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TGF-β1 Mediates Glucose-evoked Up-regulation of Connexin-43 Cell-to-cell Communication in HCD-cells

Claire E. Hills[1], Rosemary Bland[2], Jeanette Bennett[2], Pierre M. Ronco[3] and Paul E. Squires[2]

[1]Department of Infection, Immunity and Inflammation, MSB building, University of Leicester, [2]Physiology Group, Department of Biological Sciences, University of Warwick, Coventry, [3]Unite INSERM 702; Universite Paris 6, Hopital Tenon (AP-HP), Paris

Key Words

TGF-β1 • Connexin-43 • Glucose • Collecting duct • Cell-to-cell communication

Abstract

Background/Aims: In the current study we examined if the multifunctional cytokine TGF-β1 mediated glucose-evoked increases in connexin-43(Cx43)-mediated intercellular communication in cells of the human collecting duct (HCD). Methods: RT-PCR and western blot analysis were used to confirm mRNA and protein expression of TGF-β1 and Cx43 in HCD-cells. The effect of TGF-β1 and high glucose (25mM) on Cx43 protein expression, cytoskeletal organisation and cell-cell communication was determined in the presence/absence of TGF-β1 specific immuno-neutralising antibodies. Functional cell-cell communication was determined using Ca^{2+}-microfluorimetry. Results: At 24hrs, high glucose (25mM) significantly increased Cx43 mRNA and protein expression. Changes were mimicked by TGF-β1 (2ng/ml) at low glucose (5mM). Both high glucose and TGF-β1 mediated changes were completely reversed by a pan-specific immuno-neutralising antibody to TGF-β. Furthermore, high glucose-evoked changes were inhibited by a TGF-β1-specific monoclonal antibody. Mannitol (25mM), an osmotic control for high glucose, failed to alter Cx43 expression. TGF-β1 evoked changes in Cx43 expression were biphasic. An early (4-8hr) transient decrease in expression was followed by an increase in protein expression (12-24hr). The decrease in Cx43 expression was paralleled by a transient reorganisation of the actin cytoskeleton, whilst increased Cx43 expression at 24hrs coincided with a TGF-β1 specific increase in touch-evoked transmission of Ca^{2+}-signals between coupled cells. Conclusions: High glucose evoked a TGF-β1 mediated increase in Cx43 expression and gap-junction mediated cell-cell communication in HCD-cells. These changes may maintain epithelial integrity of the collecting duct following hyperglycaemic assault as observed in diabetes.

Introduction

In renal epithelial, changes in osmolality initiate cell volume regulatory processes, that restore cell volume, and maintain both cell integrity and function. We recently

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Dr P.E. Squires
Department of Biological Sciences
The University of Warwick, Coventry, CV4 7AL (UK)
Tel.+44 24 7657-2976, Fax:+44 24 7652-3701
E-Mail P.E.Squires@warwick.ac.uk

showed that cells of the cortical collecting duct express mechano-sensitive receptors that transduce alterations in the physical environment into calcium-mediated cellular changes that act to restore cell volume [1]. High glucose and/or physical stimuli, generate rapid changes in $[Ca^{2+}]_i$ that up-regulate the functional expression of the epithelial sodium channel (ENaC). We suggested that these changes may underlie secondary hypertension associated with hyperglycaemia and glucosuria, as seen in patients with poorly managed diabetes [2].

Efficient and appropriate re-absorption of sodium within the cortical collecting duct depends upon an integrated response of the entire epithelium. The co-ordination of cellular activity is therefore a pre-requisite for the maintenance of cell homeostasis. In an *in vitro* model of the human cortical collecting duct (HCD-cells) Ca^{2+}-transients evoked by mechanical stimulation propogate between cells via connexin-43 (Cx43) - mediated gap-junctions [1]. Gap-junctions allow for the direct transfer of ions and small molecules between coupled cells, enabling individual cells to monitor and adapt their function in line with the activity of their immediate neighbours. Although HCD-cells express several connexin isoforms, Cx43 is the most abundantly expressed in the collecting duct [3] and Cx43 expression increases further in response to high glucose [1]. The variability of connexin expression in response to high glucose has been described in other cell types, including retinal [4, 5] and cardiovascular tissue [6]. Loss of compensatory mechanisms may result in overt complications associated with diabetic nephropathy and end stage renal disease and deregulation of Cx expression is associated in the pathogenesis of some renal [7] and vascular diseases [8]. Whilst the question of how elevated glucose alters connexin expression remains unanswered, glucose is known to increase the activity of the multifunctional cytokine Transforming Growth Factor-β (TGF-β) [9-14].

