Regulatory Functions of the Juxtaglomerular Apparatus

BY

RUISHENG LIU
The tubuloglomerular feedback mechanism is an important regulator in the juxtaglomerular apparatus and it detects flow dependent alterations in luminal NaCl concentration ([NaCl]) at the macula densa (MD) cell site via a Na⁺-K⁺-2Cl⁻ cotransporter. Signals are sent by the MD to adjust the afferent arteriole tone and altering release of renin. This signaling mechanism is unclear but MD cell calcium concentration, release of ATP and nitric oxide (NO) might be important.

In cultured rat glomerular mesangial cells the NO production was measured using confocal microscopy and calcium responses to ATP was measured with fura-2 using imaging techniques. NO from spermine-NONOate and L-arginine could resensitize, desensitized ATP receptors in a cGMP independent way. In mesangial cells from spontaneously hypertensive rats (SHR) less NO effect was found on ATP receptor de/resensitization indicating an impaired NO release or effect.

The macula densa cells were studied using microperfusion techniques with confocal and video imaging systems. Changes in [Ca²⁺], from exposed macula densa plaques were assessed upon addition of agonists added to bath. The order of efficacy of agonists was UTP = ATP >> 2MesATP = ADP. Dose response curve for ATP added in bath showed an EC₅₀ of 15 µM. Macula densa cell volume and NO concentration increased considerably with increasing luminal [NaCl] indicating an important role for NO in the signaling process to counteract a vasoconstrictor response and reset the sensitivity of the tubuloglomerular feedback mechanism.

In conclusion, the results showed 1). NO can increase the P2Y receptor resensitization in rat glomerular mesangial cells, acting through a cGMP-independent pathway. 2) An impaired NO generation/effect on P2Y receptors in mesangial cells from SHR rats. 3) Macula densa cells possess P2Y₂ purinergic receptors on basolateral and that activation of these receptors results in the mobilization of Ca²⁺. 4) Increased luminal [NaCl] delivery increased cell volume and the NO productions in the macula densa cells.

Key words: Mesangial, desensitization, nitric oxide, confocal, calcium, P2Y receptor, macula densa, NaCl, luminal, microperfusion, angiotensin.
This thesis is based on the following studies, which will be referred to their Roman numbers:


II. The effects of nitric oxide on P2Y receptor resensitization in spontaneously hypertensive rat mesangial cells. Liu R., and Persson A.E.G. *Submitted*


IV. Changes of nitric oxide concentration in macula densa cells caused by changes in luminal NaCl concentration. Liu R., Pittner J., and Persson A.E.G. *Submitted*

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<td>juxtaglomerular apparatus</td>
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<td>TGF</td>
<td>tubuloglomerular feedback</td>
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<td>[NaCl]</td>
<td>sodium chloride concentration</td>
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<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
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<td>NO</td>
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<td>PMT</td>
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<td>SD</td>
<td>sprague dawley</td>
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<td>SHR</td>
<td>spontaneously hypertensive rats</td>
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<td>WKY</td>
<td>Wistar Kyoto rat</td>
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<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
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<td>ODQ</td>
<td>1H-(1,2,4)oxadiazolo(4,3-α)quinoxalin-1-one</td>
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<td>2-methylthio-ATP</td>
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<td>IBS</td>
<td>isolation buffer solution</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>cTAL</td>
<td>cortical thick ascending limb</td>
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<td>ROIs</td>
<td>regions of interest</td>
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<tr>
<td>[Ca^{2+}]_i</td>
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1. INTRODUCTION

1.1. Juxtaglomerular apparatus-background:

The juxtaglomerular apparatus (JGA) consists of the macula densa (MD), afferent and efferent arterioles, and the mesangium. The JGA is found at the hilus of the renal glomerulus where the thick ascending limb of the loop of Henle of the same nephron changes its morphologic characteristics as it comes in contact with the vascular pole of the glomerulus. These modified cells are MD cells. The MD can sense the sodium chloride concentration ([NaCl]) and fluid load in distal tubule, then regulate the afferent arteriole tone and renin release (Schnermann et al. 1973; Bell and Navar 1982). The glomerular arterioles (afferent arteriole in particular) present a unique morphological characteristic, which consists of the appearance of cells exhibiting both smooth muscle and endocrine features. These cells synthesize, store and release renin. They are more abundant as the arterioles approach the hilus of the glomerulus. Between the two arterioles as they enter the glomerulus there is a group of cells located in a region known as the mesangium containing mesangial cells. Mesangial cells are smooth muscle-like pericytes that abut and surround the filtration capillaries within the glomerulus. Structural and functional studies suggest that mesangial cells play an important role in regulation of glomerular microcirculation and filtration rate in both a static and dynamic fashion (Deen et al. 1972; Brenner et al. 1996).

In 1925, Ruyter (Ruyter 1925) described myoepitheloid cells located in the afferent arteriole just before its penetration into the glomerulus and suggested that these cells might contribute to the regulation of blood flow through the glomerulus. In the 1930’s, the JGA became the object of intensive study for two prominent investigators, Goormaghtigh (Goormaghtigh 1932) and Zimmermann (Zimmermann 1933). They both foresaw a functional role for the JGA in the regulation of glomerular blood flow. There is now much
agreement that the interrelationship of tubular and vascular elements in the JGA reflects a functional connection between the vascular system and the electrolyte handling tubular system of the kidney. However, the available experimental data, although accumulating rapidly, is not sufficiently clear to give a complete and unified description of the function of the JGA.

