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## **Autonomic Receptor-Mediated Regulation of Production and Release of Nitric Oxide in Normal and Malignant Human Urothelial Cells**

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**Running title:** *Nitric oxide production and release in human urothelial cells*

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*Abstract:* In the urinary bladder, the main source of NO seems to be the urothelium and the underlying suburothelium. In this study, we aimed to characterize how receptors in the human urothelium regulate the production and release of NO. For this, we cultured two human urothelial cell lines - the normal immortalized cell line UROtsa and the malignant cell line T24. These were treated with an array of agonists and antagonists with affinity for

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adrenergic, muscarinic and purinergic receptors. The production of NO and expression of nitric oxide synthase (NOS) was studied by immunocytochemistry and western blotting. The amount of released NO was measured indirectly by detecting nitrite using amperometry and a Griess reaction kit. The results showed that NO, endothelial NOS and inducible NOS were predominantly produced and expressed in the close vicinity of the nucleus in untreated human urothelial cells. Upon treatment with a beta-adrenoceptor agonist, but not any of the other agonists or antagonists, the pattern of NO production changed, showing a more even production throughout the cytosol. The pattern of expression of endothelial NOS changed in a similar way upon dobutamine treatment. The release of nitrite, as a measurement of NO, increased following treatment with dobutamine from  $0.31\pm 0.029$  to  $1.97\pm 0.18$  nmol and  $0.80\pm 0.12$  to  $3.27\pm 0.24$  nmol in UROtsa and T24, respectively. In conclusion, our results show that the expression of NOS and production of NO as well as the release of NO from human urothelial cells is regulated by beta-adrenoceptor activation.

As recently reviewed [1], numerous studies have been published which detail the various roles of nitric oxide (NO) in the urinary bladder. When comparing data from these studies, they appear to be somewhat contradictory, since beneficial effects according to one study can be contradicted by data from another. This has led to difficulties when trying to propose pharmacological treatments that regulate NO. Despite the dissonance among data in the literature, NO has been shown to be involved in several bladder diseases, including bladder cancer [2, 3]. NO is also involved in inflammatory diseases in the urinary bladder [4-6], even though it is still somewhat unclear whether the increase in NO/NOS synthase (NOS) is a secondary consequence of inflammation or an integral part of the protective inflammatory response [7]. While immunohistochemical staining has revealed the presence of endothelial and inducible NOS (eNOS and iNOS) in the sub-urothelium [8], the main source of NO in the bladder seems to be the urothelium [9-11]. This is especially evident during certain disease states in which the levels of expression of NOS can be altered [8, 12].

The urothelium consists of 3-5 layers of transitional epithelial cells that line the major parts of the renal pelvis, ureters, urinary bladder and urethra. The innermost, apical, surface of the urothelium is covered by a thick layer of glycoproteins and basolaterally the suburothelium can be found, which harbours nerves, blood vessels, connective tissue and various other interstitial cells. Release of NO from the urothelium can be induced by bladder wall

distension, changes in pH or composition of urine or by activation of a number of receptors present in the urothelium. An array of receptors including muscarinic, adrenergic, purinergic and vanilloid receptors are expressed in the urothelium (for full review, see Winder *et al.*, 2014). Release of urothelial nitric oxide by activation of adrenoceptors has previously been demonstrated in rats [14]. The same group later showed that activation of beta-adrenoceptors also increased urothelial eNOS in rats [15]. During certain disease states, *i.e.* cystitis, it has been shown that NO can be released upon activation of muscarinic receptors of the M5 subtype [11]. These receptors are mainly expressed in the urothelium [8], however, it has not been entirely proven that the urothelium is the source of NO. Tentatively, the activation of urothelial muscarinic M5 receptors could lead to the release of an unidentified substance that in turn causes release of NO in the suburothelium.

Apart from its involvement during cystitis, several studies have shown that NO can regulate contraction of the bladder smooth muscle [16], in particular by mediating relaxation of the bladder neck [17]. Even though expression of all three subtypes of beta-adrenoceptors have been identified in the human bladder, there seems to be a predominant expression of  $\beta_1$ - and  $\beta_2$ -adrenoceptors in the urothelium and, conversely, of  $\beta_3$ -adrenoceptors in the detrusor [18]. Since activation of  $\beta_3$ -adrenoceptors in endothelial cells causes release of NO and subsequent vasorelaxation [19], it has been assumed that treatment of overactive bladder (OAB) with a  $\beta_3$ -adrenoceptor agonist causes relaxation of the detrusor in a similar fashion. However, this remains to be proven.

Since NO has been shown to be involved in the onset and development of several urinary tract diseases, pharmacological treatment regulating the levels of NO, especially in the urothelium, has been proposed. It is possible that pharmacological treatments affecting the lower urinary tract that are already used in clinical practice, *i.e.* treatment against overactive bladder, affect levels of NO. However, until more precise mechanisms of production and release of NO have been unraveled, it will not be possible to develop drugs that specifically and selectively affect levels of NO in humans without causing significant side effects. Most previous studies on NO examine expression and effects of NOS and not NO *per se*. Further, to our knowledge, the release of NO from the human urothelium has not previously been quantified. The aim of the current study therefore was to quantitatively and qualitatively examine how the production and release of NO in a normal and a malignant human urothelial cell line is regulated by activation of autonomic receptors. This was achieved by treating the cells with the non-selective muscarinic agonist methacholine, the non-selective muscarinic

antagonist atropine, the P2 purinoceptor agonist adenosine 5'-triphosphate (ATP), the P1 purinoceptor agonist adenosine, the alpha-adrenoceptor agonist phenylephrine, the beta-adrenoceptor agonist dobutamine, the alpha-adrenoceptor antagonist phentolamine or the beta-adrenoceptor antagonists propranolol ( $\beta_{1/2}$ -selective) or L-748,337 ( $\beta_3$ -selective) for either 24 or 72 hr, in order to study short- and long-term exposure, respectively, and examine how the treatments affected production and release of NO.