TGF-β is a ubiquitous cytokine that has a broad spectrum of biological functions and actions in a variety of cell types [15-17]. The prosclerotic properties of TGF-β have been linked to excessive matrix deposition in db/db diabetic mice [18] and in patients presenting with nephropathy [19], and the cytokine is thought to play a key role in the development and progression of diabetic glomerulopathy [20-21]. Elevated TGF-β increases the synthesis of fibronectin, laminin and collagen in both glomerular mesangial cells and tubular cells [22]. Furthermore, TGF-β has been linked to epithelial mesenchymal transition (EMT) [23], a process by which epithelial cells into α-smooth muscle actin (SMA)-positive, fibroblast-like cells. The phenotypic alterations in renal proximal tubular cells that arise as a consequence of TGF-β-induced EMT, can be reflected in a decreased expression of inter-cellular adhesion molecules [24-25], re-arrangement of actin microfilaments [24, 26-28] and disassembly of tight junction complexes [29]. It has been suggested that inter-cellular adhesion precedes and may even be a prerequisite in forming fully functional gap junctions [30]. In pancreatic β-cells we recently published data demonstrating that one class of Ca^{2+}-dependent adhesion molecules, the catenins, bind to the cytoskeleton [31] and facilitate gap junction assembly [30, 32-35]. It therefore seems likely that phenotypic alterations in the cytoskeleton and thus cell shape may affect gap-junction-mediated cell-to-cell interactions in some cell types. To date however, there is no evidence for glucose-evoked EMT in the cortical collecting duct and the effect of TGF-β on gap-junction mediated cell-cell communication has not yet been examined. In the current study we have assessed temporal effects of TGF-β1 and high glucose on Cx43 expression, cell architecture and cell-cell communication in human cortical collecting duct (HCD) cells.

Materials and Methods

Cell Culture

HCD cells were derived from normal human kidney cortex and immortalized with SV-40 virus. Clones were selected using the monoclonal antibody, Ab272, which specifically recognizes collecting duct principal cells. HCD cells (passages 18-30) were maintained in DMEM/Hams F-12 medium (GIBCO, Invitrogen), supplemented with 2% fetal calf serum (FCS), glutamine (2mmol/L), 15mmol/L HEPES, transferrin (5μg/mL), Na_2SeO_3 (5ng/mL), insulin (5μg/mL) and dexamethasone ($5x10^{-8}$M). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Prior to treatment, cells were cultured in DMEM/ Hams F-12 low glucose (5mM) for 48 hours. Basal (5mM) glucose culture media was generated as described previously [2]. For TGF-β1 experiments, cells were cultured in either low (5mM) or high (25mM) glucose containing un-supplemented DMEM/F12 for 48hr. Cells were treated with or without TGF-β1 (2ng/ml) and pre-incubated with either a TGF-β pan-specific neutralising antibody (10μg/mL; R&D Systems Inc, Minneapolis, USA) or a monoclonal TGF-β1-specific immuno-neutralising antibody (10μg/mL; R&D Systems Inc, Minneapolis, USA) for 1hr. In all experiments, cells were serum starved overnight before agonist stimulation. For immuno-cytochemistry and microfluorimetry experiments, cells were seeded onto 3-Aminopropyltriethoxy-silane (APES) (Sigma, Poole, UK) treated coverslips and used within 2 days of plating.

mRNA	Primers (5'→ 3')	Direction	Annealing temp (°C)	Product size (*bp*)
TGF-β1	AGAGGTCGCTTGGGAATTTT	F	58	341
	TTTGGAAACCATGTCAAGCA	R		
Cx43	CCCGTGAGAACACCAAGTTT	F	58	597
	TCACTCCAGGGCATTTCTTC	R		

Table 1. Polymerase chain reaction (PCR) primers used to amplify TGF-β1 and Cx43 mRNA. F = forward primer, R = reverse primer.

Analysis of mRNA expression

RNA was prepared from 80% confluent HCD cells by acid-guanidinium extraction using a genelute mammalian total RNA miniprep kit (Sigma) following the manufactures instructions. Complementary DNA was synthesized by reverse transcription using a Promega Reverse Transcription System following an adapted method. Briefly, 1μg of total RNA and 0.5μg of random hexamers, in a final volume of 11μL, were incubated at 70°C for 5min, and then allowed to cool slowly to 25°C. Primer extension was then performed at 37°C for 60min following the addition of 1x (final concentration) reaction buffer, containing 50mmol/L Tris-HCl (pH 8.3), 50mmol/L KCl, 10mmol/L $MgCl_2$, 10mmol/L dithiothreitol and 0.5mmol/L spermidine, 1mmol/L (final concentration) of each dNTP, 40uL of rRNAsin ribonuclease inhibitor and 15uL of AMV reverse transcriptase in a final volume of 30μL. The RT mixture was heated to 95°C for 5min, then 4°C for 5min. An aliquot of 4μL was used in subsequent polymerase chain reaction (PCR) reactions.

PCR amplification of cDNAs

Amplification of specific cDNAs was carried out using the primers listed in Table 1. PCR reactions (20μL) were set up containing 1.5mmol/L $MgCl_2$, 0.2mmol/L of each dNTP, 0.5μM of each primer and 1U of Taq DNA polymerase (Promega). Amplification of samples was performed using an initial denaturation step of 95°C (5min) followed by either 35 cycles (TGF-β1) and 30 cycles (Cx43) consisting of one minute of denaturing at 95°C, one minute of annealing at the required temperature and a one minute extension at 72°C. A final elongation step of 72°C for seven minutes was included in all PCR amplifications.

