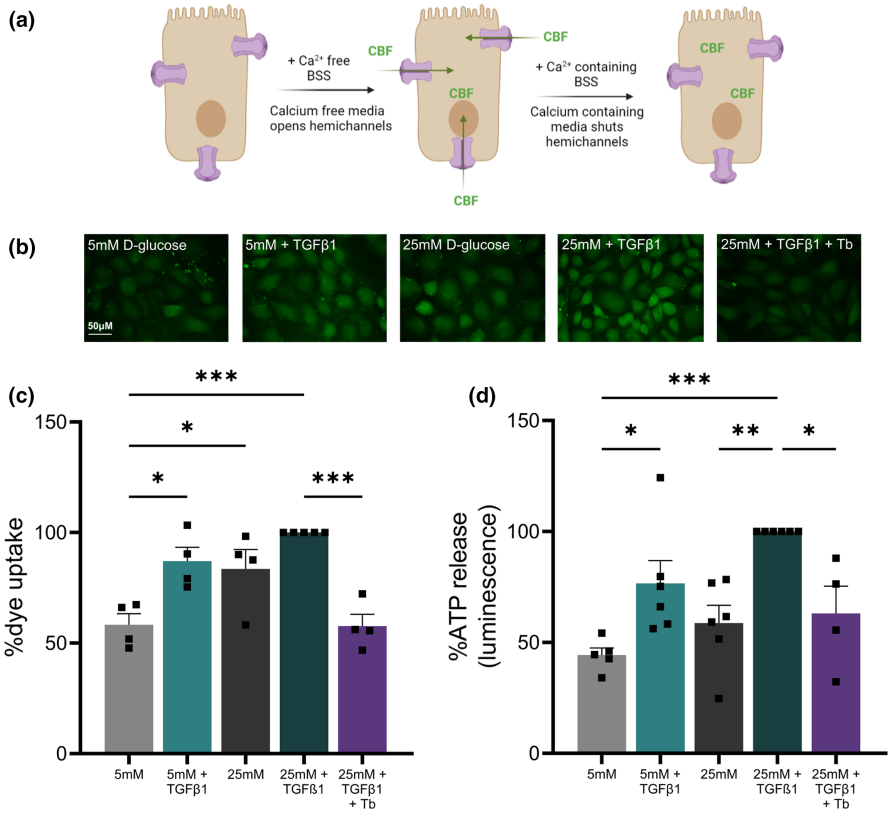

FIGURE 1 Tonabersat negates TGFβ1/glucose-evoked increases in hemichannel activity in (HK2) proximal tubule epithelial cells. Cells were cultured for 48 h in either low (5 mM) or high (25 mM) glucose ± TGFβ1 (10 ng/mL) ± Tonabersat (Tb; 100 μM). Panel (a) outlines the protocol used to measure Cx43 hemichannel-mediated carboxyfluorescein (CBF) dye uptake (BSS is balanced salt solution), while panel (b) provides representative images for dye uptake in each condition. Carboxyfluorescein studies (c) assessed changes in hemichannel mediate, and an ATPlite assay used luminescence as a direct measure of ATP release (d). Data represents mean ± SEM, $n = 4–6$, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

mediated. As shown in Figure 2, Tonabersat decreased high glucose and TGFβ1-induced increases in Cx43 expression by 46 ± 10.3% ($p < 0.01$), while fibronectin and collagen I expression were decreased by 35.7 ± 4.8%, ($p < 0.01$) and 40.6 ± 5.4%, ($p < 0.001$), respectively. There was no effect on N-cadherin.

3.3 | Tonabersat blocks hemichannel-mediated ATP release in renal fibroblasts

Tubulointerstitial fibrosis (TIF) is the key underlying pathology of diabetic kidney disease and develops in response to activation of multiple cell types in and around the proximal tubules.[23] Medullary fibroblasts contribute to the onset and progression of TIF in diabetic nephropathy. With previous studies linking the heterozygous $Cx43^{+/-}$ UUO mouse to decreased fibroblast activation in the kidney cortex,[6] we wanted to assess (i) if renal fibroblasts (TK173) have functional hemichannels and (ii) if Tonabersat could block effects of glucose and TGFβ1 on Cx43 hemichannel-mediated release of ATP. Renal fibroblasts were treated in low (5 mM) and high (25 mM) glucose with TGFβ1 (10 ng/ml) ± Tonabersat (10 μM) at high glucose for 48 h. Carboxyfluorescein measured hemichannel-mediated dye uptake and ATP luminescence assays quantified ATP release.

As shown in Figure 3a,b, high glucose (25 mM) augmented TGFβ1-evoked dye uptake by 28.1% (compared high glucose alone; $p < 0.05$). The combined effect of glucose and cytokine was completely negated when cells were co-incubated with Tonabersat (49.8 ± 4.6% reduction; $p < 0.001$). The ability of Tonabersat to reduce dye uptake in response to TGFβ1 and high glucose was paralleled by a 43 ± 5.9% decrease in ATP release (Figure 3c; $p < 0.05$).

