Connexin-mediated cell communication in the kidney, a potential therapeutic target for future intervention of diabetic kidney disease?

Joan Mott Prize Lecture

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Abstract:

The ability of cells to communicate and synchronise their activity is essential for the maintenance of tissue structure, integrity and function. A family of membrane-bound proteins called connexins are largely responsible for mediating the local transfer of information between cells. Assembled in the cell membrane as a hexameric connexon, they function either as a conduit for paracrine signalling, forming a trans-membrane hemi-channel or, if aligned with connexons on neighbouring cells, form a continuous aqueous pore, or gap junction, which allows for the direct transmission of metabolic and electrical signals. Regulation of connexin synthesis and activity is critical to cellular function and a number of diseases are attributed to changes in the expression and/or function of these important proteins. A link between hyperglycaemia, connexin expression, altered nucleotide concentrations and impaired function, highlights a potential role for connexin-mediated cell communication in complications of diabetes. In the diabetic kidney, glycaemic injury is the leading cause of end stage renal failure, reflecting multiple aetiologies including glomerular hyperfiltration, albuminuria, increased deposition of extracellular matrix, and tubulointerstitial fibrosis. Loss of connexin-mediated cell-to-cell communication in diabetic nephropathy may represent an early sign of disease progression, however, our understanding of the process remains severely limited. This review focusses on recent evidence demonstrating that glucose-evoked changes in connexin mediated cell communication and associated purinergic signalling, may contribute to the pathogenesis of kidney disease in diabetes, highlighting the tantalising potential of targeting these proteins as a novel therapeutic intervention.

Short title:

Connexins, connexons and cell communication in the diabetic kidney

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Introduction:

Renal function depends on a high level of co-operativity between cells of the nephron. For cells to function efficiently and adapt appropriately in times of stress, they must not only interact with each other, but also with their immediate environment. Most cells communicate with their neighbours via gap junctions, or paracrine signalling [1-3]. Hemi-channels form as a result of hexameric clustering of membrane-bound proteins called connexins, which together form a trans-membrane pore more commonly termed a connexon [1]. When hemi-channels align with those on adjacent cells they 'dock' to form a gap junction. This junction facilitates the transmission of metabolic and electrical signals directly between cells, enabling cells to entrain their activity and synchronise tissue function. In the absence of neighbouring partners, uncoupled hemi-channels permit local paracrine release of nucleotides, including adenosine triphosphate (ATP) and its metabolites (adenosine diphosphate, ADP; adenosine monophosphate, AMP; and adenosine) (Figure 1) [2, 4].

Hemi-channel mediated release of nucleotides has multiple functions in the kidney, including regulation of renal blood flow, glomerular filtration rate and the regulation of renal tubular transport [reviewed in 3]. Derived from purinergic-mediated signals and activated in response to nucleotide binding to membrane-bound G-protein coupled purino-receptors (P1/adenosine and P2/ATP and ADP) on adjacent cells [4], increased hemi-channel activity and high levels of extracellular ATP have recently been linked to inflammation and fibrosis [5-7]. Aberrant connexin expression and the subsequent loss of cell function has been suggested to underpin the pathophysiology of multiple disease states, including diabetes, where glycaemic injury has been shown to decrease gap junction conductance and hemi-channel activity [8-10]. In light of this, connexins have received considerable attention in recent years, with suggestions that these proteins may represent a promising future therapeutic target for treatment of disease [11], including diabetic kidney disease; the leading cause of End Stage Renal Failure.

Chronic kidney disease has been described as a global pandemic, with an estimated 850 million people worldwide thought to be affected by some form of kidney malfunction [12]. Diabetes is the biggest contributor to this statistic [12] with renal complications of the disease accounting for approximately 21% of deaths in patients with type1 diabetes, and approximately 11% in those with type2. Categorised into five stages and associated with a number of complex metabolic and inflammatory changes, diabetic nephropathy develops in response to defects at the molecular, cellular and tissue level in the vasculature, glomerulus and tubulointerstitium [13]. Whilst regulating blood sugar and blood pressure slow disease progression, many patients still develop renal failure even in the face of good glycaemic control. Consequently, as the incidence of diabetes continues to increase, there is a desperate need to improve therapeutic intervention to combat the long-term complications

of this lifelong metabolic disease. Changes in the expression profile of proteins involved in cell communication and associated downstream, purinergic signalling, may contribute to the development and progression of renal disease and thus represent a viable future target for intervention. In the current article, we review the expression, localisation and function of connexins, purinergic receptors and purinergic signalling in the diabetic kidney, and explore their underlying role as linked to the pathology of renal complications of diabetes.

Connexins: The building blocks of cell-cell communication

A family of trans-membrane proteins, connexins (CX) are designated by a numerical suffix that refers to their molecular weight. Expression may be ubiquitous, e.g. CX32 is expressed on various cell types including cardiac tissue, kidney and hepatocytes, or tissue specific, e.g. CX62 is only expressed in retinal tissue [14]. With a short half-life of only a few hours, connexins are continually synthesised and degraded, with biosynthesis and export to the cell membrane synonymous to that of other membrane-bound proteins [15], ultimately allowing for acute regulation of channel activity and intercellular communication [15].

All connexins share common structural features and are composed of four trans-membrane helices, interconnected by two extracellular loops and one intracellular loop, a cytoplasmic -NH2 and a -COOH terminal region [16]. The highly conserved amino terminal tail incorporates a putative calmodulin-binding motif necessary for both membrane insertion and, together with the -COOH terminal region, regulation of channel conductance, the latter of which is controlled by post-translational modification and phosphorylation of either serine/threonine or tyrosine residues [17-18]. When connexins combine, they oligomerise into hexameric channels. Comprised of six individual connexins, these trans-membrane aqueous pores, 1-2nm in diameter, are permeable to a variety of small ions and molecules, including Ca^{2+}, and IP_3 and ATP [19].

Local release of ATP from hemi-channels was first identified by Gordan *et al,* who proposed that cellular efflux of the nucleoside triphosphate occurred in response to altered plasma membrane topography [20]. Further studies supported the notion that extrusion of ATP into the extracellular environment is a consequence of pore formation in the plasma membrane, pores later identified as “hemi-channels” [21]. Numerous connexins have since been implicated in the release of ATP, including CX26, CX32, and CX43 [reviewed in 22], and it is now widely accepted that hemi-channel release of purines, including ATP, adenosine and pyrimidine, can serve as paracrine signals for intercellular communication. These locally released purinergic signals bind one of three classes of receptor (metabotropic P1 receptors, P2Y metabotropic and P2X ionotropic receptors), activation of which initiates downstream signals linked to regulation of renal function [3]. More recently, a role for

extracellular nucleotides and paracrine signalling in organ fibrosis has been highlighted [5-7]. Not surprisingly, strict control of connexin activity is imperative in maintaining cellular function and is further supported by the observation that there are a large number of diseases where mutations and/or single nucleotide polymorphisms result in inflammation [23-24], ischaemia [25] and essential- [26] or renin-induced hypertension [27]. Consequently, hemi-channel gating is regulated in response to various stimuli including changes in cell proliferation, intracellular calcium, increased apoptosis, post-translational modification, changes in pH and in cell volume [reviewed in 1].

Connexin-mediated cell communication in the diabetic kidney

With the incidence of diabetic nephropathy doubling over the last decade, it now accounts for approximately 50% of patients presenting with end stage renal failure [28-29]. Although the aetiology of type1 and type2 diabetes are notably distinct, glucose-evoked changes in the kidney are almost indistinguishable and often lead to a loss of renal function prompting the need for dialysis or transplantation. Diabetic nephropathy is characterised by structural and functional changes, specifically in the glomerulus, tubulointerstitium and vasculature, where glycaemic injury encompasses structural abnormalities ranging from hypertrophy, thickening of the glomerular basement membrane, tubular atrophy and interstitial fibrosis [30]. Ultimately, these changes contribute to increased glomerular filtration rate, proteinuria, systemic hypertension and overall, loss of renal function [30].

Our knowledge of connexin-mediated cell-to-cell communication in the kidney is limited. Whilst studies on renal vasculature have attributed a role for connexins in the regulation of blood pressure [31-34], we lack a basic understanding of the effects of connexin-mediated cell communication and associated purinergic signalling in tubular epithelia. Whilst a link between connexins and renal damage has recently emerged, how connexins contribute to the underlying pathology of chronic kidney disease remains to be confirmed. Of the known 21 mammalian connexin isoforms, only nine are expressed within the human kidney (CX26, CX30.3, CX31, CX32, CX37, CX40, CX43, CX45 and CX46 [reviewed in 35]). A summary of their physiological functions in the kidney are listed in Table 1.