Analysis of protein expression

The preparation of cytosolic proteins and their subsequent separation by gel electrophoresis and electro-blotting onto Immobilon P membrane (Millipore, Watford,UK) were as described previously [36]. Briefly proteins (5μg) were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (4.5% stacking gel, 7.5% or 10% resolving gel) at 200v for 50min in electrophoresis buffer containing 25mmol/L Tris, 192mmol/L glycine and 0.1% (wt/vol) SDS. Proteins were transferred onto Immobilin P membrane in transfer buffer (25mmol/LTris, 192mmol/L glycine, 20% (vol/vol) methanol) for 1hr at 100v; 4°C. Following protein transfer, membranes were blocked in PBS-T (PBS plus 0.1% Tween-20) containing 20% (wt/vol) nonfat milk powder (Marvel, Premier Brands, Stafford, UK) for 1hr at 25°C and then washed with PBS-T for 15min. Filters were analyzed with specific polyclonal antibodies against human TGF-β1 (Santa Cruz), and anti-human Cx43 (Santa Cruz) at a dilution of 1:500 and 1:200 respectively in PBS-T. After three 10min washes in PBS-T, the membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated) anti-rabbit (Cx43 diluted 1:30,000) and anti-sheep (TGF-β1 diluted 1:40,000) in PBS-T (0.05%) for 60min at 25°C followed by three 10min washes in PBS-T. Specific proteins were detected using ECL detection reagent chemiluminescence system (Amersham Biosciences) and were visualized after exposure of membranes to X-ray film for 1-10min. Control experiments were included where primary antibody was omitted, and filters were exposed to secondary antibody and ECL detection.

Immunocytochemistry

Cells were allowed to grow to 80% confluence on APES treated coverslips and then fixed in 4% paraformaldehyde. Non-specific binding was prevented by blocking for 1hr at 25°C in PBS + 0.01% triton X-100 containing 10% normal goat serum. Following three 10min washes with PBS, the nuclear stain DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; 1mmol/L) was added to each coverslip for 3min. After washing with PBS (3x5min), cells were incubated with the cytoskeletal stain TRITC-conjugated-Phalloidin (Sigma) diluted at 1:100 in PBS supplemented with 0.01% Triton X-100 for 1 hour at 25°C. After 1hr, cells were washed 3x10min and coverslips mounted in citifluor (glycerol/PBS solution: Agar Scientific) on glass slides.

Single-cell microfluorimetry

HCD cells seeded and grown overnight on APES-coated coverslips were loaded for 30min at 37°C with 2.5μM of the Ca^{2+} fluorophore fura-2/AM (Sigma). Coverslips were washed and placed in a steel chamber, the volume of which was approximately 500μl. A single 22mm coverslip formed the base of the chamber, which was mounted into a heating platform on the stage of an Axiovert 200 Research Inverted microscope (Carl Zeiss, Welwyn Garden City, UK). All experiments were carried out at 37°C using unsupplemented DMEM-Ham's F-12 medium as the standard extracellular medium. Cells were illuminated alternatively at 340 and 380nm using a Metaflour imaging workbench (Universal Imaging, Marlow, Bucks, UK). Emitted light was filtered by using a 510nm long-pass barrier filter and detected using a Cool Snap HQ CCD camera (Roper Scientific). Changes in the emission intensity of fura-2 expressed as a ratio of dual excitation were used as an indicator of changes in $[Ca^{2+}]_i$ with the use of established procedures. Data were collected at 3s intervals for multiple regions of interest in any one field of

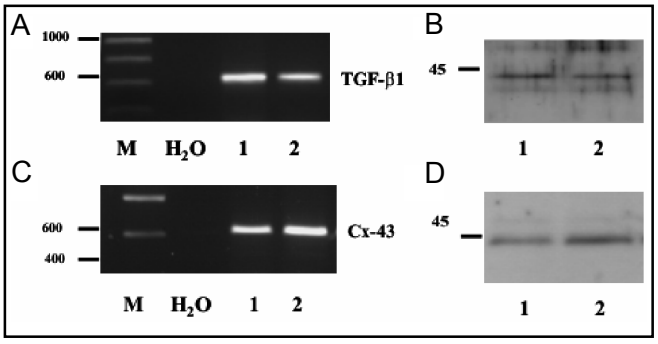

Fig. 1. Expression of TGF-β1 and Cx43 mRNA and protein in HCD-cells. Panels A and C represent RT-PCR analyses using primers specific for human TGF-β1 and Cx43. PCR products of 341bp and 597bp were observed in two separate RNA preparations (1 and 2) corresponding to mRNA expression for TGF-β1 and Cx43 in HCD cells. Negative controls included both water and samples in which AMV enzyme had been omitted from the reverse transcription reaction (data not shown). Western blot analyses of HCD cell lysates (panels B and D, 5μg protein/lane) using an antibody against human TGF-β1 (panel B) and Cx43 (panel D) confirmed the presence of the protein in HCD cells. A protein band of approximately 45kDa (TGF-β1), and 43kDa (Cx43) was detected. Controls included TGF-β1 and Cx43 antibody pre-absorbed with a 10-fold excess of immunizing peptide. (Data not shown).

view. All records have been corrected for background fluorescence (determined from cell-free coverslip).

Mechanical stimulation of HCD cells

Individual cells within a cluster (6-12 cells/cluster) of fura-2-loaded cells were stimulated via touch using a femtotip electrode delivery system (Eppendorf, Hamburg, Germany). Maintained fura-2 fluorescence confirmed the integrity of the cell membrane.