### **Methods and materials**

Two human urothelial cell lines, UROtsa and T24, were used in this study. The UROtsa cell line is an immortalized cell line derived from the ureter urothelial lining and shares several characteristics with cells that are found in the intermediate layers of the normal human urothelium [20, 21]. The T24 cell line is a non-tumourigenic transitional carcinoma cell line, which is frequently used to study urothelial cancer cell proliferation [22-25]. The UROtsa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Stockholm, Sweden) and the T24 cells in McCoy's 5A medium (Sigma-Aldrich) with 10% foetal bovine serum and 1% penicillin/streptomycin in a humidified chamber at 37°C in a 5/95% CO<sub>2</sub>/O<sub>2</sub> atmosphere. Medium was renewed three times weekly, and the cells were passaged once a week or when they reached a confluency of 80-90%. Experiments were conducted on cells in passage 5-12.

During splitting, trypsin is stressful for the cells and as a response to this stress they initially decrease in size and become rounded. Occasionally, such a stress response was still observed in cells 4 hr after a split, but not after 24 or 72 hr. Round cells observed at a later time point were instead likely undergoing apoptosis. Careful notice was taken to only include fully recovered cells in the evaluation process.

For visualization of NO-production, cells of both the UROtsa and T24 cell lines were trypsinized and cultured in 3 mL of the corresponding cell media in 60 mm non-cytotoxic tissue culture dishes (Sarstedt, Newton, USA). Once the cells reached a confluency of 70%, they were treated with either phosphate-buffered saline (PBS; serving as control), the beta-adrenoceptor agonist dobutamine ( $1 \times 10^{-4}$  M; Tocris Bioscience, Bristol, UK), the alpha-adrenoceptor agonist phenylephrine ( $5 \times 10^{-4}$  M; Sigma-Aldrich), the non-selective muscarinic agonist methacholine ( $1 \times 10^{-4}$  M; Sigma-Aldrich), the P2 purinoceptor agonist adenosine 5'-triphosphate (ATP;  $5 \times 10^{-5}$  M; Sigma-Aldrich), the P1 purinoceptor agonist adenosine ( $5 \times 10^{-5}$

M; Sigma-Aldrich), the non-selective muscarinic antagonist atropine ( $1 \times 10^{-4}$  M; Sigma-Aldrich), the alpha-adrenoceptor antagonist phentolamine ( $5 \times 10^{-5}$  M; Sigma-Aldrich), the  $\beta_{1/2}$ -selective adrenoceptor antagonist propranolol ( $1 \times 10^{-5}$  M; Sigma-Aldrich) or the  $\beta_3$ -selective adrenoceptor antagonist L-748,337 ( $1 \times 10^{-5}$  M; Tocris Bioscience). The chosen drug concentrations were based on pilot studies and previous viability data from MTT cell proliferation assays [26]. After 24 hr, the cells were treated with 1,2-diaminoanthraquinone (DAQ; 1.5  $\mu$ g/mL) for 30 min. DAQ actively scavenges NO, forming a red fluorophore which can be visualized with a rhodamine filter under a fluorescent microscope. During the final 20 min. of DAQ staining, for staining of the nucleus, DAPI (Life Technologies, Carlsbad, USA; 1 drop/mL of media) was added. Next, the cells were rinsed with pre-heated ( $37^\circ\text{C}$ ) PBS and fixed for 15 min. at room temperature with 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M phosphate buffer (500  $\mu$ L/well) containing Alexa Fluor 488 phalloidin (Life Technologies), which stains actin filaments allowing for visualization of the cytoskeleton. After fixation, the cells were rinsed two times with PBS and were subsequently stored in 2 mL PBS until further use.

For visualization of NOS and receptor protein expression, cells of both the UROtsa and T24 cell lines were trypsinized and plated in 12-well plates (VWR International, Leuven, Belgium) fitted with a cover glass (15x15 mm; Mänzel, Braunschweig, Germany) at 300,000 cells/mL in the wells containing cells that were cultured for 24 hr and 100,000 cells/mL in the wells containing cells that were cultured for 72 hr. Pilot experiments were conducted in order to assure that the amount of plated cells would generate a similar final amount of cells per cover glass after 24 and 72 hr, respectively. After a recovery period of 4 hr, the cells were treated for 0, 24 or 72 hr with either PBS (serving as control), dobutamine ( $1 \times 10^{-4}$  M) or methacholine ( $1 \times 10^{-4}$  M). In the wells containing cells that were treated for 72 hr, the media (including drugs) was renewed every 24 hr. After the treatment period, the cells were rinsed with preheated PBS and fixed for 15 min. at room temperature with 4% PFA in 0.1 M phosphate buffer (500  $\mu$ L/well). Thereafter, the cover glasses (still placed in the 12-well plates) were rinsed for 3 x 10 min. in PBS. This was followed by a 1-hr incubation in 5% normal goat or horse serum (Vector Laboratories, Burlingon, CA, USA) and 0.25% Triton X-100 in PBS (350  $\mu$ L/well). The cells were subsequently incubated overnight with primary antibodies (500  $\mu$ L/well) against eNOS (1:2000, mouse; AB76198, Abcam, Cambridge, UK), iNOS (1:2000, mouse; AB178945, Abcam), muscarinic M1 (1:2000, goat; AB77098, Abcam), muscarinic M2 (1:2000, rat, MAB367, Merck-Millipore, Temecula, CA),