Of the CX isoforms, high levels of expression of CX37, CX40 and CX43 are found in endothelial cells of the rat renal afferent arteriole, whilst post-glomerular endothelial cells of the efferent arteriole appear to express only CX43 [35]. The expression and localisation of particular subtypes can change. In the streptozotocin (STZ) treated mouse model of type1 diabetes, CX40 expression in smooth muscle cells of the afferent arteriole and inside the glomerulus increases, whilst endothelial expression of CX43 is decreased in the efferent arterioles [36-37]. These changes probably reflect the role of CX40 in regulating renin-dependent hypertension. Located in the juxtaglomerular

apparatus of the kidney, and part of the renin-angiotensin-aldosterone system involved in regulating blood pressure, renin-secreting cells are highly electrically coupled via CX40 mediated gap junctions [38-39]. Chronic changes in blood pressure necessitate recruitment of new renin-secreting cells from transformed smooth muscle cells associated with the pre-glomerular arterioles. This transformation is accompanied by increased expression of CX40 at the expense of CX45 [40]. Enhanced CX40 expression is thought to increase cell-to-cell coupling between renin-secreting cells and their neighbours, including endothelial, smooth muscle and mesangial cells [40]. Loss of function defects in CX40 causes translocation of renin-secreting cells from the media layer of the afferent arteriole into the periglomerular interstitium. This translocation is thought to reduce negative feedback control by high blood pressure on renin secretion and further exacerbate hypertension. Although apparently counter-intuitive, these findings have been elegantly demonstrated in the CX40 knockout mouse model ($CX40^{-/-}$), which develops hypertension [41]. Reasons for the shift of renin-secreting cells in the absence of CX40 and the subsequent loss of blood pressure regulation in response to enhanced renin secretion are unknown, however the work of Machura K *et al.* (2015), suggests that re-localisation of cells probably arises as a consequence of disrupted connexin-mediated cell communication rather than enhanced activation of the renin angiotensinogen system [40]. Moreover, recent work by Haefliger JA *et al.* demonstrates that induction of renin-dependent hypertension, induced by clipping 1 renal artery in the 2-kidney, 1-clip (2K1C) model, is associated with increased aortic expression of CX43 [42]. A follow up study by the same group, was later to reveal that replacement of CX43 by CX32 is associated with decreased expression and secretion of renin, negating renin-dependent hypertension as previously observed in the wild-type 2K1C model [43]. Contrary to these findings, results from mice exhibiting deleted CX43 expression in both the endothelium and renin-secreting cells [44] fail to confirm a role for CX43 in either renin secretion or expression in mice maintained on a normal, or low salt diet ± an angiotensin I-converting enzyme inhibitor [44].

In addition to a role for CX40 and CX43, connexin 37 has also been shown to be localised to renin-secreting cells and has also been linked to regulation of blood pressure. Studies using normotensive wild-type and CX37-deficient ($CX37^{-/-}$) mice confirmed that the knockout mice were less hypertensive than their wild-type counterparts when infused with Angiotensin II (Ang II) over a 2-4-week period. Further clarification using the 2K1C renin-dependent model of hypertension, confirmed that $CX37^{-/-}$ mice rapidly recover a normal blood pressure, in spite of increased plasma renin levels. In contrast, in the renin-independent model of hypertension, mice remained hypertensive, suggesting that loss of CX37 most likely influences expression of proteins implicated in the Ang II pathway, notably the Angiotensin II receptor type II (AT2R) [45]. Moreover, studies using Ang II treated mesangial cells and an Ang II-induced mouse model of hypertension, confirmed renal damage as induced by activation of

several metabolic pathways, including increased CX43 expression which appeared dependent on activation of the RhoA/Rho-associated protein kinase (ROCK) pathway. Interestingly, incubation with an inhibitor of the RhoA/ROCK pathway; Fasudil, 2 weeks prior to the 4-week treatment time point returned CX43 expression levels to near basal and reduced the level of renal damage as observed to wild-type mice. However, mice remained hypertensive. Collectively, these data suggest a role for CX37 and CX43 in Ang II-induced renal damage [46].

Nitric oxide (NO) has been linked to the pathogenesis of disease in early diabetes [47-49]. Although intra-renal NO production is increased in the early phases of diabetic nephropathy, a progressive decline in NO production, specifically NO bioavailability in the kidney, is observed with advanced renal failure [50]. To decipher the interplay between connexins and endothelial nitric oxide synthase (eNOS), a nitric oxide synthase responsible for NO generation, a recent study by Le Gal L *et al.* utilised CX40-null mice combined with either the 1-Kidney, 1-clip (1K1C) procedure (a model of volume dependent hypertension), or the 2-kidney, 1-clip (2K1C) procedure (a model of renin-dependent hypertension) [26]. In wild-type 1K1C mice, interactions between CX40, CX37 and eNOS were enhanced resulting in increased NO release. However, in mice lacking CX40, eNOS levels were decreased and an interaction between CX40 and eNOS was absent [26]. These data strongly suggest a role for CX40 mediated cell communication in regulation of NO synthesis, the effect of which manifests itself through loss of NO-induced vasodilation, and has been observed in other secondary complications of diabetes [51]. An additional role for NO in regulation of gap junction mediated intercellular communication (GJIC) in the mesangium has also been proposed. Yao *et al.* recently confirmed that increased NO augments CX43 mediated GJIC via protein kinase A [52]. Their observations are the first to suggest that diminished NO may, in part, mediate loss of CX43 mediated cell communication in the mesangium in diabetic nephropathy. The effects on overall mesangial cell function are yet to be clarified, however studies using the Zucker Lean and Zucker Diabetic Fat rat model of type2 diabetes, confirm both increased phosphorylation and inactivation of CX43 and reduced CX37 expression in renin-secreting cells, changes which were subsequently matched to increased glomerular filtration rate (GFR) and impaired auto-regulation [53].

Secondary to changes in the renal vasculature, an early hallmark of diabetic nephropathy is glomerulopathy, an important structural change characterised by thickening of the glomerular basement membrane, mesangial expansion, and development of morphological lesions in the arterioles, tubules and interstitium. Mesangial expansion is the structural parameter that best correlates with GFR and is also closely related to the presence of proteinuria and hypertension [54-55]. Glomerular mesangial cells are highly coupled by CX43 containing gap junctions and various studies have examined a role for glucose in regulation of this protein and associated downstream

signalling molecules. The data provide a more detailed insight into how glucose-evoked alterations in renal connexins may, in part, contribute to loss of cell function. Furthermore, loss of CX43 expression has been linked to the G1 (senescence) phase of the cell cycle, a state of arrested cell growth linked to mesangial cell hypertrophy and accumulation of the extracellular matrix [56]. The effect was reversed when CX43 was over-expressed and the PTEN/Akt/mTOR pathway activated [57]. These data have been corroborated by Ya-Nan-Guo *et al*, who suggested that AMP-activated protein kinase (AMPK) mediated inhibition of mTOR, negates the loss of CX43 mediated signalling [58].

Knowledge of how connexins mediate their effects through interaction with associated downstream signalling molecules is improving, as is our understanding of how connexin expression/function becomes compromised in disease. Connexins are multifunctional proteins that contribute to a large number of cell functions including, proliferation, cell adhesion and cell migration. Phosphorylated on tyrosine residues Tyr265 and Tyr247 by the non-receptor tyrosine kinase c-Src, phosphorylation of CX43 inactivates the channel and reduces GJIC [59-60]. Elevated in patients with diabetes, c-Src has been linked to the pathogenesis of diabetic nephropathy [61]. Studies by Xie *et al.* confirm that inhibition of c-Src, not only attenuates the up-regulation of glucose-induced intercellular adhesion molecule-1 (ICAM-1), transforming growth factor-beta 1 (TGF-β1) and fibronectin expression in glomerular mesangial cells, but it also promotes Nuclear Factor-kappa B (NFκB) activation, leading to renal inflammation. These effects were negated when CX43 was over-expressed [62]. Whilst NFκB is increased in kidneys of STZ-diabetic rats and glucose treated glomerular mesangial cells, the mechanisms by which this transcription factor mediates its effects remain elusive. The small GTPase RhoA, is a member of the Ras superfamily [63]. Rho-associated protein kinase (ROCK) is a serine/threonine kinase and downstream target of RhoA. Signalling via RhoA/ROCK regulates multiple cell functions, including cell migration, contraction, adhesion, gene expression and cell cycle progression. Furthermore, RhoA/ROCK signalling has been linked to the pathogenesis of diabetic nephropathy, with ROCK inhibitors Y27632 and Fasudil negating the accumulation extracellular matrix and preventing a loss of renal function [64]. In addition to its role in controlling deposition of fibrotic material and up-regulation of pro-fibrotic factors in the kidney, recent studies by Xie *et al.* identified that low levels of CX43 induce NFκB activation in response to high glucose [65]. They reported that up-regulation of CX43 inhibited the nuclear translocation of the NFκB p65 subunit, an effect attenuated when RhoA/ROCK signalling was blocked. Furthermore, increased F-actin accumulation and an enhanced association between CX43 and tight junction protein zona occludens-1 (ZO-1) in glucose treated cells was observed [65]. Xie *et al.* concluded that activated RhoA/ROCK signalling mediates CX43 degradation and nuclear translocation of NFκB p65, by promoting association between ZO-1 and CX43, which ultimately triggers CX43 endocytosis and NFκB activation in glucose treated cells, subsequently resulting in inflammation. These studies confirm that high