Transmission velocity experiments

HCD cells were seeded and grown to 50% confluence on APES-coated coverslips in the previously described standard culture medium. After an overnight period of serum starvation, the medium was then removed and changed to a test medium containing 5 or 25 mmol/l glucose plus/minus TGF-β1 and/or TGF-β1 specific neutralising antibody. After a 48hr exposure to each test medium, individual cells within a cluster (6-12 cells/cluster) of fura-2-loaded cells were stimulated via touch using a femtotip electrode delivery system. The rate of transmission of a touch-evoked Ca^{2+} signal between individual HCD cells was assessed. Maintained fura-2 fluorescence confirmed the integrity of the cell membrane.

Data Analysis

Autoradiographs were quantified by densitometry (TotalLab 2003). Statistical analysis of data was performed using a one-way ANOVA test with a Tukey's Multiple Comparison

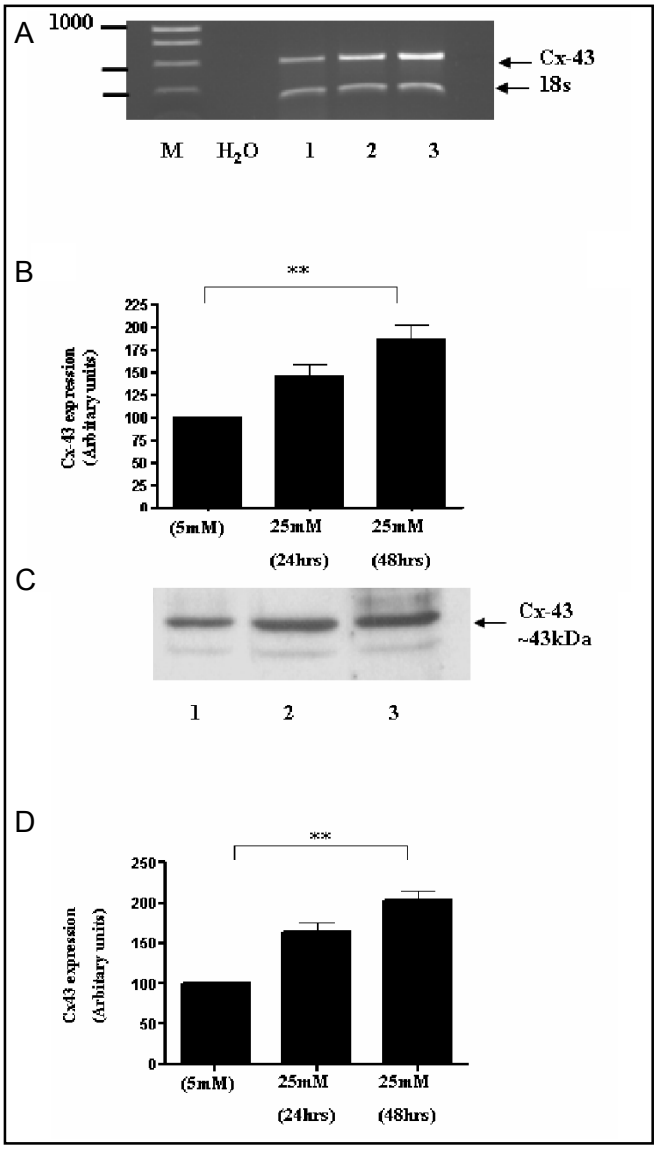

Fig. 2. Cx43 mRNA and protein expression are glucose dependent. Panel A represents RT-PCR analyses using primers specific for human Cx43. Exposure of HCD cells to high (25mM) glucose increased Cx43 mRNA at both 24 (panel A, band 2) and 48hrs (panel A, band 3) compared to low glucose control (5mM; panel A, band 1). Negative controls included both water and samples in which AMV enzyme had been omitted from the reverse transcription reaction (data not shown). Blots (n=3) were quantified by densitometry and expressed as a % of low glucose control (panel B). Differences between control and each test condition was compared statistically where, ** $P<0.01$. Western blot analyses of HCD cell lysates (panels C and D, 5μg protein/lane) using an antibody against Cx43, confirmed that exposure to high (25mM) glucose increased Cx43 protein expression at both 24 (panel C, band 2) and 48hrs (panel C, band 3) compared to low glucose control (5mM; panel C, band 1). Blots (n=3) were quantified by densitometry and expressed as a % of low glucose control (5mM; panel D). Differences between control and each test condition was compared statistically where, ** $P<0.01$.