3.4 | Tonabersat inhibits TGFβ1/glucose-induced changes in Cx43, N-cadherin and ECM localisation and expression in renal fibroblasts

Having ascertained that TGFβ1 stimulates Cx43 hemichannel activity in renal fibroblasts, an effect augmented in the presence of high glucose, we investigated if blocking this activity could confer any phenotypic protection. Immunocytochemistry (Figure 4a) and western blotting (Figure 4b–f) were used to determine TGFβ1-induced changes in protein localisation and expression at low (5 mM) and high (25 mM) glucose, before assessing the ability of Tonabersat to dampen the combined effect of glycaemic and cytokine challenge in TK173 fibroblasts.

The pro-fibrotic cytokine TGFβ1 had negligible effects on localisation of candidate proteins, but increased expression in both low (5mM) and high (25mM) glucose

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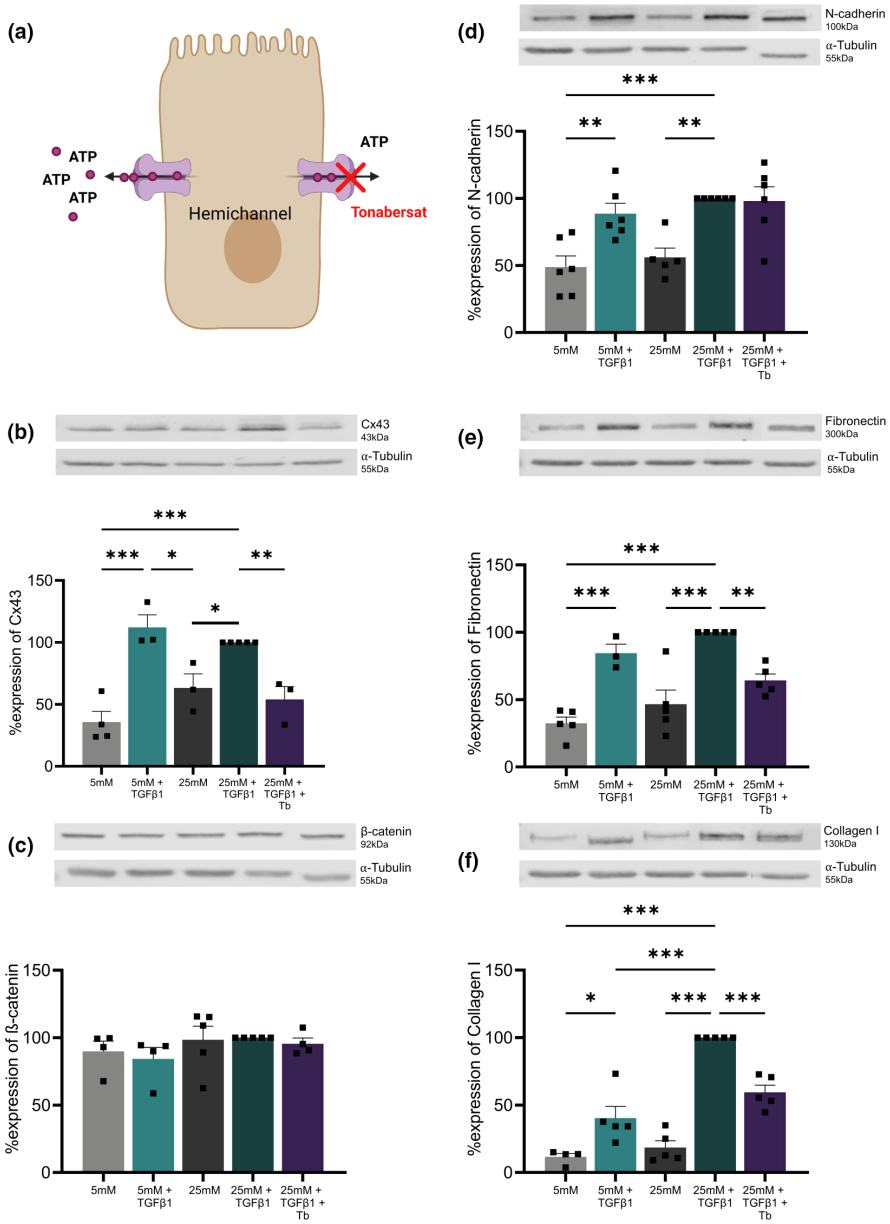