glucose impairs CX43 expression, with subsequent cell cycle arrest, inhibition of proliferation, promotion of protein synthesis, hypertrophy of the mesangium and NFκB mediated inflammation. Whilst these findings suggest that targeting CX43 may protect against mesangial cell hypertrophy and expansion in the diabetic kidney [65], it is important to remember that mesangial cells and their matrix constitute the main body of the glomerulus and work closely with both neighbouring podocytes and endothelial cells in ensuring appropriate glomerular function is maintained [66]. Consequently, alterations to either structural or behavioural characteristics will undoubtedly impinge on the behaviour of other cell types and may contribute to initial diagnosis of nephropathy as evidenced by detection of albuminuria [67].

Located within the glomerulus, podocytes have long cytoplasmic processes, which extend from the main cell body and divide into individual foot processes (pedicels). In healthy kidneys, the distance between these adjacent foot processes varies from 25nm to 60nm, a gap bridged by a thin membrane more commonly referred to as the "slit diaphragm". Initiated in response to podocyte injury, breakdown of this selective filtration barrier results in albumin appearing in the urine [68]. Although we lack definitive information of how connexins are regulated within podocytes, evidence suggests that these trans-membrane proteins may represent viable markers for assessment of early podocyte damage in diabetic kidney disease. Connexin 43 is expressed on podocytes in both normal and diseased kidney [69-70], and a role for connexins in podocyte injury has been suggested. Studies by Yaoita E *et al.*, demonstrated increased expression of CX43 in the early nephrotic stage of puromycine aminonucleoside (PAN) induced nephrosis [71], effects mediated, in part, by generation of superoxide and NADPH oxidase 4 (NOX4) [71]. Corroboration for the role for CX43 as a marker of podocyte damage has been further provided by Sawai *et al.,* who demonstrated that expression of CX43 in renal biopsies and podocytes from patients with overt nephropathy, was reduced. This loss of expression would impair podocyte cell communication and contribute to the loss of barrier function and ultimately albuminuria [72]. Furthermore, they hypothesised that altered association between CX43, tight junctions and cytoskeletal proteins, are responsible for disrupting the orientation of slit diaphragm components, thus contributing to loss of the selective filtering process. The findings support the use of CX43 as a potential marker in assessing both podocyte damage and renal function in diabetic nephropathy.

Whilst the structural and functional disturbances discussed above are synonymous with early stages of diabetic nephropathy, tubulointerstitial fibrosis represents the final common pathway of chronic renal failure and exhibits a positive correlation to the onset of end-stage renal disease and entry onto the renal transplantation programme [73]. Recent findings from our laboratory confirm that tubular CX43 and CX26 expression are increased in biopsy material from people with diabetic nephropathy

[74], and work by Abed A *et al.* reported that patients with chronic kidney disease (CKD) exhibit elevated levels of CX43. Using a mouse model of hypertension-induced CKD, they demonstrated decreased expression of cell adhesion markers, reduced monocyte infiltration and interstitial renal fibrosis when CX43 expression was reduced by 50%. Functional and histological parameters such as glomerulosclerosis and albuminuria were also reduced [75]. Although not a model of glycaemic injury, these data, along with our *in vitro* findings, suggest that aberrant CX43 mediated cell communication may have implications for the pathology of multiple forms of CKD via a common pathway.

How aberrant CX mediated communication impacts on cell function forms the basis of our ongoing studies. In early tubulointerstitial fibrosis, exposure to glucose and downstream pro-fibrotic cytokines initiates a series of events beginning with altered expression of the epithelial adhesion protein E-cadherin [74][76]. Cadherins help form the multi-protein adherens-junction that links cell-to-cell contact to the actin cytoskeleton and associated signalling molecules. It is therefore unsurprising that intercellular adhesion is a pre-requisite for connexin oligomerisation and gap junction formation. The loss of cell adhesion is the driving force behind morphological and phenotypic changes associated with early tubular injury [74], and has been linked to glucose-evoked changes in connexin expression and GJIC [74]. Although more than a dozen fibrogenic factors affect renal function, it is widely recognised that TGF-β1 and downstream Smad signalling, represent the key pathway orchestrating renal fibrosis in diabetic nephropathy [reviewed in 77-78]. Recent studies from our laboratory, confirm that TGF-β1 evoked changes in E-cadherin mediated cell adhesion facilitate changes in CX43 expression in human proximal tubule cells via a Smad dependent signalling [5]. Whilst the functional loss in cell tethering was accompanied a loss of CX43 GJIC at both 48hrs and 7days, hemi-channel mediated ATP release was increased [5].

In addition to early changes that initiate injury in the proximal region, one of the hallmarks of tubulointerstitial fibrosis is the accumulation of the extracellular matrix (ECM) in the tubular interstitium. Under physiological conditions, increased ECM deposition is associated with wound healing [79] and thus a tight balance between synthesis and breakdown of matrix proteins ensures that once the wound is closed, further ECM production ceases. Loss of this regulation can tip the balance from repair to injury, culminating in a build-up of fibrotic material, scar formation and in the kidney, a decline in excretory function [80-82]. As a mechanical framework that provides physical support for cells, the ECM has a crucial role in regulating cellular behaviour, e.g. gene expression, cell adhesion and cell communication [83-84]. Predominantly composed of laminin, collagen, fibrinogen and fibronectin, ECM proteins can interact with cell surface receptors called integrins. Association not only provides an anchor between the cytoskeleton and the cells immediate environment, to enable traction and cell movement, but also directly regulates cell signalling. Whilst

connexin mediated cell communication is essential for cell survival and function, cell-ECM interactions facilitate the activation of growth factors and cell differentiation. Interestingly, modification and remodelling of the ECM in disease can severely impact on function, with recent studies identifying a link between high glucose, ECM remodelling and disrupted GJIC in multiple disease states, including secondary complications of diabetes [85][86]. Here, remodelling of the ECM occurs in response to prolonged alterations in the activity of matrix metalloproteinases (MMPs), zinc-dependent endopeptidases that control both synthesis and degradation of ECM components. Recent studies have confirmed that increased MMP9 induces ECM remodelling, an event linked to a down-regulation of hydrogen sulphide (H_2S) [87]. Low H_2S is associated with vascular inflammation and renal complications in diabetes [88]. It is correlated to decreased expression of CX40 and CX43 in the diabetic kidney which, along with ECM remodelling and dysregulated MMP expression, can be negated in the Akita mouse treated with NaHS, a soluble source of H_2S provided in drinking water [89]. Administration of exogenous H_2S improves renal function and reduces the degree of ECM remodelling [90][91]. Although the link between connexins and ECM remodelling in the diabetic kidney is just coming to light, our knowledge of the area in retinopathy, combined with increasing awareness of these changes in the kidney, highlights a link between connexins and ECM remodelling in diabetic kidney disease.

ATP and purinergic signalling in the diabetic kidney.

Adenosine triphosphate is a ubiquitous source of energy found in all cells. Usually present in low millimolar concentrations [92], decreases in intracellular ATP are synonymous with cell death and the dramatic release of ATP into the extracellular environment is often associated with cell injury triggered by stressors including, inflammation, hypoxia or mechanical deformation. In addition to its role as an energy supply and marker of cell damage, the controlled release of ATP and associated purines also act as autocrine/paracrine extracellular messengers capable of binding to purinoreceptors on adjacent cells to help propagate cellular activity.

In the kidney, ATP is released from a variety of sources including, erythrocytes, endothelial cells, aggregating platelets and the basolateral and apical membrane of epithelial cells [93]. With a wealth of information attributing dysregulated connexin expression to the effects of hyperglycaemia, recent studies further highlight a link between hemi-channel mediated ATP release and the progression and development of fibrosis in multiple tissue types, including the diabetic kidney [94-95] where hemi-channel mediated ATP release may, in part, contribute to the pathology of diabetic nephropathy.