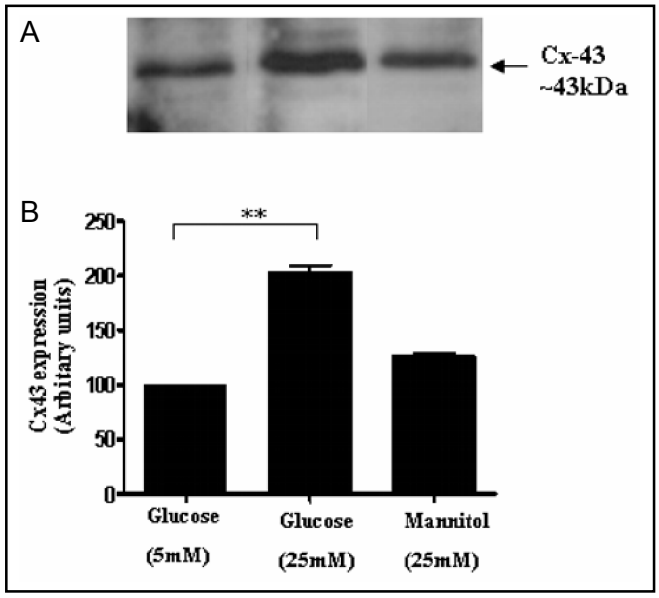

Fig. 3. Changes in osmolarity do not alter Cx43 expression. Western blot analyses of HCD cell lysates (5μg protein/lane) using an antibody against Cx43, confirmed that exposure to high (25mM) glucose increased Cx43 protein expression at 48hrs compared to low glucose control (panel A; band 2) compared to low glucose control (5mM; panel A, band 1). Equimolar concentrations of mannitol (25mM), as an osmotic control, failed to alter Cx43 expression over the same time period (panel A; band 3). Blots (n=3) were quantified by densitometry and expressed as a % of low glucose control (5mM; panel B). Differences was compared statistically where, ** P<0.01.

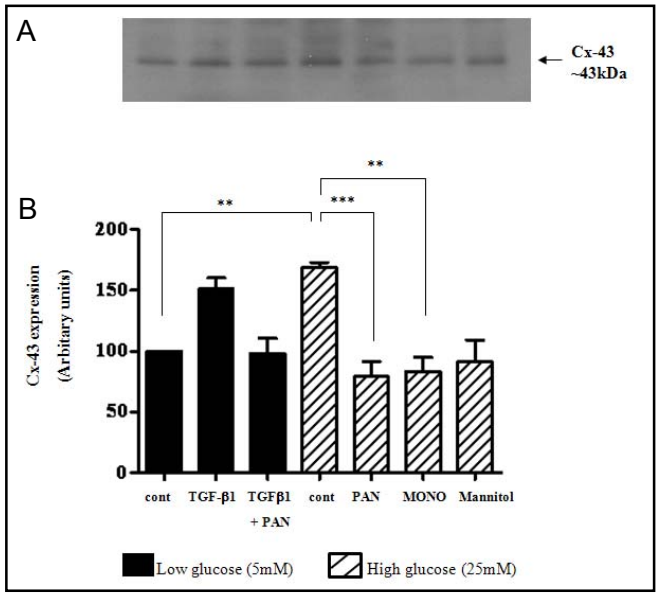

Fig. 4. TGF-β1 mediates the effects of high glucose on Cx-43 expression. HCD cells were grown (48hr) in either low glucose (5mM; solid columns) +/- TGF-β1 (2ng/mL), or high glucose (25mM; hatched columns), either in the presence or absence of a pan-specific (PAN) or monoclonal TGF-β1-specific (MONO) neutralising antibody. The expression of Cx43 was determined by immunoblotting. Upper panel (A) is a representative immunoblot showing changes in Cx43 expression under each of the conditions described in panel B. Blots (n=3) were quantified by densitometry and expressed as a % of low glucose control (5mM; panel B). Differences between control and each test condition was compared statistically where, ** P<0.01, *** P< 0.001.

post-test. Data are expressed as arithmetic mean±SEM and n denotes the number of experiments and P<0.05 signifies statistical significance.

Results

Expression of TGF-β1 and Cx43 in HCD cells

RT-PCR analysis of two RNA preparations from HCD cells revealed PCR products representative of TGF-β1 and Cx43 mRNA (figure 1A and C respectively). To confirm that the mRNA was appropriately translated, protein expression was determined by western blotting (figures 1B and D). Western blot analyses revealed bands at approximately 45kDa and 43kDa representative of those expected for TGF-β1 and Cx43.

High-glucose-evoked up-regulation of Cx43 expression

To examine the effect of glucose on Cx43 expression, HCD cells were incubated in high glucose (25mM) for 24hr and 48hr. The expression of Cx43 was assessed by RT-PCR and western blot analysis. High glucose (25mM) increased Cx43 mRNA expression (figure 2A) to 146.2±8.5% and 186.8±7.8% of low glucose control (5mM) at 24hr and 48hr respectively (figure 2B; P<0.01, n=3). Western blot analysis confirmed a corresponding increase in Cx43 protein expression (figure 2C) to 163±11% and 203±12% (P<0.01, n=3) of low glucose control (5mM: figure 2D) at 24hr and 48hr respectively. Mannitol (25mM) was used as a control for the osmotic effects of high glucose and increased Cx43 expression at 48hr by only 29±1.4% of the glucose-evoked change seen under identical experimental conditions (see figure 3, P=NS, n=3).