1.2. Tubuloglomerular feedback

The tubuloglomerular feedback (TGF) is a very important function of the JGA to regulate renal hemodynamics. This mechanism operates as a negative feedback loop, sensing changes in distal nephron fluid flow rate by detecting flow dependent alterations in luminal [NaCl] at the MD. Signals are then sent by the MD cells, which alter the afferent arteriole tones (Schnermann et al. 1973; Bell and Navar 1982; Persson et al. 1991) and release renin (Schnermann 1998). In this signals transferring, the first step is related to the NaCl transport by MD, which is relatively well understood now. NaCl uptake is mostly through the Na⁺-K⁺-2Cl⁻ cotransporter, which has been shown on a functional as well as transcriptional level (Schlatter et al. 1989; Obermuller et al. 1996). Next step is rather unclear so far. The possible mediators and modulators of the information transfer between the MD and its target cells include extracellular ion concentration, ATP, angiotensin II, adenosine, arachildonic acid metabolites and nitric oxide (NO) (Salomonsson et al. 1991; Wilcox et al. 1992; Ito and Ren 1993; Thorup and Persson 1994; Braam and Koomans 1995; Briggs and Schnermann 1996; Thorup et al. 1996; Ichihara et al. 1998; Kurtz et al. 1998; Peti-Peterdi and Bell 1999; Cruz et al. 2000; Ren et al. 2000; Wagner et al. 2000; Brown et al. 2001). It has also been found that the sensitivity of the TGF can be reset by a large number of different factors (Persson et al. 1982; Persson and Wright 1982; Schnermann et al. 1998; Persson et al. 2000).

1.3. Purinergic receptors in TGF regulation
Purinergic receptors have been identified as playing a major role within the juxtaglomerular apparatus (Schroeder et al. 2000). These receptors are activated by extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) and are important signaling molecules that mediate various biological effects in the kidney. They may serve as paracrine regulators of renal microvascular resistance (Navar et al. 1996; Osswald et al. 1997), and may modulate mesangial cell contraction, alter epithelial ion transport and influence the TGF mechanism (Franco et al. 1989; Paulais et al. 1995; Inscho et al. 1998; Fernandez et al. 2000; Gutierrez et al. 2000), via cell surface receptors for purines. Recently, it has been reported that MD cells may release ATP across the basolateral membrane via maxi-chloride channels, indicating that there may be a direct role of ATP in TGF signaling (Bell et al. 2000).

There are two main families of purine receptors, adenosine or P1 receptors, and P2 receptors, the latter recognizing primarily ATP, ADP, UTP, and UDP (Abbracchio and Burnstock 1994). Based on differences in molecular structure and signal transduction mechanisms, P2 receptors are divided into two families consisting of ligand-gated ion channel-receptors and G protein-coupled receptors termed P2X and P2Y receptors, respectively. Seven mammalian P2X receptors (P2X1-7) and five mammalian P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) have been cloned (Ralevic and Burnstock 1998). Activation of both receptors increases cytosolic calcium concentration ([Ca^{2+}]_i), the difference being that P2X receptors induce Ca^{2+} influx while P2Y receptors result in Ca^{2+} mobilization.

P2Y receptors have been reported to be expressed in numerous, if not all, nephron segments. The cortical thick ascending limb (cTAL) and collecting ducts express both P2Y1 and P2Y2 receptors (Ecelbarger et al. 1994; Paulais et al. 1995; Cha et al. 1998). In proximal tubules express the P2Y1 type (Yamada et al. 1996). In outer medullary collecting duct, P2Y1, P2Y2 and P2Y4 receptors are expressed (Bailey et al. 2000). Mesangial cells contain P2Y2 receptors (Gutierrez et al. 1999).
1.4. Receptor desensitization

Receptor response to G-protein-coupled receptors (GPCRs) agonists are usually rapidly attenuated (Freedman and Lefkowitz 1996). The mechanisms that attenuate signaling by GPCRs are of considerable interest from several viewpoints. In the healthy organism they govern the ability of cells to respond to hormones and neurotransmitters regulating intercellular signaling. Agonist removal from the extracellular fluid, receptor desensitization and receptor endocytosis, prevent uncontrolled stimulation of cells. On the other hand, receptor resensitization is also crucial, because it allows cells to maintain their ability to respond to agonists over time. Receptor desensitization, which occurs during short-term (seconds to minutes) exposure of cells to agonists, is mediated by: 1) phosphorylation, which causes uncoupling of activated receptors from G-proteins, a process that effectively terminates the signal, 2) receptor endocytosis which depletes the plasma membrane of high-affinity receptors. This receptor internalization is the first step of receptor recycling, which is a requisite for resensitization of the response. Receptor down-regulation is a loss of receptors from a cell that results from long-term (hours to days) continuous exposure to agonists (Casey 1995; Bohm et al. 1996). These regulatory mechanisms are also important from a therapeutic viewpoint (Weisman et al. 1998; Boeynaems et al. 2000). Over half of all medicines used today exert their effects through signaling pathways that involve G-proteins. Desensitization of P2Y receptors has been demonstrated in different cell lines and preparations (Weisman et al. 1998; Clarke et al. 1999). Elucidation of the mechanisms involved in P2Y receptor de- and resensitization, as well as identification of the specific enzymes that take part, will be important for the understanding of the physiological role of extracellular nucleotides and will be crucial for any possible use in therapy.