Purinergic signals bind to one of 3 receptor subclasses, the metabotropic P1 receptors, the P2Y metabotropic receptors or the P2X ionotropic receptors [96]. The P2 class of receptors can be further classified into either P2X ($P2X_{1-7}$) or P2Y ($P2Y_{1, 2, 4, 6, 11-14}$). Activated in response to various agonists, P2Y receptors couple to either cAMP or inositol triphosphate (IP_3) messenger systems, with each subtype functionally linked to different G-proteins evoking downstream activation of multiple signalling cascades. The precise location of specific receptor subtypes able to respond to these local signals is unclear. Some, including the $P2X_7$ receptor, appear up-regulated in various diseases [97-102], including diabetic nephropathy, where renal $P2X_7R$ expression has been found to be associated with severe mesangial expansion, impaired glomerular filtration (≤40ml/min/1.73sq.m.) and increased interstitial fibrosis in diabetic patients [103]. Furthermore, activation of $P2X_7R$ increased MCP-1 release in human mesangial cells when cultured under high glucose [103].

Expression of $P2X_7$ in kidneys from streptozotocin (STZ) induced diabetic rats, is elevated in glomerular podocytes, mesangial cells and endothelial cells [104]. High levels of $P2X_7$ mRNA in rat glomerulonephritis has been linked to increased IL-1β mRNA and exacerbated glomerular damage [105]. More recently, Vieira *et al.*, suggested that $P2X_7$ mediated inflammation and fibrosis in response to glycaemic injury in diabetic kidney disease [105]. In this elegant study, they demonstrated that $P2X_7$ deficient mice (-/-) are resistant to STZ-induced diabetes and fail to exhibit any alterations to blood glucose levels or pancreatic islet reduction compared to wild-type C57BL/6 mice. Not surprisingly, levels of pro-inflammatory mediators, including IL-1β, IFN-γ and NO, were unaltered in $P2X_7^{-/-}$ animals following STZ treatment. Using a $P2X_7$ antagonist, wild-type C57BL/6 mice exhibited resistance to STZ treatment. Pharmacologically blocking the receptor, or deleting its expression, is linked to a reduction in renal macrophage activity and subsequently protects against antibody-mediated glomerular inflammation [106, 107]. These data have been supported by Menzies *et al.* in a mouse model of diabetic nephropathy, which when treated with a $P2X_7R$ inhibitor (AZ11657312) exhibit reduced renal macrophage accrual [108].

Efficient and effective purinergic signalling is dependent on a balance between ATP release and breakdown. Ectonucleotidases are a class of enzymes, which metabolise nucleotides to nucleosides [109]. The contribution of ectonucleotidases in the modulation of purinergic signalling depends on the availability and preference of substrates and on the cell and tissue distribution. Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), otherwise referred to as CD39, is a human membrane-bound protein, which hydrolyses ATP and ADP to AMP, thus regulating P2Y ligand availability [110]. Studies on the CD39 null mice reveal a large degree of renal damage in the form of glomerulosclerosis [111]. The mice present with increased proteinuria, increased GFR and increased

expression of monocyte chemo-attractant protein-1 (MCP-1), all of which were more severe than changes observed in aged matched diabetic animals. The data suggests a potential protective role for the hydrolysing enzyme in glomerular inflammation. Genetic deletions of the adenosine A_{2B} receptor and CD73 (an enzyme involved in the extracellular production of adenosine) in mice, was found to induce renal nephropathy [78][110]. Studies examining a role for ATP in mesangial cell proliferation and expansion, contributory factors in the instigation of glomerulosclerosis, confirmed that ATP induced mesangial cell proliferation was mediated by P2Y receptor dependent activation of the Ras-Raf-MAPK signal transduction pathway [112] and $P2Y_4$ [113]. Recently, a role for $P2X_7$ in modulating the microvasculature response [114] and renal metabolism of extracellular adenine nucleotides in diabetic rats [114] has been examined. Findings confirmed, that in kidneys obtained from STZ-induced diabetic mice, increased levels of adenosine, ENTDPase and 5'-nucleotidase paralleled decreased levels of ATP in the renal interstitial fluid. More importantly, the effects were abolished by A438079, a $P2X_7$ receptor antagonist [114].

In tubulointerstitial fibrosis , early tubular injury occurs in response to changes in adhesion, and cell-substrate interactions [30]. Adhesiveness between cells and between cells and their surrounding substrate/ECM-matrix, allows them to adapt to their immediate environment [reviewed in 115]. Not surprisingly in diabetic nephropathy, loss of cell adhesion, increased synthesis of ECM proteins and ECM remodelling have all been linked to a loss of epithelial phenotypic stability and increased fibrosis in the proximal tubular region of the kidney [30]. Our recent studies identified that a switch in direct gap junction intercellular communication accompanies a loss of E-cadherin mediated cell adhesion and increased hemi-channel mediated ATP release. Linked to inflammation and fibrosis, incubation of proximal tubule epithelial cells with non-hydrolysable ATPγS confirmed an increase in expression of the pro-inflammatory and pro-fibrotic mediators interleukin-6 (IL-6) and fibronectin. Similarly, the ectonucleotidase apyrase, negated the IL-6 and fibronectin response to TGFβ1 at both 48hrs and 7days [5]. In addition, a direct role for ATP in ECM remodelling has been suggested by studies in rat mesangial cells demonstrating increased $P2X_4$ induced activation of the NOD-like receptor 3 (NLRP3) inflammasome, increased release of interleukins IL-1β and IL-18 and the development of tubulointerstitial inflammation [116, 117]. High glucose increased expression of NLRP3 inflammasome and interleukin IL-1β in human kidney (HK2) proximal tubular epithelial cells, with increased secretion of both interleukins IL-1β and IL-18. The effects were negated when cells were co-incubated with apyrase. Confirmation of the purinoreceptor that mediated these effects was provided when NLRP3 expression and IL-1β and IL-18 release was attenuated by co-incubating cells with the general P2X receptor antagonist TNP-ATP, or the more selective $P2X_4$ antagonist 5-BDBD, or through gene silencing of $P2X_4$ [118]. The data suggest that ATP-$P2X_4$ signalling mediates high

glucose-induced activation of the NLRP3 inflammasome, regulates IL-1 family cytokine secretion, and ultimately instigates tubulointerstitial inflammation in diabetic nephropathy. These are important findings since patients with type2 diabetes and nephropathy reportedly exhibit elevated receptor expression of $P2X_4$, $P2X_7$ and NLPR3 expression [119]. Links to the $P2X_7$ receptor have also been provided in studies using the $P2X_7$ receptor knockout mouse model of unilateral ureteral obstruction (UUO), in which animals present with progressive renal fibrosis, marked renal hemodynamic and metabolic changes, tubular injury and cell death [118]. These mice exhibit reduced myofibroblast number, collagen deposition, tubular apoptosis and macrophage infiltration. Collectively the data suggest a role for the $P2X_7$ receptor in progression of inflammation and fibrosis. In a similar study, Solini *et al.* confirmed that rat mesangial cells (RMCs) cultured in high (30mmol/L) glucose exhibit increased extracellular ATP [119]. Under both low and high glucose, exogenous application of ATP and the $P2X_7$ receptor agonist benzoylbenzoyl ATP, evoked a concentration dependent increase in the ECM markers, fibronectin, collagen IV, laminin and TGFβ. The effects were negated when RMCs cultured in high glucose were co-incubated with apyrase. Interestingly, extracellular matrix and TGFβ production remained unaltered in response to apyrase treatment in cells cultured under low glucose. A role for $P2X_7$ in mediating fibrotic changes was suggested by co-incubating with a $P2X_7$ inhibitor, which attenuated glucose-induced increases in ECM and TGFβ [119].

Aside to a potential role for hemi-channel mediated ATP release and dysregulated purinergic signalling in the early portion of the nephron, recent studies by Ponnusamy *et al.* suggest a role for nucleotides in P2R-mediated cross talk between epithelial cells and fibroblasts [120], whilst studies by Wolff *et al.* suggest a role for $P2Y_2$ receptors in TGF-β1 induced EMT in Madin Darby Kidney Cells (MDCK) [121].

Lastly, whilst a definitive link between aberrant hemi-channel mediated ATP release and the underlying pathology of diabetic nephropathy remains to be confirmed, studies utilising models of glomerulosclerosis and advanced interstitial inflammation/fibrosis (UUO), pathologies associated with diabetic kidney disease, have used pharmacological and genetic strategies to assess the implications of aberrant Cx43 mediated communication. In a model of glomerular damage, Kavvadas *et al.* confirmed increased *de novo* expression of Cx43 in podocytes, which led to cell damage and deterioration of renal function, a response which was blunted in heterogenous mice in which Cx43 expression had been genetically repressed ($Cx43^{+/-}$). More importantly, pharmacogenetic inhibition of Cx43 delayed renal structural and functional damages in mice suffering from severe GN and in cultured podocytes treated with TGF-β1 [122]. In addition and in confirming a role for Cx43 in the progression of CKD, administration of a Cx43 antisense in a mouse model of UUO attenuated E-

cadherin down-regulation and phosphorylation of the transcription factor Sp1 by the ERK pathway, resulting in decreased transcription of the type I collagen gene. Furthermore, Cx43-hemi-channel specific blocking peptide Gap19 inhibited monocyte adhesion in activated endothelium and pro-fibrotic pathways in tubular cells, thus suggesting that modulation of both connexin-mediated cell communication and purinergic signalling may represent a novel approach to preventing or attenuating fibrosis in the diabetic kidney.