FIGURE 2 Tonabersat inhibits TGFβ1/glucose-evoked increases in Cx43, adherens junction and ECM protein expression. HK2 cells were cultured for 48 h in either low (5 mM) or high (25 mM) glucose ± TGFβ1 (10 ng/mL) ± Tonabersat (Tb; 100 μM). Tonabersat blocks Cx43 hemichannel-mediated ATP release (a). Western blot examined whole cell protein expression (%) for Cx43 (b), β-catenin (c), N-cadherin (d), fibronectin (e) and collagen I (f). Data shown illustrate a representative blot and mean data ± SEM ($n = 4–6$), where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

as determined by immunocytochemistry. This effect was particularly evident with ECM proteins fibronectin and vimentin (Figure 4a). As an intermediate filament protein, vimentin was used in place of collagen I which was poorly resolved using immunocytochemistry. Tonabersat reversed the combined effects of high glucose and cytokine. Semi-quantification of protein changes using western blot determined that in the presence of high glucose, TGFβ1 increased expression of Cx43 (Figure 4b 66.1%, $p < 0.001$), β-catenin (Figure 4c 32.7%, $p < 0.05$), N-cadherin (Figure 4d 45%, $p < 0.05$), fibronectin (Figure 4e 47.6%, $p < 0.01$) and collagen I (Figure 4f 26.4%, p=NS). Tonabersat inhibited these effects for Cx43 (54.3 ± 12.6%, $p < 0.01$), N-cadherin (40.9 ± 11.8%, $p < 0.05$), fibronectin (34.3 ± 4.2%, $p < 0.05$) and collagen I (30.8 ± 3%, p=NS).

3.5 | ATP mediates TGFβ1/glucose-induced changes in Cx43, N-cadherin and ECM expression in renal fibroblasts

Having determined that high glucose in combination with TGFβ1 evokes a Tonabersat-sensitive increase in ATP release (Figure 3) and increased expression of tubular injury associated proteins (Figure 4), we further explored a downstream role for ATP-mediated purinergic signalling in driving high glucose and TGFβ1-induced changes. An ATP-diphosphohydrolase, apyrase (apy) catalyses the sequential hydrolysis of ATP to ADP and ADP to AMP, releasing inorganic phosphate and reducing the extracellular concentration of ATP ([ATP]e) to decrease activation of P2-purinergic

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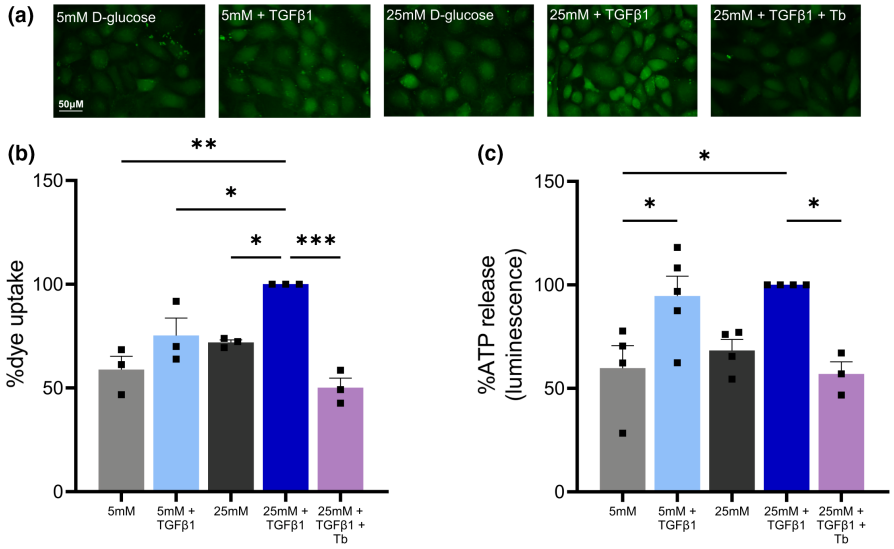

FIGURE 3 Tonabersat negates TGFβ1/glucose-evoked hemichannel mediated ATP release in (TK173) renal fibroblasts. Cells were treated with TGFβ1 (10 ng/ml) in low or high glucose ± Tonabersat (Tb; 10 μM) for 48 h. Panel (a) shows representative images for carboxyfluorescein dye uptake in each condition. Mean data (± SEM, $n = 4–6$) demonstrated that TGFβ1 increased dye uptake, an effect that was amplified when co-incubated at high glucose (panel (b)). Examining ATP release, this high glucose TGFβ1-induced response was reversed to near basal (5 mM glucose) levels when cells were pre-incubated with Tonabersat (panel (c)). Significances are represented as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

receptors (Figure 5a). TK173 cells were treated in low or high glucose with TGFβ1 (10 ng/ml) ± apyrase (100 IU/ml) for 48 h. Western blot assessed changes in whole cell protein expression. Compared to high glucose alone, TGFβ1 plus 25 mM glucose increased expression of Cx43 (Figure 5b 66.1%, $p < 0.01$), β-catenin (Figure 5c 32.7%, $p < 0.05$), N-cadherin (Figure 5d 45%, $p < 0.01$), fibronectin (Figure 5e 35.2%, $p < 0.05$) and collagen I (Figure 5f 26.4%, p=NS). These changes decreased by 51.4 ± 14.2% (Cx43; $p < 0.05$), 34.0 ± 7.0%, (N-cadherin; $p < 0.05$), 26.6 ± 1.3%, (fibronectin; $p < 0.05$), and 47.8 ± 12.3%, (collagen I; $p < 0.05$), when cells were co-incubated with apyrase. Hydrolysis of ATP failed to significantly alter TGFβ1/glucose-induced changes in β-catenin.