Glucose induced Cx43 expression is mediated by TGF-β1

To determine the role of TGF-β1 in mediating the glucose-evoked increase in Cx43 expression we examined the effect of TGF-β and TGF-β1 specific immuno-

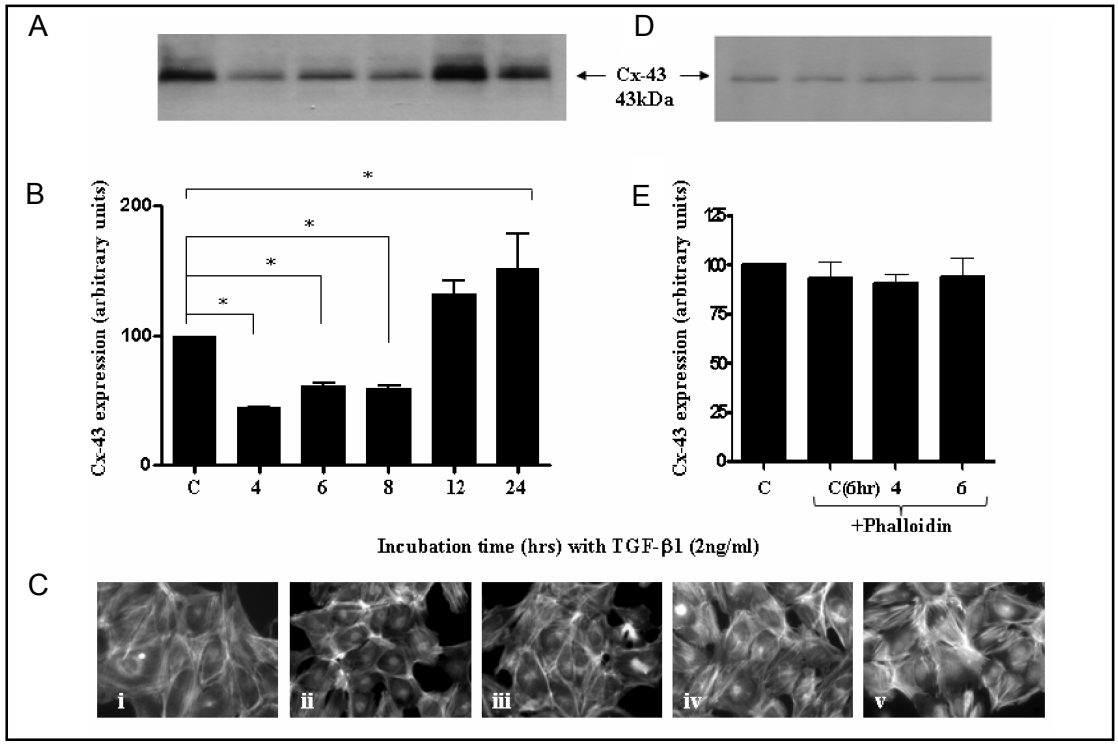

Fig. 5. TGF-β1 exerts a biphasic effect on Cx43 protein expression. HCD cells were grown in low glucose (5mM) plus TGF-β1 (2ng/ml) at 4, 6, 8, 12, and 24hrs and Cx43 expression determined by immunoblotting. Upper panel (A) is a representative immunoblot showing Cx43 expression at each of the time intervals described in panel B. Blots (n=3) were quantified by densitometry and expressed as a % of low glucose control (5mM; panel B). Differences between control and each time interval was compared statistically, where * $P<0.05$. In panel C, TRITC-conjugated phalloidin was used to visualise filamentous actin. In control low glucose (5mM) the actin cytoskeleton appears as loosely associated filaments organised throughout the cell interior (figure 5Ci). TGF-β1 (2ng/mL) stimulated the formation of peripheral stress fibres at 1 and 4hrs (figure 5Cii and iii respectively). These were prevented by co-incubation with the TGF-β1-specific immuno-neutralising antibody (figure 5Civ; 4hr incubation). At 24 hours, cytoskeletal organisation was restored to the control pattern (figure 5Cv). The effect of un-conjugated phalloidin (10μg/mL) +/- TGF-β1 (2ng/mL) on Cx43 expression in low (5mM) glucose at 4 and 6hrs is examined in panel D and E, where D shows a representative immunoblot of Cx43 expression described in E (n=3). Stabilisation of the actin cytoskeleton prevented early TGF-β1-induced changes in Cx43 expression at both 4 and 6hrs (*P*=NS).

neutralising antibodies on glucose-evoked changes in Cx43 expression (Figure 4, hatched columns). At 48hrs, high glucose (25mM) increased Cx43 expression to 169±4.3% as compared to low glucose (5mM) control ($P<0.01$). Co-incubation with either a pan-specific or TGF-β1-specific immuno-neutralising antibody negated the effect of high glucose (79±12.4%; $P<0.001$ and 83±11.6%; $P<0.01$ respectively as compared to low glucose (5mM) control; n=3).

The effect of exogenous application of TGF-β1 (2ng/mL) +/- pan-specific TGF-β immuno-neutralising antibody was also examined (Figure 4, solid columns). HCD cells were incubated for 48hrs at low glucose (5mM) +/- TGF-β neutralising antibody. TGF-β1 (2ng/ml) induced a 51% increase in Cx43 expression to 151±8.9%, whilst co-application with the pan-specific neutralising antibody reduced Cx43 expression to 98±12.7% as compared to control at low glucose. The effect of mannitol (25mM) was similar to the low glucose (5mM) control (*P=NS*).