Conclusion

To ensure efficient renal function is maintained, cells of the nephron synchronise their activity and coordinate function within different regions of the nephron through the use of connexins. Cells communicate to one another either directly, via gap junction mediated intercellular communication, or via a hemi-channel mediated paracrine release of nucleotides in to the immediate extracellular environment. In diabetic nephropathy, these small membrane-bound proteins play a vital role in orchestrating an integrated functional response against fibrotic, osmotic and metabolic assault. In the current article, we review growing evidence to suggest that dysregulated connexin mediated cell communication and altered purinergic signalling may be pivotal in the aetiology and pathogenesis of diabetic nephropathy. Our understanding of the complex interplay between connexins, nucleotide release and downstream purinergic signalling in hyperglycaemia remains rudimentary, however, it is clear that connexins offer a viable future target in the therapeutic control and treatment of this chronic metabolic condition.

Declarations of interest:

The authors confirm that they have no conflict of interest

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Figure legends

Figure 1. Connexin-mediated intercellular communication. Hemi-channels form the hexameric arrangement (connexon) of membrane bound proteins called connexins. Hemi-channels permit the efflux of small molecules (e.g. ATP) in to the intercellular micro-environment immediately surrounding cells. These local paracrine signals can be detected by receptors on adjacent cells, e.g. P2Y receptors, which help propogate and synchronise activity across cell clusters. When aligned with similar channels on neighbouring cells, hemi-channels form continuous pores or gap junction that facilitate gap-junctional intercellular communication (GJIC) of small molecules and ions.

Cx Isoform	Physiological Function
26	Tubular localisation determined but function still to be determined
30	Tubular release of ATP to regulate salt and water reabsorption in the distal nephron
37	Vascular-conducted responses, in particular arteriolar vasoconstriction
	Endothelium-derived vasodilation, via production of eNOS or hyperpolarisation
	Renal autoregulation, via tubuloglomerular feedback
	Possible tubular function, regulation by salt
40	Vascular-conducted responses, in particular arteriolar vasodilation
	Endothelium-derived vasodilation via inhibition of endothelium-derived hyperpolarising factor, production of eNOS
	Renal autoregulation via tubuloglomerular feedback and myogenic responses
	Blood pressure control via regulation of renin secretion
43	Endothelium-derived vasodilation via inhibition of endothelium-derived hyperpolarising factor, possibly via production of eNOS
	Renal autoregulation through tubuloglomerular feedback and myogenic responses
	Blood pressure control, although mechanisms are still disputed
	Tubular function, dysregulation of which is associated with fibrosis and inflammation
45	Partial renal autoregulatory function to compensate for loss of Cx40

Table 1: The physiological role of connexins in the kidney

References:

[1] Bosco D, Haefliger JA, Meda P. Connexins: key mediators of endocrine function. Physiological Reviews. 91(4):1393-445, 2011

[2] Wang N, De Bock M, Decrock E, Bol M, Gadicherla A, Vinken M, Rogiers V, Bukauskas FF, Bultynck G, Leybaert L. Paracrine signalling through plasma membrane hemichannels. Biochimica et Biophysica Acta (BBA)-Biomembranes. 31;1828(1):35-50, 2013

[3] Burnstock G, Evans LC, Bailey MA. Purinergic signalling in the kidney in health and disease. Purinergic Signalling. 10(1):71-101, 2014

[4] Sáez JC, Leybaert L. Hunting for connexin hemi-channels. FEBS Letters. 17;588(8):1205-11, 2014

[5] Hills C, Price GW, Wall MJ, Kaufmann TJ, Chi-Wai Tang S, Yiu WH, et al.: Transforming Growth Factor Beta 1 Drives a Switch in Connexin Mediated Cell-to-Cell Communication in Tubular Cells of the Diabetic Kidney. Cell Physiol Biochem 45(6): 2369-2388, 2018

[6] Dosch M, Zindel J, Jebbawi F, Melin N, Sanchez-Taltavull D, Stroka D, et al.: Connexin-43-dependent ATP release mediates macrophage activation during sepsis. Elife. 8: e42670, 2019

[7] Becker LV, Passos DF, Leal DBR, Morsch VM, Schetinger MRC: ATP signaling and NTPDase in Systemic Lupus Erythematosus (SLE). Immunobiology. 224(3)419-4262019, 2019

[8] Kuo C, Green CR, Rupenthal ID, Mugisho OO: Connexin43 hemichannel block protects against retinal pigment epithelial cell barrier breakdown. Acta Diabetol. doi: 10.1007/s00592-019-01352-3, 2019

[9] Hernández-Guerra M, Hadjihambi A, Jalan R: Gap junctions in liver disease: Implications for pathogenesis and therapy. J Hepatol 70(4): 759-772, 2019

[10] Sáez JC, Contreras-Duarte S, Gómez GI, Labra VC, Santibañez CA, Gajardo-Gómez R, et al.: Connexin 43 Hemichannel Activity Promoted by Pro-Inflammatory Cytokines and High Glucose Alters Endothelial Cell Function. Front Immunol 9: 1899, 2018

[11] Laird DW, Lampe PD: Therapeutic strategies targeting connexins. Nat Rev Drug Discov 17: 905-921, 2018

[12] Neuen BL, Chadban SJ, Demaio AR, Johnson DW, Perkovic V: Chronic kidney disease and the global NCDs agenda. BMJ Glob Health 2(2): e000380, 2017

[13] Rossing P, Frimodt-Møller M: Diabetic Nephropathy, Pathophysiology and Clinical Aspects pp 21–32, 2018

[14] Oyamada M, Takebe K, Oyamada Y. Regulation of connexin expression by transcription factors and epigenetic mechanisms. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1828(1):118-33, 2013

[15] Laird DW. Life cycle of connexins in health and disease. Biochemical Journal. 394(3):527-43, 2006

[16] Oshima A. Structure and closure of connexin gap junction channels. FEBS Letters. 588(8):1230-7, 2014

[17] Kronengold J, Srinivas M, Verselis VK. The N-terminal half of the connexin protein contains the core elements of the pore and voltage gates. The Journal of Membrane Biology. 245(8):453-63, 2012

[18] Lampe PD, Lau AF. The effects of connexin phosphorylation on gap junctional communication. The International Journal of Biochemistry & Cell Biology. 36(7):1171-86, 2004

[19] Nielsen MS, Nygaard Axelsen L, Sorgen PL, Verma V, Delmar M, Holstein‐Rathlou NH. Gap junctions. Comprehensive Physiology. 2(3):1981-2035, 2012

[20] Gordon JL. Extracellular ATP: effects, sources and fate. Biochemical Journal, 233(2):309, 1986

[21] Burnstock G. Purinergic signalling: past, present and future. Brazilian Journal of Medical and Biological Research. 42(1):3-8, 2009

[22] Wang N, De Bock M, Decrock E, Bol M, Gadicherla A, Vinken M, Rogiers V, Bukauskas FF, Bultynck G, Leybaert L. Paracrine signaling through plasma membrane hemichannels. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1828(1):35-50, 2013

[23] Rhett JM, Yeh ES. The Potential for Connexin Hemichannels to Drive Breast Cancer Progression through Regulation of the Inflammatory Response. Int J Mol Sci. 19(4), 2018

[24] Tsuchida S, Arai Y, Kishida T, Takahashi KA, Honjo K, Terauchi R, Inoue H, Oda R, Mazda O, Kubo T. Silencing the expression of connexin 43 decreases inflammation and joint destruction in experimental arthritis. Journal of Orthopaedic Research. 31(4):525-30, 2013

[25] Sahu G, Bera AK. Contribution of intracellular calcium and pH in ischemic uncoupling of cardiac gap junction channels formed of connexins 43, 40, and 45: a critical function of C-terminal domain. PloS One. 8(3):e60506, 2013

[26] Le Gal L, Alonso F, Wagner C, Germain S, Haefliger DN, Meda P, Haefliger JA. Restoration of connexin 40 (Cx40) in renin-producing cells reduces the hypertension of Cx40 null mice. Hypertension. 63(6):1198-204, 2014

[27] Firouzi M, Kok B, Spiering W, Busjahn A, Bezzina CR, Ruijter JM, Koeleman BP, Schipper M, Groenewegen WA, Jongsma HJ, de Leeuw PW. Polymorphisms in human connexin40 gene promoter are associated with increased risk of hypertension in men. Journal of Hypertension. 24(2):325-30, 2006

[28] Koye DN, Magliano DJ, Nelson RG, Pavkov ME. The Global Epidemiology of Diabetes and Kidney Disease. Adv Chronic Kidney Dis. (2):121-132. 2018

[29] Diabetes UK. Statistics - Diabetes UK; 2012 [cited 2016 Feb 13]. Available from: https://www.diabetes.org.uk/About_us/What-we-say/Statistics/.