Known to mediate inflammation and downstream fibrosis, activation of the P2X7 receptor (P2X7R) has been associated with tubular injury in an in vivo model of diabetic nephropathy.[10] We used the P2X7R inhibitor A438079 (50 μM) to determine the role of this receptor subtype in glucose/cytokine-induced changes in TK173 fibroblasts (Figure 5a). The P2X7R inhibitor reversed TGFβ1/high glucose-induced increases in Cx43 (Figure 5b 48.7 ± 12.7%, $p < 0.05$), N-cadherin (Figure 5d 53.1 ± 13.9%, $p < 0.01$) and collagen I (Figure 5f 62.6 ± 2.7%, $P < 0.001$), and marginally reduced expression of β-catenin (Figure 5c 21.8 ± 14.2%) and fibronectin (Figure 5 e 26.3 ± 1.9%).

3.6 | Tonabersat partially negates epithelial cell secretome-mediated changes on fibroblast hemichannel number and protein expression

While tubular epithelial cells are often regarded as the initial drivers of renal injury, fibroblasts are considered the major ECM producing cell type. Importantly, they are recruited and activated in response to sustained tubular injury. Regulated by soluble ligands, for example, ATP, crosstalk between the tubular epithelia and fibroblasts can orchestrate many forms of disease progression[24] including diabetic nephropathy.[25] To investigate the role of the cell secretome in paracrine-mediated signalling between each cell type, conditioned media transfer (indirect co-culture) was performed between tubular epithelial cells and medullary fibroblasts. In doing so, we determined the downstream effects on hemichannel activity and protein expression on both HK2 cells and TK173 cells when incubated with conditioned media of the alternate cell type (Figure 6a).

Although the transfer of high glucose conditioned media from fibroblasts evoked a Tonabersat-sensitive increase in dye uptake in tubular epithelial cells (55.9%, $p < 0.001$) as compared to 5 mM glucose control (Figure 6b,c), it had minimal effect on HK2 protein expression (see: Figure S1).

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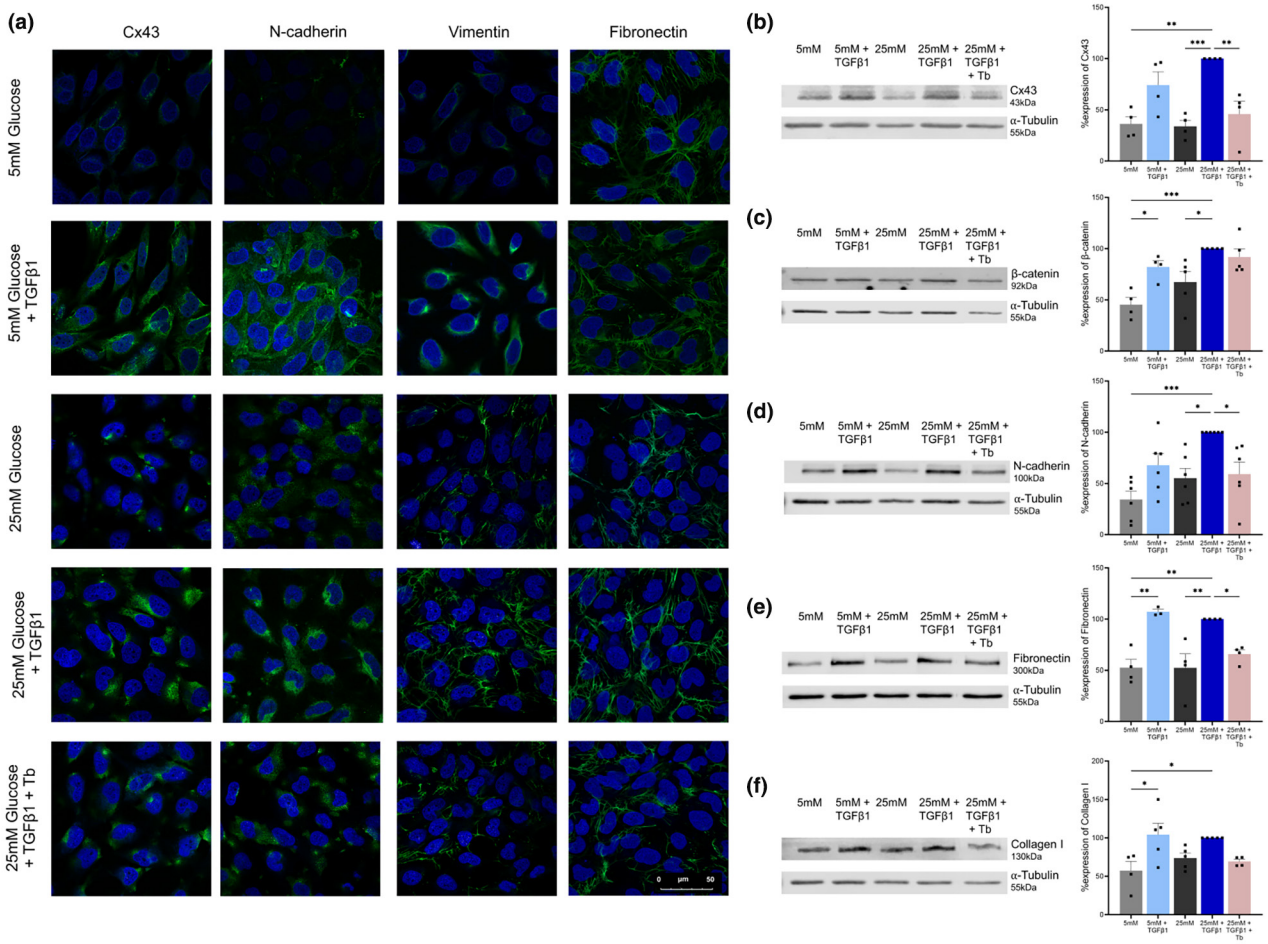