muscarinic M3 (1:1000, rabbit, AB9018, Merck-Millipore), muscarinic M4 (1:1000, rabbit, AB189432, Abcam), muscarinic M5 (1:2000, mouse, AB167567, Abcam),  $\alpha_1$  adrenergic (1:2000, rabbit, AB3462, Abcam),  $\beta_1$ -adrenergic (1:5000, rabbit, AB3442, Abcam),  $\beta_2$ -adrenergic (1:3000, rabbit, AB61778, Abcam) or  $\beta_3$ -adrenergic receptors (1:1000, chicken, AB59685, Abcam). On the following day, the cells were rinsed for 3 x 10 min. in PBS and further incubated for 2 hr with the appropriate secondary antibody (350  $\mu$ L/well; goat anti-rabbit Alexa 488 (A11034) and Alexa 568 (A11036); goat anti-mouse Alexa 488 (A11029) and Alexa 568 (A11031); goat anti-rat Alexa 488 (A11006) and Alexa 568 (A11077); goat anti-chicken Alexa 488 (A11039); and donkey anti-goat Alexa 568 (A11057); all 1:1000; Life Technologies/Thermo Fisher Scientific, Waltham, MA). Finally, the cells were rinsed for 3 x 10 min., the cover glasses with the grown cells were carefully removed from each well and placed on a non-coated glass slide, then cover-slipped using Prolong Gold antifade reagent with DAPI (P36931, Life Technologies/Thermo Fisher Scientific).

All immunostainings and DAQ stainings were visualized using a Nikon 90i fluorescence microscope with appropriate filters for Alexa 488, Alexa 568 and DAPI, fitted with a DS-Fi camera and the NIS element imaging Software v.4.40 (Nikon Corporation, Tokyo, Japan). In order to compare the staining intensities between the treatment groups, all images were captured using the same settings, including exposure time, contrast settings and digital gain.

NO is formed by conversion of L-arginine by NOS. In a biological environment, NO is rapidly converted to nitrite and nitrate [27]. Therefore, a common way to quantitatively measure release of NO is by measuring the total amount of nitrite and nitrate. Currently, supernatant samples were collected and two different methods to quantitatively measure the release of NO from the urothelial cells were utilized.

Cells of both cell lines were plated in T25 flasks (VWR International) at 100,000 and 180,000 cells/mL of UROtsa and T24 medium, respectively. After a recovery period of 24 hr, a confluency of approximately 90% was obtained. Pilot experiments were conducted in order to find the optimal seeding density. At this seeding density, the cells are not fully contact-inhibited but cannot proliferate to a significant degree, which yields metabolically active cells that do not replicate extensively. Subsequently, the cells were treated for 0, 24 or 72 hr with either PBS (serving as control), dobutamine ( $1 \times 10^{-4}$  M), methacholine ( $1 \times 10^{-4}$  M), propranolol ( $1 \times 10^{-7}$  -  $1 \times 10^{-5}$  M) or L-748,337 ( $1 \times 10^{-7}$  -  $1 \times 10^{-5}$  M). Supernatant samples for the measurement of nitrite levels were collected at each time point. In the flasks that contained cells that were treated for 72 hr, the media (including drugs) was renewed every 24

hr. A careful cell count was performed after each treatment in order to ensure that significant proliferation or cell death had not occurred.

The Griess diazotization reaction is a common way to quantitatively determine levels of NO by measuring nitrite and nitrate [27]. For this purpose, a Griess reagent kit (Thermo Fisher Scientific) was used. Briefly, the kit utilizes a two-step reaction in which first the nitrate content of all samples is reduced to nitrite by addition of vanadium-HCl (200 mg vanadium in 25 mL 1 M HCl; 130  $\mu$ L of vanadium-HCl per sample). Then, 20  $\mu$ L of the Griess reagent, which was made by mixing equal volumes of N-(1-naphthyl)ethylenediamine and sulfanilic acid, was added to all samples in separate wells of a 96-well microplate. The mixture was thereafter incubated for 30 min. at room temperature, letting the components undergo the Griess reaction. A spectrophotometric microplate reader (Spectramax 340pc, Molecular Devices, Synnavale, CA) was used to measure the absorbance of the nitrite-containing samples relative to the reference samples at a wavelength of 548 nm.

Although sensors exist for the monitoring of NO, these are often utilized directly for real-time monitoring of production from tissues or cells. In our study, we wanted to gain an understanding of how release of NO varied following pharmacological interventions over longer time frames (e.g. 24-72 hr), and therefore the net amount of nitrite production provides a suitable stable indirect marker of NO production. Composite MWCNT microelectrodes (for details, see Fagan-Murphy *et al.* (2016)) were used for the detection of nitrite. Initially, the oxidation peak potential for nitrite was obtained using differential pulse voltammetry. This was used to define the voltages utilized for amperometric recordings. For measurements, all samples were collected as previously described and amperometric measurements were carried out for 30 sec. at 0.8 V and 1.2 V against a Ag/AgCl reference electrode. A stainless-steel rod was utilized as the counter electrode. The difference in the current between the two voltages was used to determine the amount of nitrite present in the various samples. To convert from current values to molar amount, the following conversion formula can be used: amount (pmoles) = current value (nA) x 83.717.