[30] Liu BC, Tang TT, Lv LL, Lan HY: Renal tubule injury: a driving force toward chronic kidney disease. Kidney Int 93(3): 568-579, 2018

[31] Hills CE, Price GW, Squires PE. Mind the gap: connexins and cell–cell communication in the diabetic kidney. Diabetologia. 58(2):233-41, 2015

[32] Oppermann M, Carota I, Schiessl I, Eisner C, Castrop H, Schnermann J. Direct assessment of tubuloglomerular feedback responsiveness in connexin 40-deficient mice. American Journal of Physiology-Renal Physiology. 304(9):F1181-6, 2013

[33] Gerl M, Vöckl J, Kurt B, van Veen TA, Kurtz A, Wagner C: Inducible deletion of connexin 40 in adult mice causes hypertension and disrupts pressure control of renin secretion. Kidney Int 87(3): 557-63, 2015

[34] Abed A, Dussaule JC, Boffa JJ, Chatziantoniou C, E Chadjichristos C. Connexins in renal endothelial function and dysfunction. Cardiovascular & Haematological Disorders-Drug Targets (Formerly Current Drug Targets-Cardiovascular & Hematological Disorders). 14(1):15-21, 2014

[35] Hanner F, Sorensen CM, Holstein-Rathlou NH, Peti-Peterdi J. Connexins and the kidney. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 298(5):R1143-55, 2010

[36] Zhang J, Hill CE. Differential connexin expression in preglomerular and postglomerular vasculature: accentuation during diabetes. Kidney International. 68(3):1171-85, 2005

[37] Zhang JH, Kawashima S, Yokoyama M, Huang P, Hill CE. Increased eNOS accounts for changes in connexin expression in renal arterioles during diabetes. The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology. 288(9):1000-8, 2006

[38] Krattinger N, Capponi A, Mazzolai L, Aubert JF, Caille D, Nicod P, Waeber G, Meda P, Haefliger JA. Connexin40 regulates renin production and blood pressure. Kidney International. 72(7):814-22, 2007

[39] Takenaka T, Inoue T, Kanno Y, Okada H, Meaney KR, Hill CE, Suzuki H. Expression and role of connexins in the rat renal vasculature. Kidney International. 73(4):415-22, 2008

[40] Machura K, Neubauer B, Müller H, Tauber P, Kurtz A, Kurtz L. Connexin 40 is dispensable for vascular renin cell recruitment but is indispensable for vascular baroreceptor control of renin secretion. Pflügers Archiv-European Journal of Physiology. 467(8):1825-34, 2015

[41] Krattinger N, Capponi A, Mazzolai L, Aubert JF, Caille D, Nicod P, Waeber G, Meda P, Haefliger JA. Connexin40 regulates renin production and blood pressure. Kidney International. 72(7):814-22, 2007

[42] Haefliger JA, Castillo E, Bergonzelli GE, Sutter E, Nicod P, Waeber B, Meda P. Hypertension increases connexin43 in a tissue-specific manner. Circulation. 18;95(4):1007-14, 1997

[43] Haefliger JA, Krattinger N, Martin D, Pedrazzini T, Capponi A, Döring B, Plum A, Charollais A, Willecke K, Meda P. Connexin43-dependent mechanism modulates renin secretion and hypertension. The Journal of Clinical Investigation. 116(2):405-13, 2006

[44] Gerl M, Kurt B, Kurtz A, Wagner C. Connexin 43 is not essential for the control of renin synthesis and secretion. Pflügers Archiv-European Journal of Physiology. 466(5):1003-9, 2014

[45] Gómez GI, Velarde V, Sáez JC Role of a RhoA/ROCK-Dependent Pathway on Renal Connexin43 Regulation in the Angiotensin II-Induced Renal Damage. Int J Mol Sci. 7;20(18), 2019

[46] Gonzalo I. Gómez, Paola Fernández, Victoria Velarde and Juan C. Sáez Angiotensin II-Induced Mesangial Cell Damage Is Preceded by Cell Membrane Permeabilization Due to Upregulation of Non-Selective Channels Int J Mol Sci. 19(4): 957, 2018

[47] Assmann TS, Brondani LA, Bouças AP, Rheinheimer J, de Souza BM, Canani LH, Bauer AC, Crispim D. Nitric oxide levels in patients with diabetes mellitus: A systematic review and meta-analysis. Nitric Oxide. 2016 Dec 30;61:1-9

[48] Prabhakar SS. Pathogenic role of nitric oxide alterations in diabetic nephropathy. Current Diabetes Reports. 5(6):449-54, 2005

[49] Awad AS, Webb RL, Carey RM, Siragy HM. Renal nitric oxide production is decreased in diabetic rats and improved by AT1 receptor blockade. Journal of Hypertension. 22(8):1571-7, 2004

[50] Mumtaz FH, Dashwood MR, Khan MA, Thompson CS, Mikhailidis DP, Morgan RJ. Down-regulation of nitric oxide synthase in the diabetic rabbit kidney: potential relevance to the early pathogenesis of diabetic nephropathy. Current Medical Research and Opinion. 20(1):1-6, 2004

[51] Cohen RA. Role of nitric oxide in diabetic complications. American Journal of Therapeutics. 12(6):499-502, 2005

[52] Yao J, Hiramatsu N, Zhu Y, Morioka T, Takeda M, Oite T, Kitamura M. Nitric oxide-mediated regulation of connexin43 expression and gap junctional intercellular communication in mesangial cells. Journal of the American Society of Nephrology. 16(1):58-67, 2005

[53] Takenaka T, Inoue T, Okada H, Ohno Y, Miyazaki T, Chaston DJ, Hill CE, Suzuki H. Altered gap junctional communication and renal haemodynamics in Zucker fatty rat model of type 2 diabetes. Diabetologia. 54(8):2192-201, 2011

[54] Dalla Vestra M, Saller A, Mauer M, Fioretto P. Role of mesangial expansion in the pathogenesis of diabetic nephropathy. Journal of Nephrology. 14:S51-7, 2000

[55] Wolf G. Molecular mechanisms of diabetic mesangial cell hypertrophy: a proliferation of novel factors. Journal of the American Society of Nephrology. 13(10):2611-3, 2002

[56] Zhang X, Chen X, Wu D, Liu W, Wang J, Feng Z, Cai G, Fu B, Hong Q, Du J. Downregulation of connexin 43 expression by high glucose induces senescence in glomerular mesangial cells. Journal of the American Society of Nephrology. 17(6):1532-42, 2006

[57] Liu L, Hu X, Cai GY, Lv Y, Zhuo L, Gao JJ, Cui SY, Feng Z, Fu B, Chen XM. High glucose-induced hypertrophy of mesangial cells is reversed by connexin43 overexpression via PTEN/Akt/mTOR signaling. Nephrology Dialysis Transplantation. 1:gfr265, 2011

[58] Guo YN, Wang JC, Cai GY, Hu X, Cui SY, Lv Y, Yin Z, Fu B, Hong Q, Chen XM. AMPK-mediated downregulation of connexin43 and premature senescence of mesangial cells under high-glucose conditions. Experimental Gerontology.51:71-81, 2014

[59] Giepmans BN, Hengeveld T, Postma FR, Moolenaar WH. Interaction of c-Src with Gap Junction Protein Connexin-43. Role in the regulation of cell-cell communication. Journal of Biological Chemistry. 276(11):8544-9, 2001

[60] Gilleron J, Fiorini C, Carette D, Avondet C, Falk MM, Segretain D, Pointis G. Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen. J Cell Sci. 121(24):4069-78, 2008

[61] Suzaki Y, Yoshizumi M, Kagami S, Nishiyama A, Ozawa Y, Kyaw M, Izawa Y, Kanematsu Y, Tsuchiya K, Tamaki T. BMK1 is activated in glomeruli of diabetic rats and in mesangial cells by high glucose conditions. Kidney International. (5):1749-60, 2004

[62] Xie X, Lan T, Chang X, Huang K, Huang J, Wang S, Chen C, Shen X, Liu P, Huang H. Connexin43 mediates NF-κB signalling activation induced by high glucose in GMCs: involvement of c-Src. Cell Communication and Signaling. 11(1):1, 2013