TGF-β1 has a biphasic effect on Cx43 expression in HCD-cells

In other cell systems, the effects of TGF-β are reportedly rapid in onset. To resolve temporal effects of exogenous TGF-β1 we examined the effects of the cytokine (2ng/mL) on Cx43 expression at 4, 6, 8, 12 and 24hrs. Over this time course, TGF-β1 evoked a biphasic response on protein expression. At 4, 6 and 8hrs

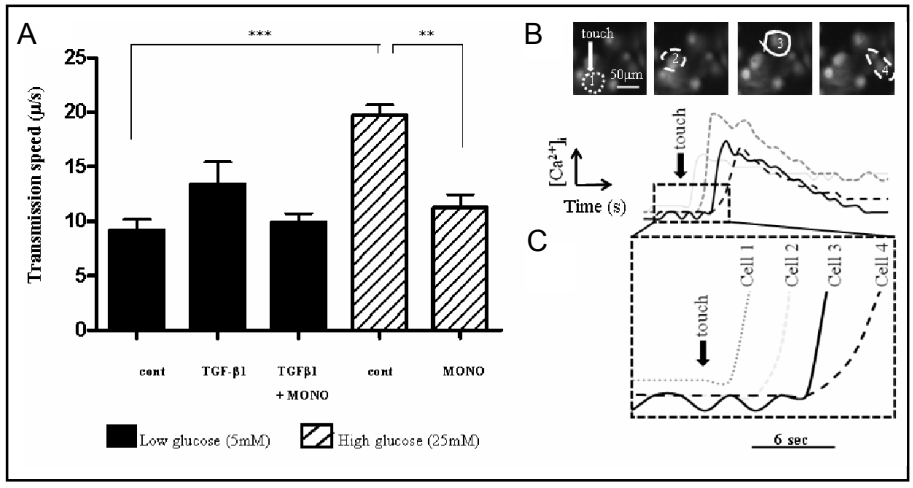

Fig. 6. Glucose-evoked increases in cell-cell communication are TGF-β1-dependent. HCD cells were grown (48hr) in either low glucose (5mM; solid columns) +/- TGF-β1 (2ng/mL), or high glucose (25mM; hatched columns), either in the presence or absence of a TGF-β1-specific (MONO) neutralising antibody. Individual Fura-2-loaded cells were mechanically stimulated and the speed of transmission (µm/s) of touch-evoked Ca^{2+}-transient between coupled cells was determined (panel B; region of interest (ROI) 1-4 overlays 4 individual cells within a coupled cell cluster). The time point for the initial rise in $[Ca^{2+}]_i$ for each ROI was determined and related to the distance between individual cells (panel C). Mean data±SEM (n=4 separate experiments for each treatment, are shown in panel A). High glucose (25mM; hatched columns) significantly increases the transmission of Ca^{2+}-transients between cells (*** $P< 0.001$), whilst co-incubation with TGF-β1-specific neutralising antibody negates the response (** $P<0.01$). A similar trend is shown for TGF-β1 application, though this did not attain significance.

TGF-β1 reduced Cx43 expression by 56±1.7%, 32±8.8% and 41±3.9% respectively, as compared to a low glucose (5mM) control (figure 5; $P<0.05$ in each case). At 24hrs Cx43 expression had significantly increased compared to control (152±17.7%, $P<0.05$, figure 5). Incubation of HCD cells with vehicle had no effect on Cx43 expression (data not shown).

Recent reports have indicated that TGF-β1 induces very rapid actin polymerization and formation of actin stress fibres in various cell models [37, 38]. To examine the potential role of cytoskeletal re-arrangement on the temporal variability of Cx43 expression we performed a parallel series of experiments to assess the effects of the cytokine on the filamentous actin. In figure 5C(i), phalloidin staining detected a loose cytoplasmic organisation of actin at low (5mM) glucose. Addition of TGF-β1 (2ng/mL) evoked a general increase in actin stress fibres around the periphery of the cells at 1 and 4hrs (figure 5Cii and iii respectively). The appearance of TGF-β1-induced peripheral stress fibres was prevented by co-incubation with the TGF-β1-specific immuno-neutralising antibody (figure 5Civ; 4hr incubation). At 24 hours, cytoskeletal organisation was restored to a low glucose (5mM) non-stimulated pattern (figure 5Cv). To confirm that the transient reduction in TGF-β1-induced Cx43 expression was correlated to cytoskeletal re-organistaion, cells were incubated at low (5mM) glucose plus or minus phalloidin (10µg/mL) in order to stabilise the actin cytoskeleton (figure 5D and E). Neither phalloidin alone at 6hrs (93±8%, figure 5E; C(6hr)), nor TGF-β1 (2ng/mL) plus phalloidin at 4 and 6hr altered Cx43 expression compared to low glucose control (88±5% and 94±10% respectively, P=NS). DMSO (0.01%) vehicle had no effect on Cx43 expression or cytoskeletal organisation (data not shown).

TGF-β1 mediates glucose-evoked increases in inter-cellular communication

We have previously shown that glucose-evoked increases in Cx43 expression are matched to an increase in gap-junction-mediated intercellular communication (GJIC [1]). In this earlier study we demonstrated that HCD-cells express the mechano-sensitive transient receptor potential channel (TRPV4), evoking transient, propagating Ca^{2+}-signals in response to touch. To confirm that glucose induced cell-to-cell coupling is TGF-β1 dependent, Ca^{2+}-microfluorimetry was used to assess the effect of high glucose (25mM) or TGF-β1 (2ng/mL), either alone or co-applied with a TGF-β1 specific neutralising antibody, on the transmission of mechanically evoked Ca^{2+}-signals between coupled HCD cell clusters (figure 6). Following 48hr incubation, the velocity of touch-evoked Ca^{2+}signals between adjacent cells was elevated by both TGF-β1 (145±14.9%) and high glucose (214±4.5%; $P<0.001$, see figure 6), as compared with that of cells cultured in low (5mM) glucose. The dependence of the glucose-evoked response on TGF-β1 was confirmed using co-incubation of the TGF-β1-specific antibody, which returned both TGF-β1 stimulated and high glucose-stimulated increases in GJIC back to control, low

glucose, levels (107±8% and 122±10%; $P<0.01$). These data suggest that both increased Cx43 expression and increased cell-to-cell coupling exhibited by HCD cells in response to high glucose is a TGF-β1 dependent process.