FIGURE 4 Tonabersat reverses TGFβ1/glucose-evoked changes in ECM and adherens protein expression and localisation in (TK173) renal fibroblasts. In panel (a) immunocytochemistry revealed the effect of the pro-fibrotic cytokine TGFβ1 (10 ng/ml) on the localisation of Cx43, N-cadherin, vimentin and fibronectin in low (5 mM) and high (25 mM) glucose ± Tonabersat (Tb; 10 μM) in TK173 fibroblasts. Western blot examined whole cell protein expression (%) of Cx43 (b), β-catenin (c), N-cadherin (d), fibronectin (e) and collagen I (f). Representative blots and mean data ± SEM ($n = 4–6$) are shown, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Conditioned media from high glucose-treated proximal tubule epithelial cells (HK2) evoked a Tonabersat-sensitive increase in dye uptake in (TK173) renal fibroblasts of 54.5% ($p < 0.001$), compared to low glucose control (Figure 6d,e). Contrary to the lack of effect that fibroblast (TK173) conditioned media had on tubule epithelial (HK2) phenotype, the effects of HK2 conditioned media on TK173 dye uptake, were paralleled by changes in Cx43 expression and markers of the ECM and adherens junction. Western blot determined that high glucose conditioned media from epithelial cells increased expression of Cx43 (47.7%, Figure 6f; $p < 0.001$), N-cadherin (24.1%, Figure 6h; $p < 0.05$) and fibronectin (60.5%, Figure 6i; $p < 0.01$) in fibroblasts compared to unconditioned 25 mM glucose media. Tonabersat negated this effect by 33.9 ± 11.4%, $p < 0.01$ (Cx43), 25.3 ± 8.9%, $p < 0.05$ (N-cadherin) and 53.4 ± 7.7%, $p < 0.05$ (fibronectin). There were minimal effects on β-catenin (Figure 6g) or collagen I (Figure 6j).

4 | DISCUSSION

The current study demonstrates that the pro-fibrotic cytokine TGFβ1 increases Cx43 hemichannel-mediated ATP release in TK173 renal fibroblasts and HK2 tubular epithelial cells, events which lead to autocrine and paracrine effects and were amplified by high glucose. Local increases in ATP independently initiate P2X7R-mediated changes in adherens junction and ECM protein expression on both resident epithelial cells and fibroblasts (see Figure 7). Our data support earlier studies under euglycaemic conditions demonstrating that TGFβ1 evokes Cx43-mediated changes in expression of markers of tubular injury and fibrosis in human primary proximal tubule epithelial cells (hPTECs)[7,12,21,22] and further suggests that effects of the cytokine are compounded by high glucose. The importance of P2X7R in diabetic kidney disease was reported by Menzies et al., who showed that deficiency of P2X7R reduced macrophage infiltration and collagen

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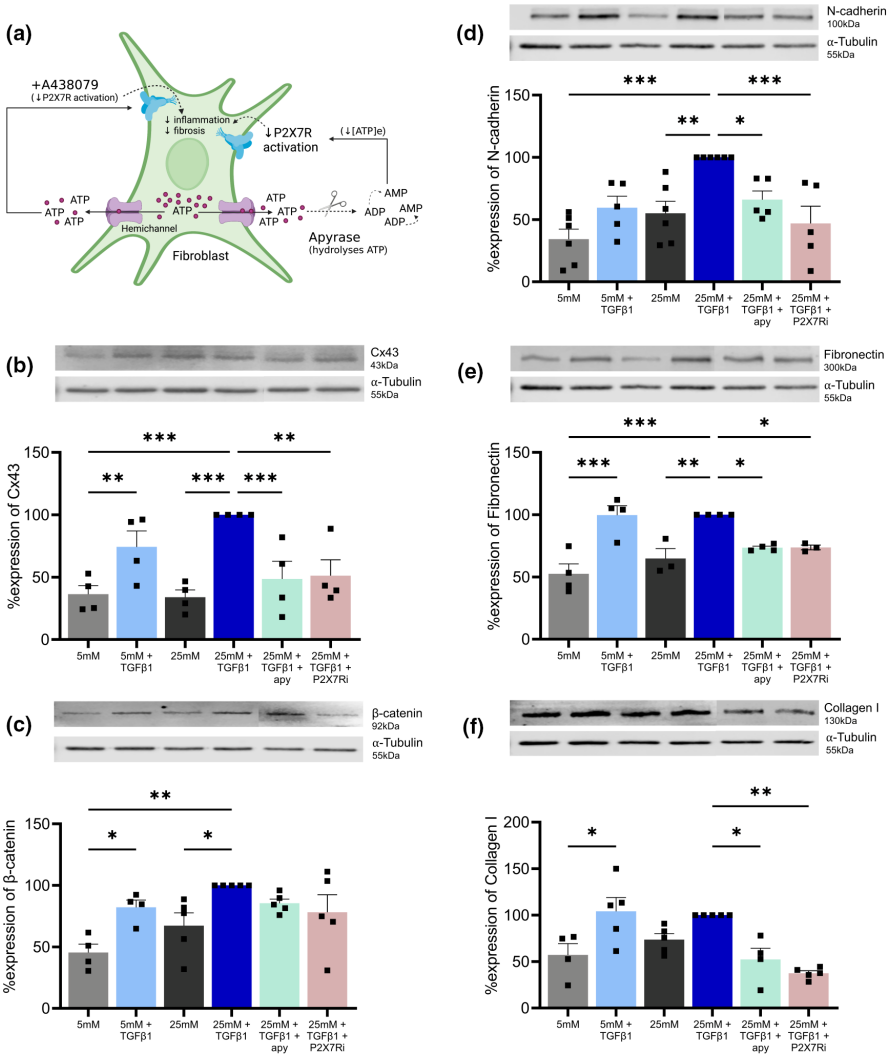