1.5. 4,5-diaminofluorescein diacetate
4,5-diaminofluorescein diacetate (DAF-2 DA) is a newly developed indicator for real-time NO measurement with a detection limit of 5 nM (Kojima et al. 1998; Nakatsubo et al. 1998; Nagano 1999; Nagata et al. 1999). DAF-2 selectively traps NO between two amino groups, and yields triazolofluorescein (DAF-2T), which emits green fluorescence when excited at 490-495 nm. DAF-2T is not formed in the absence of NO. Stable forms of NO (e.g., NO$_2^-$ and NO$_3^-$) and reactive oxygen species like superoxide (O$_2^-)$, H$_2$O$_2$, and peroxynitrite (ONOO$^-$) do not react with DAF-2 to yield a fluorescent product (Kojima et al. 1998). The fluorescence intensity is dependent on the amount of NO trapped by DAF-2. DAF-2 has been used as a specific NO indicator in different cells and tissues (Hanke and Campbell 2000; Kimura et al. 2001; Prabhakar 2001; Rhinehart and Pallone 2001).

1.6. Confocal laser scanning microscopy

The concept of confocal microscopy, first developed by Minsky, was patented in 1957. The first purely analogue mechanical confocal microscope was designed and produced by Eggar and Petran a decade later. It was not until the late seventies, with the advent of affordable computers and lasers, and the development of digital image processing software, that the first single-beam confocal laser scanning microscopes were developed in a number of laboratories and applied to biological and materials specimens. The first commercial confocal laser scanning microscopy (CLSM) systems were produced in the late eighties. During the last decade the availability of CLSM systems of ever-increasing power and sophistication has revolutionized the science of microscopy as applied to cell and developmental biology, physiology, cytogenetics, diagnostic pathology, and the material sciences (Plesch and Klingbeil 1988; Carlsson and Liljeborg 1989; Fabian et al. 1990; Goldstein et al. 1990).

The principle of confocal microscopy and of CLSM in particular, is based upon a simple optical principle. In conventional fluorescence microscopy the image quality suffers from fluorescence emission from parts of the specimen outside the plane of focus (or focal
Figure 1. Optical principle of CLSM

Detector → Slit
Dichroic mirror
Laser
Specimen
Lens and objectives
Emission light
Excitation light
plane). This leads to considerable blurring of the images, making the fine details inside a specimen difficult to resolve. In CLSM the specimen is illuminated in the focal plane by using suited optics. A tiny diaphragm (slit) is placed in front of photomultiplier tubes (PMT) to ensure that all out of focus fluorescence light does not reach the detector. By proper optical alignment of the illumination and detection light paths only emitted light is collected from the optical plane that is in focus. In this way, optical slices (sections) can be cut through the specimen by adjusting the z-position of the microscope table. This distinct CLSM property is called “optical sectioning” and results in a high axial resolution. By adjusting the size of the slit, the thickness of the optical section can be adjusted (Figure 1).

CLSM has exhibited several advantages over conventional optical microscopy. The most important of these stem from the fact that out-of-focus blur is essentially absent from confocal images, giving the capability for direct non-invasive serial optical sectioning of intact and even living specimens (Sheppard and Shotton 1997). This leads to the possibility of generating three-dimensional images of thick transparent objects such as biological cells and tissues. In a similar way, it allows profiling of the surfaces of 3-D objects and multi-layer structures such as integrated circuits, again, by a non-contacting and non-destructive method (Quesada et al. 1991; Weaver et al. 1991; Hardman and Spooner 1992; Dailey and Smith 1993; Zhu et al. 1994; Belichenko and Dahlstrom 1995; Feng et al. 1995; Huser et al. 1996; Wedekind et al. 1996; Favard et al. 1999; Ono et al. 2001; Zucker and Price 2001).

2. AIMS OF PRESENT STUDY

The aims of the present study were: 1). To characterize the effect of NO on the desensitization-resensitization cycle of the ATP-induce $[Ca^{2+}]_i$ response in Sprague Dawley (SD) and spontaneously hypertensive rats (SHR). 2). To study the existence and type of purinergic receptors expressed by MD cells and to determine, functionally, if purinergic
receptors were preferentially located at the apical or basolateral surfaces of MD cells. To measure the NO concentration in MD caused by the luminal [NaCl] alterations.

3. MATERIALS AND METHODS

3.1. Isolation and culture of mesangial cells

Rat glomerular mesangial cells were cultured as described previously in our lab (Kurtz et al. 1982; Gutierrez et al. 1999). In short, both kidneys from male SD, SHR and WKY rat (120-170 g) were removed and decapsulated under sterile conditions, respectively. Cortical tissue was cut away from the medulla and minced in isolation buffer solution (IBS) containing 5mM KCl, 2mM CaCl2, 130mM NaCl, 10mM glucose, 20mM sucrose and 10mM Tris, (pH 7.4, osmolality 290 mOsm). Glomeruli were isolated by sequential sieving and collected on a 50 μm sieve. The glomeruli were then incubated with 0.1% collagenase in IBS for 30 min at 37 °C to remove epithelial cells and obtain glomerular cores consisting mostly of mesangium and capillary loops (Striker et al. 1980). The glomeruli suspension was centrifuged at 2200 rpm for 7 min at room temperature. The pellet was resuspended with 10ml RPMI 1640 medium, supplemented with 18% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 0.66 U/ml insulin. The RPMI 1640 medium contained D-valine instead of L-valine. D-valine inhibits fibroblast growth (Ausiello et al. 1980). Aliquots of the glomeruli were transferred to 25 cm² tissue flasks containing 6 ml supplemented RPMI 1640 medium. The flasks were incubated at 37 °C and 5% CO2 in a humidified atmosphere in a CO2-controlled incubator for 3-6 weeks. The medium was changed every third day. Epithelial and endothelial specific staining (cytokeratin and factor VIII, respectively) in the confluent cultures of mesangial cells were negative, excluding any contamination.