Discussion

Diabetic nephropathy is a leading worldwide cause of chronic kidney disease (CKD) and end-stage renal disease. Elevated glucose is a major contributing factor in the development of diabetic nephropathy [39, 40], and in the kidney it has been suggested that glucose mediates many of its cellular effects via the Transforming Growth Factor-beta-1 (TGF-β1) [10-13]. A cytokine with powerful fibrogenetic effects, TGF-β is up-regulated in models of diabetes and diabetic nephropathy [20, 21, 41]. Abnormalities in TGF-β1 signalling have been discovered in a wide variety of disorders, including autoimmune diseases, malignancies, and chronic renal disease with increased receptor expression having been described in experimental renal disease models including membranous nephropathy, obstructive nephropathy, and diabetic nephropathy.

In the current study we have examined the possibility that TGF-β1 mediates glucose-evoked increases in Cx43 and cell-to-cell communication in a model system of the human collecting duct. Confirming previous data [1] we have demonstrated that HCD cells express both Cx43 and TGF-β1 and that the expression of both mRNA and protein is glucose-dependant, significantly increasing over 24-48hr in 25mM glucose. The effect of high glucose was not osmotically-dependent as equimolar mannitol failed to alter Cx43 expression. Conversely, immuno-neutralising TGF-β, and more specifically the TGF-β1 isoform, prevented glucose-evoked changes in Cx43 expression. TGF-β1 significantly increased Cx43 expression at 24hrs, but the effect was biphasic, being preceded by an initial short-term (<8hrs), transient decrease in protein expression.

The localisation, activity and trafficking of membrane bound proteins is mediated through interactions with actin, actin-binding proteins and/or scaffolding proteins [reviewed in 42]. Therefore, alterations in the architectural framework of cells would have clear repercussions for the clustering, retention and activity of membrane bound cell adhesion and coupling proteins. Consequently, it could be argued that changes in the arrangement of the actin cytoskeleton could alter gap junction expression and function. Interestingly, in the current study, the short-term TGF-β1-induced loss of Cx43-expression was matched temporally to a rapid, but transient re-organisation of the actin cytoskeleton into peripheral stress fibres. These changes were reversible over time (24hrs) and could be blocked by TGF-β1-specific neutralising antibody. Changes in TGF-β expression have been associated with numerous phenotypic changes including re-organisation of the actin cytoskeleton in both proximal tubular epithelial cells [24, 25] and mesangial cells [27]. TGF-β-induced changes in actin, and thus cell shape, could have dramatic implications for cell architecture, protein expression, cell-to-cell communication and ultimately epithelial function. Previous studies have shown that TGF-β remodels actin microfilaments in proximal tubular cells, causing disassembly of tight junction complexes [29], and the present study suggests that TGF-β1 also affects Cx43 expression. Early TGF-β1 induced architectural changes serve to strengthen the cell membrane, but result in a transient decrease in Cx43-mediated cell contact. Whilst initial TGF-β1-induced down-regulation of Cx-43 expression may reflect a latency period involving re-arrangement of the actin cytoskeleton, it seems likely that up-regulation of Cx43 at 24 and 48hrs reflects stabilisation of the cytoskeleton and recovery, allowing restoration and enhancement of protein expression, in the continuous presence of the cytokine. The dependence of the transient loss in Cx43 expression on changes to cytosolic filamentous actin was confirmed using phalloidin to stabilise the actin cytoskeleton. In these experiments, where phalloidin alone and vehicle had no effect on protein expression, the previous short-term loss in TGF-β1-induced Cx43 expression was ablated.

Cx43 represents the major gap-junction connexin in the nephron [3]. Consequently, up-regulation of Cx43 expression would be expected to correlate to an increase in GIJC. Previous data from our laboratory have demonstrated the expression of mechano-sensitive TRPV4 in HCD cells [1]. By physically touching an individual fura-2-load HCD cell we are able to elicit an increase in $[Ca^{2+}]_i$. This Ca^{2+}-transient then flows through Cx-mediated gap junctions to elicit a synchronised response in neighbouring cells. By measuring the time of onset of our touch-evoked rises in $[Ca^{2+}]_i$ and measuring the distance between coupled cells we can determine the transmission speed of direct cell-to-cell communication. Through the use of exogenous TGF-β1 and TGF-β1-specific neutralising antibodies our data suggest that high glucose significantly increases cell-cell communication via a TGF-β1-specific mechanism.

As previously suggested [1], this improvement in the coupling of responses across the epithelium of the collecting duct may have a protective role in ensuring epithelial integrity and function under conditions of hyperglycaemia associated with diabetes.

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