[63] Sit ST, Manser E. Rho GTPases and their role in organizing the actin cytoskeleton. Journal of Cell Science. 124(5):679-83, 2011

[64] Xie X, Peng J, Chang X, Huang K, Huang J, Wang S, Shen X, Liu P, Huang H. Activation of RhoA/ROCK regulates NF-κB signaling pathway in experimental diabetic nephropathy. Molecular and Cellular Endocrinology. 369(1):86-97, 2013

[65] Xie X, Chen C, Huang K, Wang S, Hao J, Huang J, Huang H. RhoA/rho kinase signaling reduces connexin43 expression in high glucose-treated glomerular mesangial cells with zonula occludens-1 involvement. Experimental Cell Research. 327(2):276-86, 2014

[66] Schlöndorff D, Banas B. The mesangial cell revisited: no cell is an island. Journal of the American Society of Nephrology. 20(6):1179-87, 2009

[67] Siddiqi FS, Advani A. Endothelial-podocyte crosstalk: the missing link between endothelial dysfunction and albuminuria in diabetes. Diabetes. 62(11):3647-55, 2013

[68] Brinkkoetter PT, Ising C, Benzing T. The role of the podocyte in albumin filtration. Nature Reviews Nephrology. 9(6):328-36, 2013

[69] Hillis GS, Duthie LA, Brown PA, Simpson JG, Macleod AM, Haites NE. Upregulation and co‐localization of connexin43 and cellular adhesion molecules in inflammatory renal disease. The Journal of Pathology. 182(4):373-9, 1997

[70] Hillis GS, Duthie LA, Mlynski R, McKay NG, Mistry S, MacLeod AM, Simpson JG, Haites NE. The expression of connexin 43 in human kidney and cultured renal cells. Nephron. 75(4):458-63, 1997

[71] Yaoita E, Yao J, Yoshida Y, Morioka T, Nameta M, Takata T, Kamiie JI, Fujinaka H, Oite T, Yamamoto T. Up-regulation of connexin43 in glomerular podocytes in response to injury. The American Journal of Pathology. 161(5):1597-606, 2002

[72] Sawai K, Mukoyama M, Mori K, Yokoi H, Koshikawa M, Yoshioka T, Takeda R, Sugawara A, Kuwahara T, Saleem MA, Ogawa O. Redistribution of connexin43 expression in glomerular podocytes predicts poor renal prognosis in patients with type 2 diabetes and overt nephropathy. Nephrology Dialysis Transplantation. 21(9):2472-7, 2006

[73] Hodgkins KS, Schnaper HW. Tubulointerstitial injury and the progression of chronic kidney disease. Pediatric nephrology. 27(6):901-9, 2012

[74] Siamantouras E, Hills CE, Squires PE, Liu KK.: Quantifying cellular mechanics and adhesion in renal tubular injury using single cell force spectroscopy. Nanomedicine 12(4): 1013-1021, 2016

[75] Abed A, Toubas J, Kavvadas P, Authier F, Cathelin D, Alfieri C, Boffa JJ, Dussaule JC, Chatziantoniou C, Chadjichristos CE. Targeting connexin 43 protects against the progression of experimental chronic kidney disease in mice. Kidney International. 86(4):768-79, 2014

[76] Hills CE, Siamantouras E, Smith SW, Cockwell P, Liu KK, Squires PE: TGFβ modulates cell-to-cell communication in early epithelial-to-mesenchymal transition. Diabetologia 55(3): 812-24, 2012

[77] Rauchman M, Griggs D: Emerging strategies to disrupt the central TGF-β axis in kidney fibrosis. Transl Res 209: 90-104, 2019

[78] Hills CE, Squires PE. The role of TGF-β and epithelial-to mesenchymal transition in diabetic nephropathy. Cytokine & Growth Factor Reviews. 22(3):131-9, 2011

[79] Hirschberg R. Wound healing in the kidney: complex interactions in renal interstitial fibrogenesis. Journal of the American Society of Nephrology. 16(1):9-11, 2005

[80] Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC. Structural-functional relationships in diabetic nephropathy. Journal of Clinical Investigation. 74(4):1143, 1984

[81] Steffes MW, Østerby R, Chavers B, Mauer SM. Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. Diabetes. 38(9):1077-81, 1989

[82] Mason RM, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. Journal of the American Society of Nephrology. 14(5):1358-73, 2003
83

[84] Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. Journal of cell science. 123(24):4195-200, 2010

[85] El-Sabban ME, Abi-Mosleh LF, Talhouk RS. Developmental regulation of gap junctions and their role in mammary epithelial cell differentiation. Journal of mammary gland biology and neoplasia. 8(4):463-73, 2003

[86] Moon J, Knack HP, Roy S. Effect of inhibiting high glucose-induced upregulation of ECM on connexin 43 (Cx43) expression in retinal endothelial cells. Investigative Ophthalmology & Visual Science. 52(14):3554, 2011

[87] Kundu S, Pushpakumar SB, Tyagi A, Coley D, Sen U. Hydrogen sulfide deficiency and diabetic renal remodeling: role of matrix metalloproteinase-9. American Journal of Physiology-Endocrinology and Metabolism. 304(12):E1365-78, 2013

[88] Szabo C. Roles of hydrogen sulfide in the pathogenesis of diabetes mellitus and its complications. Antioxidants & redox signaling. 17(1):68-80, 2012

[89] Kundu S, Pushpakumar S, Sen U. MMP-9-and NMDA receptor-mediated mechanism of diabetic renovascular remodeling and kidney dysfunction: hydrogen sulfide is a key modulator. Nitric Oxide. 46:172-85, 2015

[90] Zhou X, Feng Y, Zhan Z, Chen J. Hydrogen sulfide alleviates diabetic nephropathy in a streptozotocin-induced diabetic rat model. Journal of Biological Chemistry. 17;289(42):28827-34, 2014

[91] Ha H, Lee HB. Reactive oxygen species and matrix remodeling in diabetic kidney. Journal of the American Society of Nephrology. 14(s3):S246-9, 2003

[92] Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG, Ashcroft FM. A novel method for measurement of submembrane ATP concentration. Journal of Biological Chemistry. 275(39):30046-9, 2000

[93] Schwiebert EM, Zsembery A. Extracellular ATP as a signaling molecule for epithelial cells. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1615(1):7-32, 2003

[94] Lu D, Soleymani S, Madakshire R, Insel PA. ATP released from cardiac fibroblasts via connexin hemichannels activates profibrotic P2Y2 receptors. The FASEB Journal. 26(6):2580-91, 2012

[95] Ferrari D, Gambari R, Idzko M, Müller T, Albanesi C, Pastore S, et al. Purinergic signaling in scarring. The FASEB Journal. 30(1):3–12, 2016

[96] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacological reviews. 1;50(3):413-92, 1998

[97] Burnstock G, Knight GE: The potential of P2X7 receptors as a therapeutic target, including inflammation and tumour progression. Purinergic Signal 14: 1–18, 2018

[98] Koo TY, Lee JG, Yan JJ, Jang JY, Ju KD, Han M, et al.: The P2X7 receptor antagonist, oxidized adenosine triphosphate, ameliorates renal ischemia-reperfusion injury by expansion of regulatory T cells. Kidney Int 92(2): 415-431, 2017

[99] Arulkumaran N, Sixma ML, Pollen S, Ceravola E, Jentho E, Prendecki M, Bass PS, Tam FWK, Unwin RJ, Singer M. P2X7 receptor antagonism ameliorates renal dysfunction in a rat model of sepsis. Physiol Rep. doi: 10.14814/phy2.13622, 2018

[100] Rabadi M, Kim M, Li H, Han SJ, Choi Y, D'Agati V, Lee HT. ATP induces PAD4 in renal proximal tubule cells via P2X7 receptor activation to exacerbate ischemic AKI. Am J Physiol Renal Physiol. 314(2):F293-F305, 2018

[101] Geraghty NJ, Watson D, Sluyter R. Long-term treatment with the P2X7 receptor antagonist Brilliant Blue G reduces liver inflammation in a humanized mouse model of graft-versus-host disease. Cell Immunol. 336:12-19, 2019

[102] Crabbé M, Van der Perren A, Bollaerts I, Kounelis S, Baekelandt V, Bormans G, Casteels C, Moons L, Van Laere K. Increased P2X7 Receptor Binding Is Associated With Neuroinflammation in Acute but Not Chronic Rodent Models for Parkinson's Disease. Front Neurosci. 13:799, 2019

[103] Menzies RI, Booth JWR, Mullins JJ, Bailey MA, Tam FWK, Norman JT, Unwin RJ. Hyperglycemia-induced Renal P2X7 Receptor Activation Enhances Diabetes-related Injury. EBioMedicine. 19:73-83, 2017