3.2.Glomerulus preparation:
Female New Zealand White rabbits weighing 1.0 - 1.5 kg were killed and the left kidney removed and cut into several 1.5 - 3 mm transversal slices. The slices were placed in a chilled low NaCl buffer solution, containing: 35 mM NaCl, 1.3 mM CaCl₂, 1 mM MgSO₄, 1.6 mM K₂HPO₄, 5 mM glucose, 20 mM Hepes with pH adjusted to 7.4 and addition of sucrose sufficient to achieve an osmolality at 290 mOsm. Glomeruli with attached cortical thick ascending limb (cTAL) and containing the MD plaque were isolated by microdissection at 4°C under a dissection microscope (Wild, Switzerland). In some experiments, the cTAL was carefully removed leaving the MD plaque attached to the glomerulus. In other experiments, the cTAL was left intact for microperfusion studies.

3.3. Microperfusion method:

Individual cTAL with attached glomeruli were dissected and perfused as described previously (Kirk et al. 1985; Gonzalez et al. 1988). The cTAL was cannulated and perfused with the low NaCl buffer solution. The preparation was bathed continuously with Ringer solution at a rate of 6-7 ml/min. MD cells were loaded with the fluorescent probe from the luminal side.

3.4. Confocal settings:

The image size was set to 640X480 pixels. The confocal slit was set at a width of 25 nm. Photobleaching was kept to a minimum by maintaining laser intensity at below 30% of maximum and using a shutter so that the preparation was exposed to laser light only during the collection of images. Data collection, with the Noran Odyssey confocal system is controlled by a Silicon Graphics workstation. Image acquisition was limited to 30 frames/s and, when necessary, image noise was reduced by averaging or summing 16-32 individual images. Sampling time for each pixel was 100 ns.
3.5 Fluorescence loading:

3.5.1. Fura-2 and Fura Red:

The fluorescent Ca\(^{2+}\) indicators fura-2 was used for video imaging and Fura Red for confocal microscopy. The fura-2 loaded cells were excited alternately at 340 nm and 380 nm, and the emission was measured at 510 nm. The intensity of 340/380 emission ratio was used to determine the intracellular calcium concentration after calibration in vitro. Fura Red loaded MD-glomerular preparations were transferred to the confocal system. Fura Red was excited at 488 nm with an argon-ion laser, while emitted fluorescence was recorded at wavelengths of >600 nm through a 550 nm long pass barrier filter. Relative changes of [Ca\(^{2+}\)]\(_i\) were calculated by a normalization procedure to obtain a ‘pseudo ratio’ : \((F - F_{\text{rest}})/F_{\text{rest}}\).

3.5.2. Calcein

Calcein was excited at 488 nm with an argon-ion laser, while emitted fluorescence was recorded at wavelengths of 510 to 530 nm. Square-shaped regions of interest (ROIs) were set inside the cytoplasmic area of MD cells and the mean intensity within the ROIs was recorded every 5 s. Relative changes of cell volume and NO concentration changes were calculated by a normalization procedure to obtain a ‘pseudo ratio’ \( (F-F_{\text{rest}})/F_{\text{rest}}\).

3.5.3 DAF-2

Excitation and recording wavelength in confocal system were similar to that in calcein measurement. Calculations were based on the following equations.

\[
V_{c1} \times C_{c1} = V_{c2} \times C_{c2}
\]

Where \(V_{c1}\) is cell volume and \(C_{c1}\) is concentration of calcein during resting condition; \(V_{c2}\) and \(C_{c2}\) are changed cell volume and changed concentration of calcein, respectively. The concentration of calcein is proportional to its fluorescent intensity, therefore, the ratio of concentrations is equal to the ratio of intensities. Assuming that \(V_{c1}\) is 1, the changed cell...
volume could be expressed with the changed calcein intensity ($F_{c2}$) and the intensity at basal level ($F_{c1}$).

$$V_{c2} = V_{c1} \cdot \frac{C_{c1}}{C_{c2}} = \frac{F_{c1}}{F_{c2}}$$  \hspace{1cm} (II)

Delta relative changes of cell volume can be calculated as:

$$\frac{(V_{c2} - V_{c1})}{V_{c1}} = \frac{F_{c1}}{F_{c2}} - 1$$  \hspace{1cm} (III)

The triazolofluorescein of DAF-2 (DAF-2T) is the fluorescent form after DAF-2 selectively traps NO between two amino groups. The relative changes of the amount of DAF-2T in MD cells can be calculated by the changed cell volume ($V_{d2}$) multiplied with changed concentration ($C_{d2}$) of DAF-2T divided by the basal level of cell volume ($V_{d1}$) multiplied with concentration ($C_{d1}$) of DAF-2T.

$$\frac{(V_{d2} \cdot C_{d2})}{(V_{d1} \cdot C_{d1})}$$  \hspace{1cm} (IV)

Since $V_{d2}$ and $V_{c2}$ are the same, and $V_{d1}$ is regarded as 1, the $C_{d2} /C_{d1}$ could be expressed by the ratio of DAF-2T intensities in changed conditions ($F_{d2}$) and in basal level ($F_{d1}$). The delta relative changes of DAF-2T amount can be expressed:

$$\frac{[(V_{d2} \cdot C_{d2}) - (V_{d1} \cdot C_{d1})]}{(V_{d1} \cdot C_{d1})} = \frac{V_{c2}}{C_{c2}} \cdot \frac{(F_{d2} / F_{d1}) - 1}{(V_{d1} \cdot C_{d1})}$$  \hspace{1cm} (V)

Equations III and V were used to calculated the changes of cell volume and DAF-2T amount in MD cells.