To confirm the immunocytochemical changes regarding NOS expression, UROtsa cells were cultured in a T75 flask and treated with dobutamine ( $1 \times 10^{-4}$  M), methacholine ( $1 \times 10^{-4}$  M) or PBS for 24 or 72 hr. The cells were subsequently lysed in phosphate EDTA buffer containing 5  $\mu$ M sodium fluoride, 1.5 mM aprotinin, 0.1 mM leupeptin, 0.2  $\mu$ M sodium vanadate, 0.1 mM pepstatin, 5 mM CHAPS, 4 mM Pefablock SC, 6.4 mM deoxycholic acid and 2.5% triton X-100. The lysate was thereafter freeze-thawed for three cycles at  $-80^{\circ}$ C and

centrifuged at 10,000 x g for 10 min. The pellet was discarded and the Pierce BCA protein assay kit (Thermo Fisher Scientific) was used for protein determination of the supernatant. Briefly, the protein assay utilizes the reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  by protein in an alkaline medium. The formation of  $\text{Cu}^{1+}$  can be detected by adding a reagent containing bicinchoninic acid, which forms a purple-coloured reaction product. The protein content of each sample was quantified by comparison to a standard curve of diluted bovine serum albumin. The quantified protein samples were mixed with NuPAGE LDS sample buffer (Thermo Fisher Scientific) and heated to 70°C for 10 min. to allow for denaturation of the proteins. After dilution with distilled water, 12.5 µg of protein from each sample was loaded onto a NuPAGE Novex 4-12% bis-tris gel (Thermo Fisher Scientific). For determination of the molecular weights of the proteins, two wells were loaded with MagicMark (5 µL; Thermo Fisher Scientific). The PowerPac high-current power supply (Bio-Rad, Hercules, CA) and XCell SureLock mini-cell electrophoresis system (Thermo Fisher Scientific) were used for the immunoblotting procedure upon which the protein samples were electrophoretically separated and subsequently transferred to a PVDF membrane (Thermo Fisher Scientific). After the transfer, the membrane was blocked for one hour with TBS-T containing 5% non-fat milk. Thereafter, the membrane was incubated overnight at 4°C with a polyclonal primary antibody against  $\beta_1$ -adrenoceptors (1:4000; AB3442; Abcam),  $\beta_2$ -adrenoceptors (1:2000; AB61778; Abcam),  $\beta_3$ -adrenoceptors (1:1000; AB59685; Abcam), eNOS (1:1000; AB76198; Abcam), iNOS (1:1000; AB178945; Abcam) or beta-actin (1:2000; Sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA). All antibodies were diluted in TBS-T containing 5% non-fat milk. The following day, the membranes were incubated for one hour at room temperature with a HRP-conjugated secondary antibody (1:4000 in TBS-T containing 5% non-fat milk) targeted against the species in which the primary antibody was raised (Goat ant-rabbit, #656120; Goat anti-mouse, #626520; and Goat anti-chicken, #31401; Thermo Fisher Scientific). Finally, the ECL Select western blotting detection reagent (GE Healthcare Life Sciences, Marlborough, MA) was used to develop the membrane and the chemiluminescence was detected in a Fujifilm LAS-1000 Intelligent Dark Box with Image Reader Pro v2.6 software (Fujifilm Corporation, Tokyo, Japan). After development, the membrane was occasionally stripped of antibody by incubation for 15 min. with Restore Western Blot stripping buffer (GE Healthcare Life Sciences). No membrane was stripped more than twice.

All values are expressed as mean±SEM. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Tukey correction for multiple comparisons. P-

values <0.05 were regarded as statistically significant. Statistics and graphs were generated and parameters computed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA) and Adobe Illustrator CS6, v. 16.0.0 (Adobe Systems, San Jose, USA). For publication purposes, figs. 1 and 2 were digitally enhanced using Photoshop v CS6 (Adobe Systems, San Jose, USA). All images were enhanced in an identical way.

## Results

In untreated cells of both the UROtsa and T24 urothelial cell line, DAQ staining showed that NO predominantly is produced in the close vicinity of the nucleus, as compared to the rest of the cytosol (Fig. 1A, 1G). However, upon treatment with dobutamine, the pattern of production of NO was altered in both cell lines. As a result, not considering the intensity of the stain but instead the staining pattern, the production of NO seemed to be more evenly spread throughout the cytosol (Fig. 1B, 1H). The pattern of production of NO was not affected by treatment with methacholine, phenylephrine, ATP, adenosine or any of the antagonists. Separate from this, we could also observe small, rounded double-cells with a high level of NO production that were identified as dividing cells.

In accordance with the observations of the DAQ staining, we observed a similar pattern of expression of iNOS and eNOS antibody staining in untreated urothelial cells, namely a predominant expression in the close vicinity of the nucleus (Fig. 2A, 2C, 2G). Dobutamine treatment altered the pattern of expression of eNOS (Fig. 2D, 2H), and possibly also iNOS (Fig. 2B, 2H), causing a more even distribution throughout the cytosol. Visually, the data also suggest a higher expression of eNOS, and possibly lower expression of iNOS, upon dobutamine treatment in UROtsa, but not T24, cells. This was also indicated by western blot, which showed that dobutamine treatment (72 hr) of UROtsa cells seems to decrease the level of expression of iNOS but increase the level of expression of eNOS (Fig. 3).

Immunocytochemical studies revealed that both cell lines expressed adrenoceptors and muscarinic receptors. In line with previous findings in human urothelial cells [29-31], we could observe expression of  $\alpha_1$ -adrenoceptors, all three subtypes of beta-adrenoceptors ( $\beta_{1-3}$ ) and all five subtypes of muscarinic receptors (M1-M5) (*figures not shown*). Visually, the level of expression or pattern of expression of the receptors did not seem to be altered by treatment with dobutamine or methacholine. Further, western blot experiments did not show

any change in the expression of beta-adrenoceptors in either cell line after treatment with dobutamine or methacholine (*data not shown*).