FIGURE 5 Reducing activation of the P2X7 receptor reverses TGFβ1/glucose-induced changes in ECM and adherens junction protein expression in (TK173) renal fibroblasts. To reduce activation of the P2X7R, either the concentration of TGFβ1/glucose-evoked extracellular ATP ([ATP]e) was reduced by apyrase (apy), which catalyses the sequential hydrolysis of ATP to ADP and AMP, or the P2X7 receptor was inhibited using A438079. Both strategies reduced markers of TGFβ1-dependent inflammation and fibrosis (a). Western blot analysis determined whole cell protein expression (%) of Cx43 (b), β-catenin (c), N-cadherin (d), fibronectin (e) and collagen I (f) in response to TGFβ1 (10 ng/ml) in low (5 mM) and high (25 mM) glucose ± apyrase (100 IU/ml) ± the P2X7R inhibitor (P2X7Ri: A438079 (50 μM)). Representative blots are shown above mean data ± SEM, $n = 3–5$, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

IV deposition in a Type 1 mouse model of diabetic nephropathy.[10] Poorly expressed in healthy kidney tissue,[26] expression of P2X7R is markedly increased in renal biopsy material from people with diabetic nephropathy.[10,12] Pharmacologically blocking Cx43-mediated ATP release using Tonabersat and reducing markers of tubular injury in the present study corroborates data from our normoglycaemic $Cx43^{+/-}$ UUO model[12] while demonstrating that this protection is still apparent under high glucose conditions associated with DN and likely involves Cx43 hemichannel-mediated ATP release as an initiating trigger.

Lacking direct cell–cell contact, heterotypic cell-to-cell crosstalk between the tubular epithelia and fibroblasts is regulated by soluble mediators (e.g., cytokines) and has been associated with various forms of disease progression.[24,27] Although tubular epithelial cells are often perceived as the instigators of renal injury, inflammation, and downstream fibrosis in and around the tubules involves multiple cell types. Moreover, a 50% knockdown of Cx43 in the heterozygous $Cx43^{+/-}$ mouse model of unilateral ureteral obstruction, has been shown to decrease interstitial fibrosis (sirius red staining) and fibroblast accumulation (fibroblast specific protein-1 [FSP-1] staining).[6] Mediated by Tonabersat-sensitive Cx43 hemichannel ATP release, indirect co-culture studies utilising conditioned media from the alternative cell type demonstrate the degree of interdependence between tubular epithelial cells and fibroblasts. Driven by epithelial cell-mediated release of cytokines and chemokines, for example, TGFβ1, we found that the association between tubular epithelial cells and TK173 medullary fibroblasts favoured epithelial regulation of fibroblast protein expression (Figure 7). This apparent hierarchy of control has previously been reported when describing the profibrotic effects of the tubular senescence-associated secretory phenotype (SASP) and activation of fibroblasts by mouse tubular epithelial cells.[28] Moreover, studies by Zhou et al., determined that tubule-derived Wnts have an essential role in promoting fibroblast activation[27] while injured tubular epithelial cells have been shown to initiate the activation and proliferation of fibroblasts