4. RESULTS

4.1. Study I:

The present study evaluates the effect of NO on P2Y nucleotide receptor resensitization in cultured rat glomerular mesangial cells. L-arginine increased and Nω-nitro-L-arginine methyl ester (L-NAME) decreased NO production significantly (P<0.05), measured with the NO indicator DAF-2 using confocal microscopy. Calcium responses to ATP were measured with fura-2 and imaging techniques. Repeated stimulation with ATP results in receptor desensitization, characterized by lower calcium peak amplitude.
Desensitization was induced by challenging mesangial cells with four consecutive 2 min pulses of ATP (0.1 mM) separated by 4.5 min control perfusions. \([\text{Ca}^{2+}]_i\) increase evoked by second, third and fourth ATP challenges were about 40\%, 26\% and 18\% of the first challenge. The NO precursor, L-arginine (10 mM) and the NO donors, spermine-NONOate (500 \(\mu\)M) and sodium nitroprusside (SNP, 1 mM), were added before and during the fourth ATP challenge. Spermine-NONOate and L-arginine induced a recovery of the \([\text{Ca}^{2+}]_i\) response to the fourth ATP challenge (\(p<0.01, 0.05\) respectively). The NO synthase inhibitor, L-NAME (5 mM) applied together with ATP, was shown to enhance desensitization. 1H-(1,2,4)oxadiazolo(4,3-\(\alpha\))quinoxalin-1-one (ODQ, 30 \(\mu\)M), an inhibitor of guanylate cyclase, was used together with L-arginine, SNP or spermine-NONOate. There was no significant difference with or without ODQ. Neither ODQ nor 8-Br-cGMP, an analog of cGMP, at different concentrations showed effects on ATP stimulated \([\text{Ca}^{2+}]_i\). There was no elevation of \([\text{Ca}^{2+}]_i\) when the cells were challenged by different concentrations (1 \(\mu\)M, 100 \(\mu\)M, 1mM, 20 mM and 30 mM) of caffeine, caffeine plus ATP (0.1 mM) and 4-chloro-3-ethylphenol (100 \(\mu\)M, 500 \(\mu\)M and 1mM), a new agonist of ryanodine receptors.

The results indicate that: NO can increase the P2Y receptor resensitization in rat glomerular mesangial cells, acting through a cGMP-independent pathway. We found no evidence for the existence of ryanodine sensitive intracellular calcium stores in rat mesangial cells.

4.2. Study II

The first ATP-stimulated increase in \([\text{Ca}^{2+}]_i\) was significantly higher in SHR (1330.25 \(\pm\) 360.31 nM) than that in WKY (974.28 \(\pm\) 397.72 nM) (\(P< 0.05\)). The ratio of second, third and fourth ATP-stimulated increase in \([\text{Ca}^{2+}]_i\) in SHR was significant lower than that in WKY (\(P< 0.05\)). Spermine-NONOate and L-arginine could significantly increase the fourth ATP-stimulated \([\text{Ca}^{2+}]_i\) in WKY (\(P< 0.01, 0.05\) separately). In SHR, only spermine-
NONOate can significantly recover the fourth ATP-challenged \([\text{Ca}^{2+}]_i\) increase. L-NAME can greatly reduce the second, third and fourth ATP-stimulated \([\text{Ca}^{2+}]_i\) increase in WKY (P<0.01), but not in SHR. There was no significant difference in \([\text{Ca}^{2+}]_i\) increase in the presence or absence of ODQ in both WKY and SHR. The cGMP analog, 8-Br-cGMP (10^{-4} \text{M to } 10^{-6} \text{M}) \((n = 53)\), on the fourth ATP challenges did not show any significant effect on \([\text{Ca}^{2+}]_i\) stimulated by ATP. \([\text{Ca}^{2+}]_i\) was not elevated when the WKY cells were challenged by caffeine at concentrations of 1 \(\mu\text{M}, 100 \, \mu\text{M}, 1\text{mM}, 20 \text{mM} \text{ and } 30 \text{mM} \) separately for 2 min. When the cells were superfused with L-NAME, L-arginine or spermine-NONOate for a period of 5 min before and during one single ATP challenge, the responses observed in these conditions were not significant different from the response in control experiment with WKY.

In conclusion, we found 1) A higher ATP response and a faster desensitization were found in the SHR cells compared to WKY. 2) The presence of L-NAME enhance receptor desensitisation in WKY but not in SHR. 3) These effects are mediated through a cGMP-independent pathway. 4) We could not prove the existence of a ryanodine receptor to release calcium from intracellular stores in rat mesangial cells.