Measurements with an amperometric selective nitrite sensor showed that treatment with dobutamine increased the release of NO from both cell lines; from  $3.74 \pm 0.35$  nA ( $0.31 \pm 0.029$  nmol) to  $23.52 \pm 2.10$  nA ( $1.97 \pm 0.18$  nmol) and  $9.55 \pm 1.42$  nA ( $0.80 \pm 0.12$  nmol) to  $39.01 \pm 2.91$  nA ( $3.27 \pm 0.24$  nmol) in UROtsa (Fig. 4A) and T24 (Fig. 4B), respectively. In contrast, methacholine did not significantly increase the release of NO from either of the cell lines. These findings were confirmed by measurements with a Griess reagent assay (Fig. 4C and 4D). The data from the control (PBS) groups in the sensor experiments show that the production of NO is stable over time (0-72 hr) in human urothelial cells in culture. In the Griess measurement, one can observe a gradual decrease of the amount of released nitrite over time. This decrease is most likely due to a higher proliferation rate during these experiments, causing contact inhibition. However, all Griess samples were handled and collected in the same manner, providing a viable comparison between the treatment groups. Both propranolol ( $\beta_{1/2}$ -selective adrenoceptor antagonist) and L-748,337 ( $\beta_3$ -selective adrenoceptor antagonist) dose-dependently blocked the dobutamine-induced release of NO from UROtsa cells (Fig. 5A). Interestingly, propranolol blocked the dobutamine-induced NO release at a lower concentration than L-748,337. Meanwhile, while propranolol dose-dependently blocked the dobutamine-induced release of NO from T24 cells, L-748,337 could lower but not completely block the NO release in the concentrations used (Fig. 5B).

## Discussion

In the current study, we have shown that NO release from human urothelial cells, as well as production, is regulated by beta-adrenoceptor stimulation. This is in line with previous findings in other species [14, 15]. However, a similar comprehensive examination of receptor-induced regulation of nitric oxide has not previously been conducted in human urothelial cells. Contrarily, the muscarinic receptor agonist methacholine did not alter the release of NO from neither immortalized normal nor malignant human urothelial cells, which is in slight contrast with previous findings in rats [10, 32]. However, one must acknowledge the possibility that the source of NO in the previous studies could be other than the urothelium (*i.e.* the sub-urothelium). Further, some of the previous observations were made only during inflammation and seemed to be dependent on an increased expression of

muscarinic M5 receptors [11]. In the current study, no changes in autonomic receptor expression could be observed. The differences between the current and previous findings thus strengthen the proposed idea that the regulation of NO alters during certain disease states. In turn, the current and previous findings indicate that both the sympathetic and parasympathetic divisions of the autonomic nervous system play a part in the regulation of NO in the urinary bladder *in vivo*.

By utilizing DAQ, a NO scavenger, we could show that the pattern of production of NO was altered by beta-adrenoceptor activation. A similar change in pattern of production of NO has not previously been demonstrated in human urothelial cells. This is likely due to the fact that most previous studies have focused on the expression of NO synthase (NOS), and not NO *per se*. It is important to note that we have utilized DAQ staining to conduct a qualitative evaluation of the pattern of production of NO and not quantitatively measure the total amount of fluorescence. A quantitative measurement could possibly even be misleading since it has not been shown that NO can be stored intracellularly. Separate from the changes in NO production seen during receptor activation, the increase in fluorescence that is observed in dividing and apoptotic cells is likely due to the decreased surface volume of the cells, concentrating the amount of fluorescence to a smaller area while not affecting the amount of fluorescence *per se*. However, this question should be addressed in a future study in which a quantitative measurement of the amount of NO in healthy, dividing and apoptotic cells is conducted.

The fact that the dobutamine-induced release of NO could be dose-dependently blocked by the presence of propranolol or L-748,337 shows that the increased release is beta-adrenoceptor-specific. Even though an increased proliferation could be part of the cause for the increased release, careful cell counts were performed, none of which indicated a significant amount of proliferation. While propranolol is considered a  $\beta_{1/2}$ -selective antagonist, L-748,337 is mainly  $\beta_3$ -selective ( $K_i$  values of 4.0, 204 and 390 nM for  $\beta_3$ -,  $\beta_2$ - and  $\beta_1$ -adrenoceptors, respectively; [33]). Expression of all three subtypes of beta-adrenoceptors has previously been shown in the human urothelium [30, 34]. Our data show that propranolol is a more potent antagonist of dobutamine-induced release of NO than L-748,337, especially in the T24 cells (Fig. 5). Considering the selectivity profiles of beta-adrenoceptor antagonists and the fact that L-748,337 should be less selective at its highest concentration, our data might imply that the observed NO release is mainly regulated by either  $\beta_1$ - or  $\beta_2$ -adrenoceptors.

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One can assume three different possible mechanisms, or any combination of these, regarding the long-term increase in NO release upon beta-adrenoceptor activation. The first possible mechanism is an increase in the expression of beta-adrenoceptors. However, for this to occur, longer stimulation periods are usually required. Nevertheless, we examined this possibility, without finding any evidence of an increased receptor expression. The second possibility is an up-regulation of the expression of any of the NOS isoforms. Many reports have described such a mechanism during inflammatory and malignant disease states [2, 8, 35], and we could see an up-regulation of eNOS and possible down-regulation of iNOS in UROtsa cells after 72 hr of dobutamine treatment. We could not, however, see the same changes in the T24 cells. The remaining, third, possible mechanism is an increase in the activity of NOS. Considering that NO cannot be stored intracellularly, NOS activity should be reflected by changes in the levels of extracellular nitrate. Therefore, the increase we observe in NO release can be considered a demonstration of either increased NOS activity *per se*, an up-regulation of NOS expression or a combination of both.