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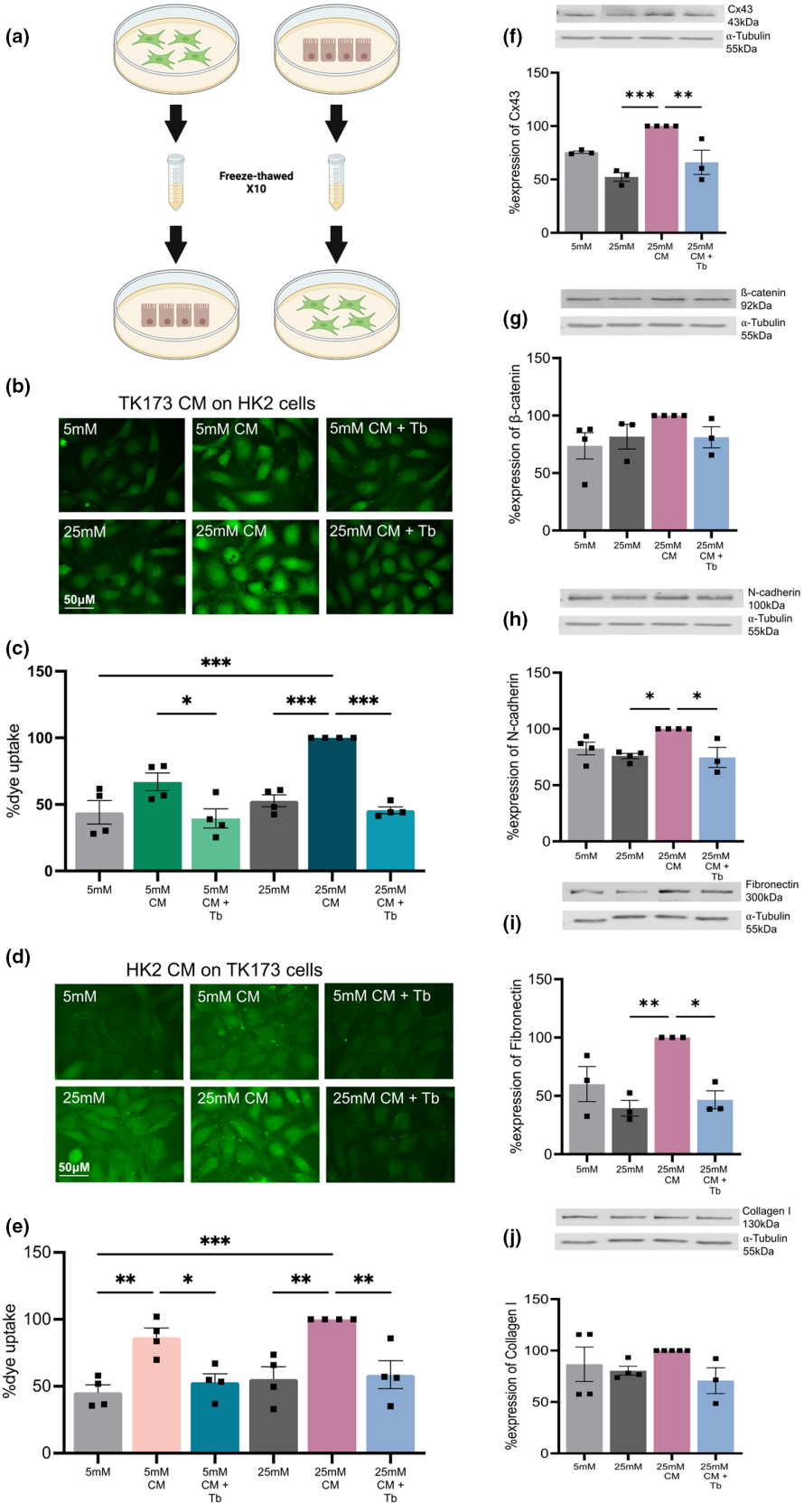

FIGURE 6 Conditioned media from high glucose-treated proximal tubule epithelial cells (HK2) evoked Tonabersat-sensitive changes in ECM and adherens junction protein expression in (TK173) renal fibroblasts. Indirect co-culture utilised 10 times freeze-thawed 48 h high glucose (25 mM)-treated conditioned media (CM) ± Tonabersat (Tb; 10 μM) to determine the effect of cell secretome on the heterotypic cell type (a). Carboxyfluorescein studies assessed changes in dye uptake when fibroblast conditioned media were added to tubular epithelial cells (b) and (c) and vice versa (d) and (e). Determined by western blotting, tubular epithelial cell secretome examined Tonabersat-sensitive changes in fibroblast whole cell protein expression (%) for Cx43 (f), β-catenin (g), N-cadherin (h), fibronectin (i) and collagen I (j). Data shown illustrate representative blots and mean data ± SEM ($n = 3–4$), where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

via direct shuttling of miR-150-containing exosomes[29] or the miR-21/PTEN/Akt pathway.[30] Building on these observations, data in the current study suggest that blocking Cx43-mediated ATP release may not only dampen homotypic PTEC-PTEC, fibroblast–fibroblast activation and ECM remodelling/deposition, but may importantly reduce epithelial–mesenchymal communication, events which predispose a profibrotic environment.[21]