4.3. Study III:

Isolated glomeruli containing MD cells, with and without the cortical thick ascending limb, were loaded with the \(\text{Ca}^{2+}\) sensitive indicators Fura Red (confocal microscopy) or Fura 2 (conventional video image analysis). Studies were performed on an inverted microscope in a chamber with a flow-through perfusion system. Changes in \([\text{Ca}^{2+}]_i\) from exposed MD plaques were assessed upon addition of adenosine, ATP, UTP, ADP or 2-methylthio-ATP (2-MeS-ATP). There was no change in \([\text{Ca}^{2+}]_i\) with addition of adenosine (10^{-7}-10^{-3} \text{ M}). UTP and ATP (10^{-4} \text{ M}) caused \([\text{Ca}^{2+}]_i\) to increase by 268 ± 40 nM; \(n = 21\) and 295 ± 53 nM; \(n = 21\), respectively, while in response to 2MesATP and ADP \([\text{Ca}^{2+}]_i\) increased by only 67 ± 13 nM; \(n = 8\) and 93 ± 36 nM; \(n = 14\), respectively. Dose response curve for ATP (10^{-7}-10^{-3} \text{ M}) added
in bath showed an EC$_{50}$ of 15 µM. No effect on MD [Ca$^{2+}$], was seen when ATP was added from the lumen. ATP caused similar increases in MD [Ca$^{2+}$], in the presence or absence of bath Ca$^{2+}$ and addition of 5 mM EGTA. Suramin (an antagonist of P2X and P2Y receptors) completely inhibited ATP-induced [Ca$^{2+}$], dynamics. ATP-Ca$^{2+}$ responsiveness was also prevented by the phospholipase C inhibitor, U-73122, but not by its inactive analog, U-73343.

These results suggest that MD cells possess P2Y$_2$ purinergic receptors on basolateral but not apical membranes and that activation of these receptors results in the mobilization of Ca$^{2+}$.

4.4. Study IV

NO concentration in MD cells was measured with confocal microscopy in isolated perfused thick ascending limb using the NO-sensitive fluorophore DAF-2 DA. Calcein was used to measure cell volume changes. The loop perfusion fluid was a modified Ringer solution containing 10, 35, or 135 mM NaCl with constant total osmolarity (290 mOsm), while the bath was perfused with the 135 mM NaCl solution. The results show that MD cell volume and NO concentration increased considerably (19 % and 28 %, respectively) with increasing luminal [NaCl]. Similar results were got with calcium free solution both in lumen and bath. 5 mM L-arginine increased (30 %) the NO concentration in the MD cells. 7-nitroindazole could totally inhibit the NO production caused by L-arginine and by increased luminal [NaCl].

In conclusion, for the first time, we could quantitatively measure MD cell volume changes caused by the changes of luminal [NaCl]. We found that increasing the luminal [NaCl] resulted in an increase in cell volume. We also found that NO formation in MD cells could be measured with DAF-2 and that NO production was increased by an increase in
luminal [NaCl]. An increased NO production will suppress the vasoconstriction induced by the TGF and at the same time reduce the TGF sensitivity.

5. DISCUSSION

5.1. NO production in mesangial cells

NO plays an important role in the physiological regulation of hemodynamics in the kidney (Tolins et al. 1990; Stockand and Sansom 1998). Mesangial cells may be exposed to NO through the high production of NO from MD cells. Once iNOS in mesangial cells is induced, it produces large amounts of NO that can influence cell and tissue function and cause damage (Palmer et al. 1988; Nathan and Xie 1994; Willmott et al. 1996). In study I, we found that L-arginine can increase NO concentration and that the increase caused by L-arginine can be inhibited with L-NAME. This indicates that NO is produced by unstimulated mesangial cells and NO production can be stimulated by the addition of L-arginine. Similar results have been reported (Kunz et al. 1994; Mohaupt et al. 1994; Datta and Lianos 1999; Guan et al. 1999; Kihara et al. 1999) by measuring nitrite and/or nitrate, which are end products of NO interaction with superoxide species. They found that there is a basal level of NO production in unstimulated mesangial cells. It has also been reported that L-NMMA can reduce the nitrite concentration in mesangial cells under basal condition (Shultz et al. 1991; Doi et al. 2000). Unstimulated mesangial cells do not express constitutive NOS. This might be due to effects of cytokines or endotoxin during cell culture. Furthermore, cultured mesangial cells in fetal calf serum (FCS) free medium showed an increased nitrite production and iNOS expression (Rodriguez-Lopez et al. 1999; Rodriguez-Lopez et al. 2000). In present study, 18% FCS was used during culture, 9% FCS was used in subculture for 24 to 48 hours, and the FCS-free solution used during the experiment. This also might be another way to enhance the activity of iNOS.
5.2. NO induce receptor desensitization in SD mesangial cells

In study I and II, the receptors were desensitized to a large extent after three ATP challenges. The first ATP-stimulated \([\text{Ca}^{2+}]_i\) increase was significantly higher in SHR than that in WKY. This may be due to the higher receptor capacity in SHR, which has also been found by others (Bottiglieri et al. 1988; Modrall et al. 1995; Ishikawa et al. 1996; Sidhu et al. 1998; Ozono et al. 2000). It might be the results of receptor upregulation. It also might be due to the difference between different strains of rats. The ratio of the second, third and fourth ATP-stimulated \([\text{Ca}^{2+}]_i\) increase was significantly lower in SHR than that in WKY. This reflected greater receptor desensitization in SHR. In the juxtaglomerular apparatus ATP can contract afferent arteriole by increasing \([\text{Ca}^{2+}]_i\) in smooth muscle cells via P2X receptors (Gutierrez et al. 1999). At the same time, ATP can stimulate NO release from the endothelial cells in the kidney via P2Y\(_2\) receptors (Fernandez et al. 2000). If less NO was produced, a mechanism to explain the blood pressure increase could be presented.