Apart from the previously described mechanisms, one must consider the presence of a NO pool, which would not require any increase in expression or activity of NOS or beta-adrenoceptors. A few reports have been published which either show or discuss the possibility of a NO pool [36]. Even though this tentatively could be part of the explanation for the increase in NO release that we observe in our acute (24-hr) experiments, the sustained increase over 72 hr indicates that it is unlikely to be the main mechanism in place.

When studying human bladder contraction, it has been shown that the main functional receptors are of the muscarinic M3 and  $\beta_3$ -adrenoceptor subtypes, with M3 activation causing contraction and  $\beta_3$  activation causing relaxation of the detrusor [37-39]. Over-active bladder (OAB) has been treated for decades with antimuscarinic drugs, with limited success. Recently, mirabegron, a selective  $\beta_3$ -agonist, was approved for the treatment of OAB in combination with antimuscarinics [40]. Early on, it was suggested that  $\beta_3$ -agonists exert their relaxatory effect via release of NO [41]. At this time, it was known that in certain disease states, *i.e.* cystitis, activation of urothelial muscarinic receptors, most likely of the M5 subtype, can cause release of NO which has an inhibitory effect on rat detrusor contraction [11]. However, despite the known relaxatory effects of beta adrenoceptor-induced release of NO in other smooth muscle tissue, *i.e.* arteries, there has not yet been proven to be a clear link between  $\beta_3$ -adrenoceptor activation, NO and detrusor relaxation [42]. Further, the urothelium seems to exert an inhibitory influence on  $\beta_3$ -adrenoceptor-induced detrusor

relaxation which is not dependent on release of NO from the urothelium [30, 43]. One possible physiological effect of beta-adrenoceptor-induced release of NO from urothelium may be as a modulator of afferent signalling [44]. However, when discussing the possible physiological importance of beta-adrenoceptor-induced NO release from the urothelium and how this might apply to current pharmacological treatments, one must take the selectivity differences between mirabegron and dobutamine into consideration. Nevertheless, this highlights the importance of future functional experiments outlining the exact role of beta-adrenoceptor-induced release of NO from the urothelium.

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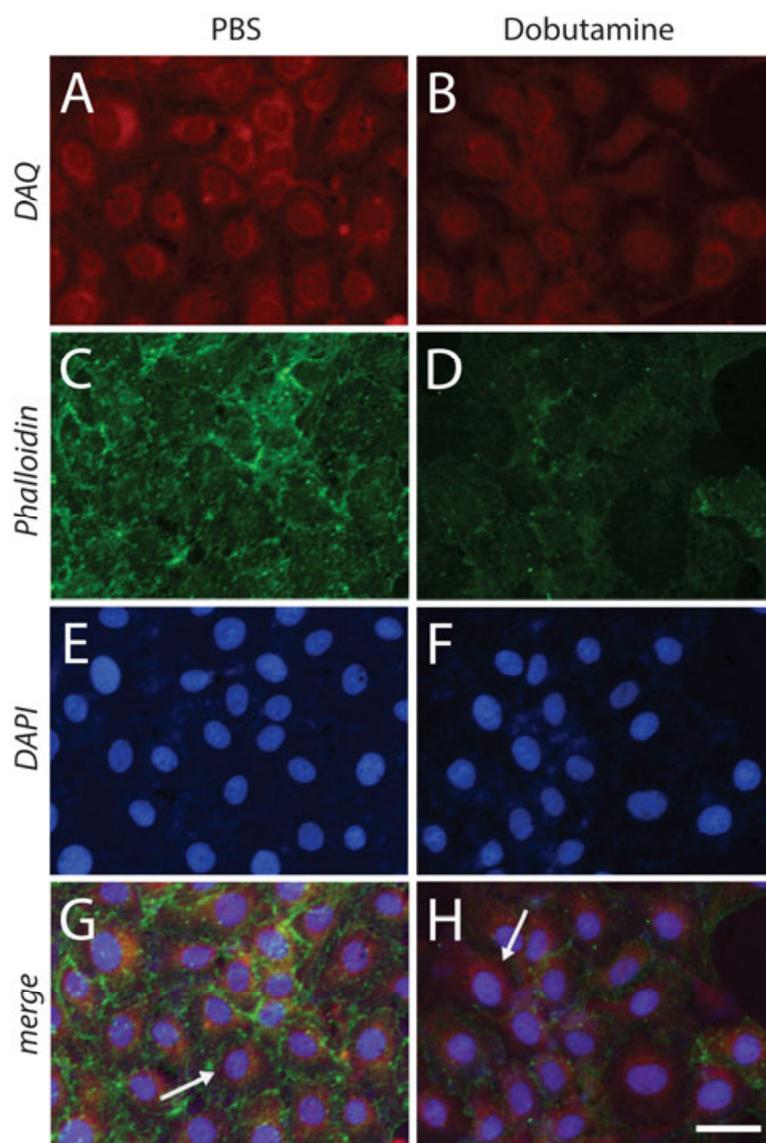
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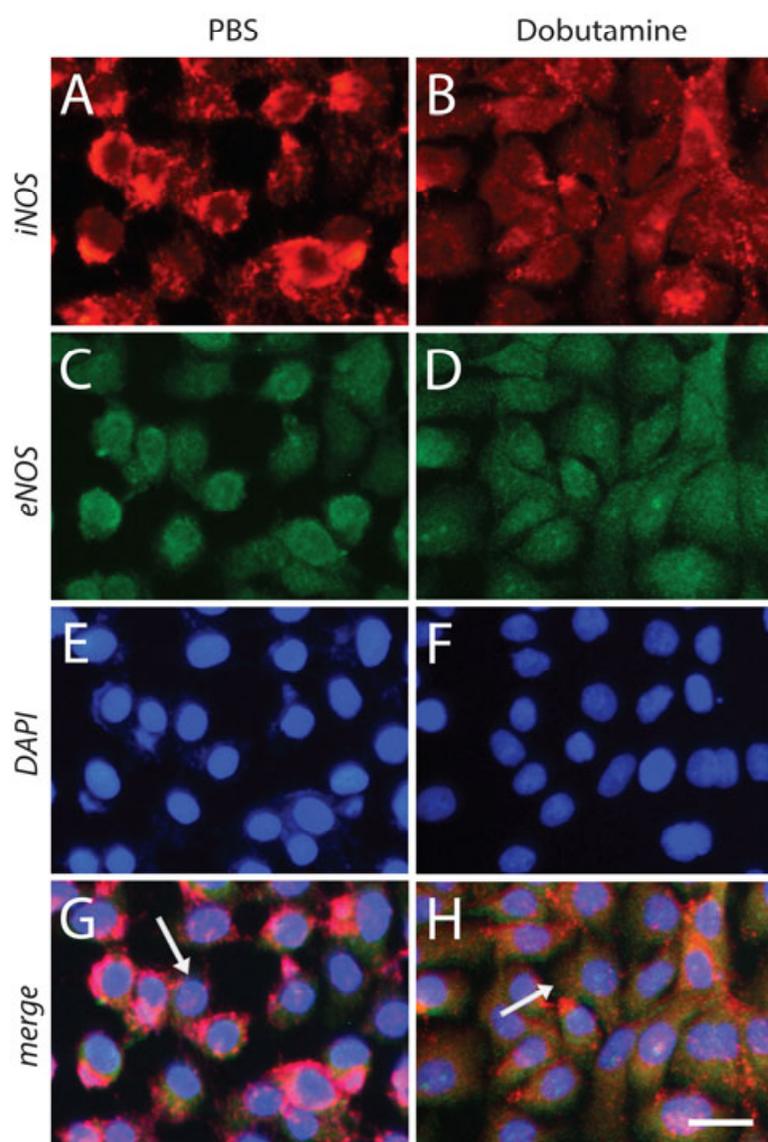
Winder et al., Figure 1