[104] Rodrigues AM, Serralha RS, Farias C, Punaro GR, Fernandes MJS, Higa EMS. $P2X_7$ receptor and klotho expressions in diabetic nephropathy progression. Purinergic Signal. 14(2):167-176, 2018

[105] Vieira FS, Nanini HF, Takiya CM, Coutinho-Silva R. P2X7 receptor knockout prevents streptozotocin-induced type 1 diabetes in mice. Molecular and cellular endocrinology. 419:148-57, 2016

[106] Taylor SR, Turner CM, Elliott JI, McDaid J, Hewitt R, Smith J, Pickering MC, Whitehouse DL, Cook HT, Burnstock G, Pusey CD. P2X7 deficiency attenuates renal injury in experimental glomerulonephritis. Journal of the American Society of Nephrology. 20(6):1275-81, 2009

[107] Ji X, Naito Y, Weng H, Endo K, Ma X, Iwai N. P2X7 deficiency attenuates hypertension and renal injury in deoxycorticosterone acetate-salt hypertension. American Journal of Physiology-Renal Physiology. 303(8):F1207-15, 2012

[108] Menzies RI, Howarth AR, Unwin RJ, Tam FW, Mullins JJ, Bailey MA. Inhibition of the purinergic P2X7 receptor improves renal perfusion in angiotensin-II-infused rats. Kidney International. 88(5):1079-87, 2015

[109] Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signalling. 1;2(2):409-30, 2006

[110] Bauerle JD, Grenz A, Kim JH, Lee HT, Eltzschig HK. Adenosine generation and signaling during acute kidney injury. Journal of the American Society of Nephrology. 1;22(1):14-20, 2011

[111] Friedman DJ, Rennke HG, Csizmadia E, Enjyoji K, Robson SC. The vascular ectonucleotidase ENTPD1 is a novel renoprotective factor in diabetic nephropathy. Diabetes. 56(9):2371-9, 2007

[112] Vonend O, Grote T, Oberhauser V, Kügelgen I, Rump LC. P2Y‐Receptors stimulating the proliferation of human mesangial cells through the MAPK42/44 pathway. British Journal of Pharmacology. 139(6):1119-26., 2003

[113] Harada H, Chan CM, Loesch A, Unwin R, Burnstock G. Induction of proliferation and apoptotic cell death via P2Y and P2X receptors, respectively, in rat glomerular mesangial cells. Kidney international. 57(3):949-58, 2000

[114] Kreft E, Kowalski R, Jankowski M, Szczepańska-Konkel M. Renal vasculature reactivity to agonist of P2X7 receptor is increased in streptozotocin-induced diabetes. Pharmacological Reports. 29;68(1):71-4, 2016

[115] Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harbor perspectives in biology. 1;3(12):a005058, 2011

[116] Solini A, Menini S, Rossi C, Ricci C, Santini E, Blasetti Fantauzzi C, Iacobini C, Pugliese G. The purinergic 2X7 receptor participates in renal inflammation and injury induced by high-fat diet: possible role of NLRP3 inflammasome activation. The Journal of Pathology. 1;231(3):342-53, 2013

[117] Chen K, Zhang J, Zhang W, Zhang J, Yang J, Li K, He Y. ATP-P2X4 signaling mediates NLRP3 inflammasome activation: a novel pathway of diabetic nephropathy. The International Journal of Biochemistry & Cell Biology. 45(5):932-43, 2013

[118] Goncalves RG, Gabrich L, Rosario A, Takiya CM, Ferreira ML, Chiarini LB, Persechini PM, Coutinho-Silva R, Leite M. The role of purinergic P2X7 receptors in the inflammation and fibrosis of unilateral ureteral obstruction in mice. Kidney international. 70(9):1599-606, 2006

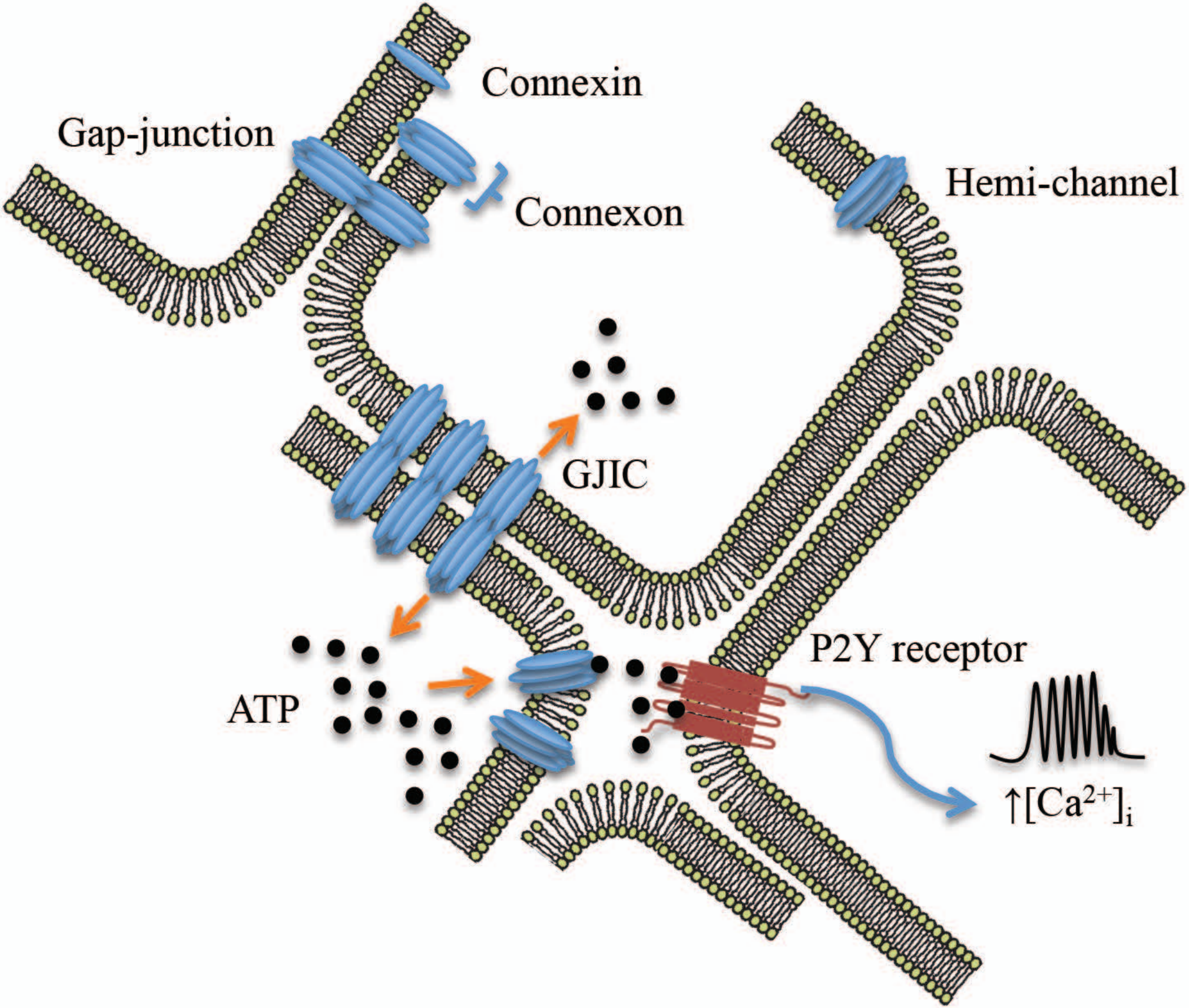
[119] Solini A, Iacobini C, Ricci C, Chiozzi P, Amadio L, Pricci F, Di Mario U, Di Virgilio F, Pugliese G. Purinergic modulation of mesangial extracellular matrix production: role in diabetic and other glomerular diseases. Kidney International. 31;67(3):875-85, 2005

[120] Ponnusamy M, Ma L, Gong R, Pang M, Chin YE, Zhuang S. P2X7 receptors mediate deleterious renal epithelial-fibroblast cross talk. American Journal of Physiology-Renal Physiology. 300(1):F62-70, 2011

[121] Wolff CI, Bundey RA, Insel PA. Involvement of P2Y Receptors in TGF {beta}-Induced EMT of MDCK Cells. The FASEB Journal. 1;22:942-5, 2008

[122] Kavvadas P, Abed A, Poulain C, Authier F, Labéjof LP, Calmont A, Afieri C, Prakoura N, Dussaule JC, Chatziantoniou C, Chadjichristos CE. Decreased expression of connexin 43 blunts the

progression of experimental GN. Journal of the American Society of Nephrology. 28(10):2915-30, 2017


Connexin
Gap-junction
Connexon
Hemi-channel
GJIC
ATP
P2Y receptor
↑[Ca2+]i