The NO synthase inhibitor, L-NAME, was shown to increase purine receptor desensitization, except in the SHR rats. An increase in NO delivery to the mesangial cells by L-arginine or NO donors resensitized the ATP receptors. Among the NO donors, the spermine-NONOate had the greatest effect, although it was only tested at a single concentration. This is consistent with other reports (Muhl et al. 1996; Kone and Baylis 1997). The results showed that in SHR there is an impairment of receptor resensitisation in the reaction to the endogenous and exogenous NO. These findings of a larger desensitization effect and impaired receptor resensitization in the SHR would be in line with a reduced NO concentration in these cells. A low NO concentration has been found to be caused by an increased formation of oxygen radicals in the juxtaglomerular apparatus sensitizing the tubuloglomerular feedback mechanism (Thorup and Persson 1996; Welch et al. 2000).
5.5. Contributions of c-GMP pathway and ryanodine receptors

NO has an inhibitive effect on Ca^{2+} release from IP3-sensitive stores (the GPCR-PLC-IP3 cascade) through a cGMP-dependent pathway. This has been demonstrated in various cell systems (Karaki et al. 1988; Clementi et al. 1995; Harvey and Burgess 1996; Stockand and Sansom 1996), a mechanism operating through the activation of protein kinase G (PKG). In general, NO stimulated activation of PKG is associated with a decrease in intracellular calcium, a mechanism consistent with decreased contractility of smooth muscle cells. The guanylate cyclase inhibitor ODQ and the cGMP analog, 8-Br-cGMP, were used to determine the contribution of the cGMP pathway. The results indicate that there are no significant differences with or without ODQ and 8-Br-cGMP. NO can also play a role in controlling Ca^{2+} release from ryanodine-sensitive intracellular calcium stores (Galione et al. 1993). However, we have failed to see calcium release response when challenging with caffeine, caffeine plus ATP and 4-chloro-3-ethylphenol, a new ryanodine receptor agonist (Shoshan-Barmatz and Ashley 1998; Westerblad et al. 1998). We found that a low dose of caffeine (100 nM) can inhibit calcium release challenged by ATP. Similar effects have been reported in the other kinds of cells and tissues through IP3 sensitive intracellular calcium stores (Bezprozvanny et al. 1994; Missiaen et al. 1998). The mechanism is not clear, probably because caffeine inhibits the binding site of IP3 receptors and deactivates the receptors (Maes et al. 1999).

5.6. NO on unstimulated receptors

It has been shown that the receptor desensitization cascade is not activated until the receptors are stimulated by agonists (Trowbridge et al. 1993). Thus, we can exclude the contribution of receptor desensitization cascade in only one ATP challenge. We found that there was no significant difference in ATP-stimulated [Ca^{2+}] increase after a 5 min perfusion of different NO donors and L-NAME in SD,WKY and SHR mesangial cells. The lack of
effects of NO before the first stimulus indicates that NO increase P2Y receptor resensitization in rat mesangial cells.

5.7. Purinergic receptor signaling at MD cells

Confocal microscopy has superior spatial and temporal resolution compared to conventional image analysis systems. In study III, we found no increase in [Ca^{2+}]_i during the administration of adenosine (10^{-7}-10^{-3} M). Earlier reports have found (Schnermann 1988; Franco et al. 1989) that adenosine A_1 receptor agonists enhance TGF responses when added from the lumen. In their studies (Schnermann 1988; Franco et al. 1989), it is possible that the A_1 receptor agonists directly affected the arteriolar smooth muscle cells. As recently reported (Gutierrez et al. 1999; Gutierrez et al. 1999), freshly dissected renal arterioles but not cultured mesangial cells respond to adenosine with an increase in [Ca^{2+}]_i. We found that in [Ca^{2+}]_i responses, the order of efficacy of agonists was UTP = ATP >> 2MesATP = ADP. This is consistent with P2Y receptor expression. Since the early maximal increase in [Ca^{2+}]_i is a good indicator of release of Ca^{2+} from intracellular pools (Sipma et al. 1999), the maximal increases in [Ca^{2+}]_i obtained with each agent was recorded in the results. Phospholipase C inhibitor, U-73122, prevented increases in [Ca^{2+}]_i, but its inactive analog, U-73343, could not. These findings strongly support our conclusion that the most important contribution to the rapid and maximal increase in [Ca^{2+}]_i was from Ca^{2+} mobilization through the PLC-IP_3 pathway and not through Ca^{2+} entry mechanisms (Watanabe and Endoh 1999; Doi et al. 2000; Mignen and Shuttleworth 2000). It also further supports the presence of P2Y receptors and not P2X receptors in MD cells. This later class of receptors exhibits properties of a non-selective cation channel that promotes increases in [Ca^{2+}]_i via Ca^{2+} entry (McCoy et al. 1999). The order of the efficacy of the nucleotides in MD cells was essentially the same as that found for P2Y_2 or/and P2Y_4 receptors (Lustig et al. 1993; Parr et al. 1994; Bowler et al. 1995; Godecke et al. 1996; Gutierrez et al. 1999). Pharmacological characterizations of P2Y_2 and P2Y_4 are almost the same, except P2Y_4 receptors are less sensitive to suramin (Bogdanov et al. 1998;
Harada et al. 2000). Our results showed that suramin could completely block the $[\text{Ca}^{2+}]_{i}$ responses induced by ATP, so we conclude the MD cells express P2Y$_{2}$ receptors.