*Fig 1. Representative immunocytochemical images of nitric oxide production in PBS (control) and dobutamine treated UROtsa cells.*

The production of NO was visualized by staining with DAQ (A, B), the cytoskeleton was visualized by staining with phalloidin (C, D) and the nucleus was visualized by DAPI-stain (E, F). The stainings show a predominant production of NO in the close vicinity of the nucleus in PBS-treated urothelial cells (G; control). The distribution pattern was altered following dobutamine treatment (H), where a more widespread production could be observed throughout the cytosol. Arrows indicate distinctive points of observation. Scale bar: 25  $\mu$ m.

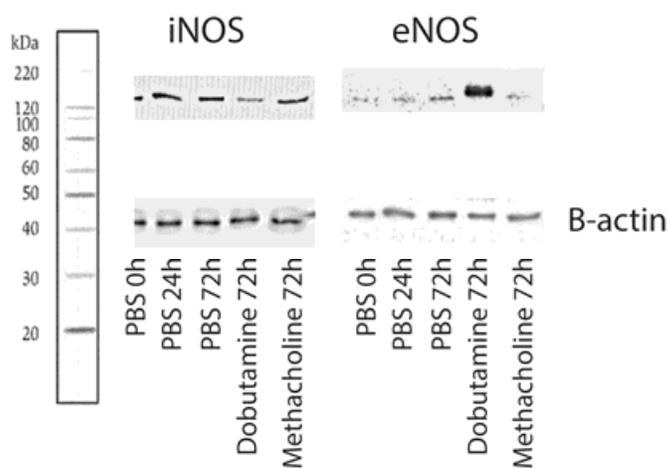
Winder et al., Figure 2



**Fig 2. Representative immunocytochemical images of nitric oxide synthase expression in PBS (control) and dobutamine-treated UROtsa cells.**