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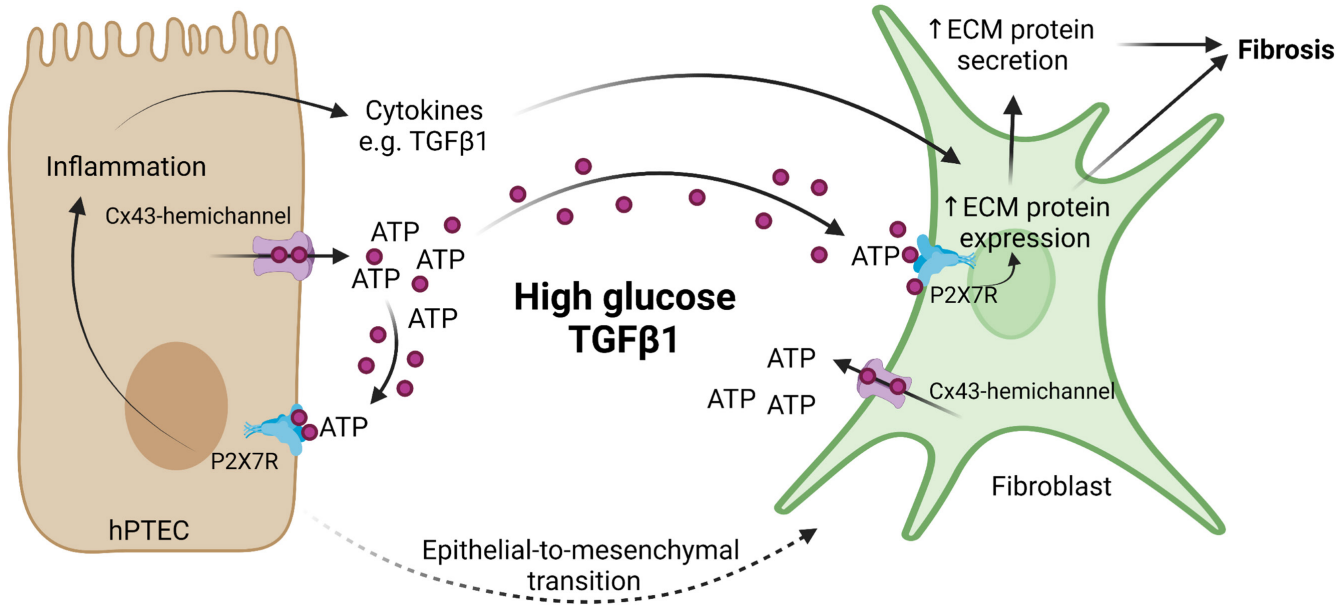

FIGURE 7 Cell–cell interactions between tubular epithelial cells and fibroblasts. Glucose and transforming growth factor beta1 (TGFβ1) increase Cx43 hemichannel-mediated ATP release from both human proximal tubule epithelial cells (hPTECs) and fibroblasts. Local increases in extracellular ATP stimulate P2X7R on both cell types driving an altered cell phenotype of tubule cells through partial epithelial-to-mesenchymal transition and myofibroblast activation. Co-culture experiments determine that an ATP-P2X7R driven response in hPTECs triggers secretion of cytokines, for example, TGFβ1, which through paracrine mediated signalling activates neighbouring fibroblasts to further increase fibroblast-derived deposition of extracellular matrix (ECM) and exacerbate increased fibrosis.

In conclusion, while this in vitro study provides a minimalistic model for the complexities of what is a debilitating complication of diabetes, the model provides an accessible and reliable tool for the in vitro study of cellular mechanisms. This is supported by the fact that our data build upon previously published observations which have informed in vivo work using the UUO mouse model of advanced interstitial inflammation and fibrosis.[12] Moreover, although recently reported that Tonabersat has homology with some other alpha group connexin isoforms (e.g., Cx40),[31] previously published in vitro[12] data using Cx43 hemichannel blocker Peptide 5 and in vivo[6] observations using the $Cx43^{+/-}$ heterozygous mouse, strongly support a pathogenic role of Cx43 hemichannels in interstitial inflammation and fibrosis, while highlighting the potential future use of Tonabersat in dampening aberrant Cx43 hemichannel-mediated purinergic communication in a model of diabetic nephropathy.

AUTHOR CONTRIBUTIONS

CEH and PES were involved in conceptualisation, supervision and project administration. BMW and CLC were involved in experimental studies. BMW, PES and CEH were involved in writing—original draft preparation. BMW, CLC, ID, PES and CEH were involved in writing. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENTS

This work was short listed for the Diabetes UK Early Career Investigator Award at the Diabetes UK Professional Conference 2022. Williams BM et al., *Diabetic Medicine*, 39 (Supplement1) A38 (P65) 2022. Schematics were created with BioRender.com.

FUNDING INFORMATION

BMW is a recipient of a PhD studentship supported by Diabetes UK and the Masonic Charitable Foundation. (18/0005919; awarded to PES & CEH).

CONFLICT OF INTEREST

The authors confirm that there are no conflict-of-interest issues.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Williams BM, Cliff CL, Demirel I, Squires PE, Hills CE. Blocking connexin 43 hemichannel-mediated ATP release reduces communication within and between tubular epithelial cells and medullary fibroblasts in a model of diabetic nephropathy. *Diabet Med*. 2022;00:e14963. doi: 10.1111/dme.14963

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