5.8. Luminal NaCl and NO production in MD

The fluorophore calcein was used to measure volume in MD cells. Calcein concentration is not significantly related to changes in measure volume in MD cells intracellular calcium and pH (Breuer et al. 1995; Crowe et al. 1995). Changes in cell volume are expected to be reflected by changes in the fluorescence intensity, with decreased intensity during cell swelling and increased intensity during cell shrinkage (Mandeville et al. 1995; Sonnentag et al. 2000). In study IV, total osmolarity was held constant. When the luminal [NaCl] solution was decreased, the MD cell volume was reduced, and when the luminal [NaCl] was increased, the cell volume was also increased.

To measure NO we used DAF-2, a single-wavelength measurement probe, which is affected by cell volume. Therefore, the amount of DAF-2T had to be corrected for upon changes in cell volume. After volume correction the concentration of DAF-2T could be determined in MD cells. The results showed that when the luminal [NaCl] was increased from 10 mM or 35 mM to 135 mM, keeping total osmolarity constant, the DAF-2T amount was increased significantly. When the luminal [NaCl] was decreased the amount of DAF-2T decreased significantly. This increase in DAF-2T could be inhibited by treatment with L-NAME or 7-nitroindazole. It is known that the fluorescence intensity of DAF-2T is pH dependent (Kojima et al. 1998; Nagano 1999). The fluorescence of DAF-2T is comparatively stable above pH 7, but its fluorescence dramatically decreases below a pH of 7. It has been reported that increased luminal [NaCl] elevats pH, while decreased luminal [NaCl] lowers the pH in MD cells through apical Na:H exchangers (Fowler et al. 1995; Peti-Peterdi and Bell 1998). In present study, when luminal [NaCl] increased, which also increase intracellular pH in MD cells, the influence of pH value on the DAF-2T intensity should be minor. While,
when luminal [NaCl] decreased, the pH in MD cells will decrease, which could possibly influence the intensity of DAF-2T significantly. As discussed above, the amount of DAF-2T during luminal [NaCl] increase reflected the NO production in MD cells. So, the NO production in MD cells increased significantly, following increase luminal [NaCl]. There are rather contradictory reports regarding the distal [NaCl] and the NO production in MD cells (Shultz and Tolins 1993; Bosse et al. 1995; Deng et al. 1995; Singh et al. 1996; Thorup and Persson 1996; Wilcox and Welch 1996; Ichihara et al. 1998; Turkstra et al. 1998; Braam 1999). Decreased luminal [NaCl] would lower the pH in MD cells (Fowler et al. 1995), which could greatly decrease the amount of DAF-2T (Kojima et al. 1998; Nagano 1999). The significant decrease of DAF-2T fluorescence in this study is probably caused by the intracellular pH fall in MD cells. With a fall in pH, it is difficult to evaluate the actual NO productions.

Constitutive NOS is dependant on intracellular calcium and calmodulin (Sasaki et al. 2000). Our data showed that with use of Ca\(^{2+}\) free solution, the changes in NO intensity were not significantly different from those with the normal Ca\(^{2+}\) solution. Intracellular calcium changes, caused by alterations in luminal [NaCl] in the MD cells, would be abolished if calcium free solutions were used in bath and lumen (Salomonsson et al. 1991; Peti-Peterdi and Bell 1999). However, it has been reported that increased shear stress on endothelial cells can increase NO release in an non-calcium-dependent way (Fleming et al. 1998; Fulton et al. 1999). A similar mechanism might exist in the MD cells. In addition, changes in [Ca\(^{2+}\)]\(_i\), caused by alterations of the luminal [NaCl] was only 20 to 40 nM (Salomonsson et al. 1991; Peti-Peterdi and Bell 1999). Such a small change might not be enough to overwhelm other influential factors. Furthermore, the local NO production could also be regulated below the level of NOS activity. The release of NO from the MD cells upon increased [NaCl] could, however, be an important mechanism to counteract the TGF mediated vasoconstriction in the afferent arteriole and also to reset the sensitivity of the TGF mechanism to a lower level.
SUMMARY:

- NO can increase the P2Y receptor resensitization in rat glomerular mesangial cells, through a cGMP-independent pathway.
- We found no evidence for the existence of ryanodine sensitive intracellular calcium stores in rat mesangial cells.
- A higher ATP response and a faster desensitization were found in the SHR cells compared to WKY.
- The presence of L-NAME enhances receptor desensitization in WKY but not in SHR. These effects are mediated through a cGMP-independent pathway.
- We found that MD cells possess P2Y2 purinergic receptors on the basolateral but not apical membranes and that activation of these receptors results in mobilization of Ca$^{2+}$.
- We could quantitatively measure the MD cell volume changes with calcein and NO formation in MD cells with DAF-2, caused by the changes of luminal [NaCl], using confocal microscopy.
- Increasing the luminal [NaCl] resulted in an increase in MD cell volume and NO production. An increase in NO would oppose vasoconstriction and reset the TGF mechanism to a lower level.
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