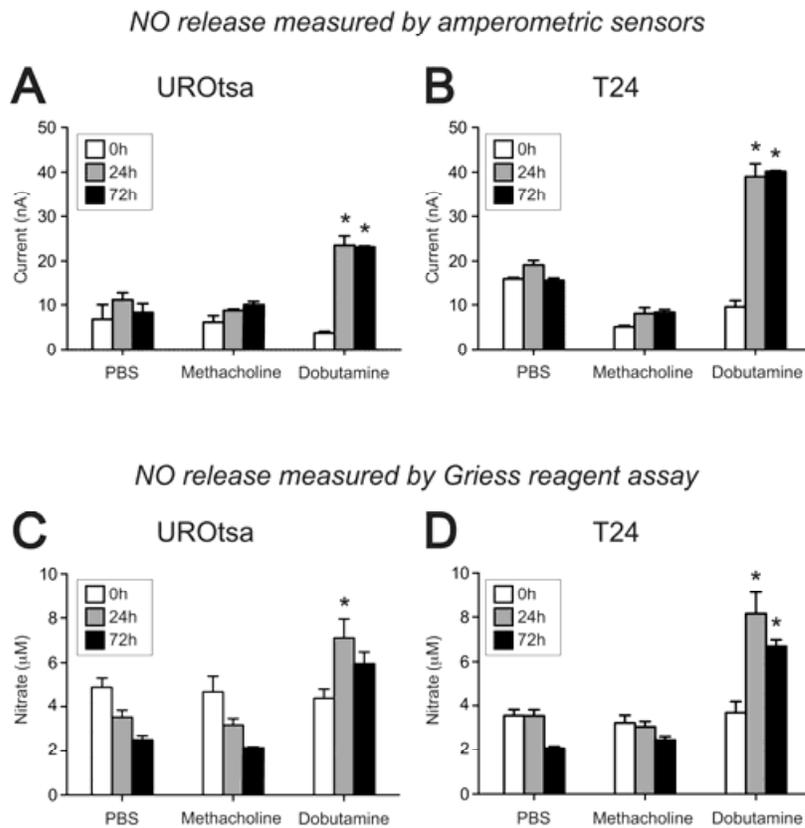
The expression of iNOS was visualized in red (A, B), the expression of eNOS was visualized in green (C, D) and the nucleus was visualized in blue by DAPI-stain (E, F). The iNOS and eNOS expression, similar to NO in the DAQ staining, show a predominant expression in the close vicinity of the nucleus in PBS-treated (control) cells (A, C, G). The distribution pattern of eNOS, and possibly also iNOS, was altered following dobutamine treatment, showing a more even distribution pattern throughout the cytosol (B, D, H.) Arrows indicate distinctive points of observation. Scale bar: 25  $\mu$ m.

### Winder et al., Figure 3



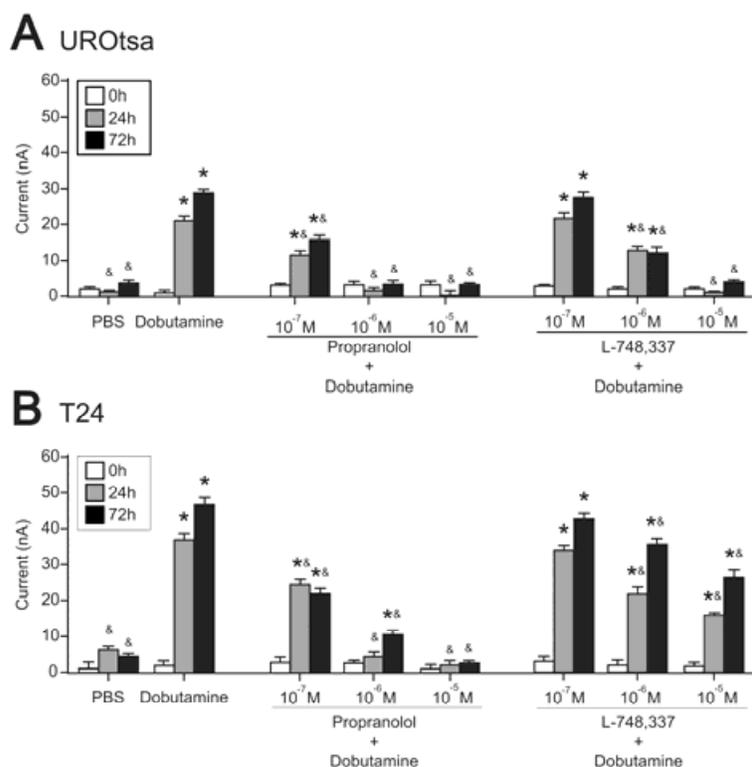
**Fig 3. Western blot image of nitric oxide synthase (NOS) expression in human urothelial cells.**

iNOS (top left panel) and eNOS (top right panel) expression in UROtsa cells after PBS (control), dobutamine or methacholine treatment. Bottom panels show the corresponding beta-actin expression in the two blots. The beta-actin blots were run in succession after stripping the membranes of the iNOS and eNOS antibodies.



**Fig 4. Receptor-induced nitric oxide (NO) release from human urothelial cells.**

Measurement of NO release from UROtsa and T24 cells after 0, 24 and 72 hr of treatment with PBS, methacholine or dobutamine utilizing amperometric sensors (A and B) and Griess reagent assay (C and D). While the methacholine treatment did not affect the NO release, the dobutamine-treated UROtsa (A) and T24 (B) cell lines displayed a significantly increased release at 24 hr, which was sustained over 72 hr. These changes were also confirmed by the Griess reagent assay in both cell lines (UROtsa, C; T24, D). \* denotes a significant increase compared to the corresponding 0-hr time-point. Two-way ANOVAs followed by a Tukey post-hoc test, A:  $F(2,30)=28.85$ ,  $n=6$ ,  $p<0.0001$ ; B:  $F(2,30)=72.52$ ,  $n=6$ ,  $p<0.0001$ ; C:  $F(2,27)=5.19$ ,  $n=4$ ,  $p=0.012$ ; and D:  $F(2,27)=9.67$ ,  $n=4$ ,  $p=0.0007$ .



**Fig 5. Inhibition of receptor-induced nitric oxide (NO) release from human urothelial cells.**

Utilizing amperometric sensors, the effect of selective beta-adrenoceptor antagonists on dobutamine-induced NO release was evaluated in both UROtsa (A) and T24 (B). In both UROtsa and T24 cells, the data demonstrate concentration-dependent inhibition of NO release in the presence of the  $\beta_{1/2}$ -selective adrenoceptor antagonist propranolol as well as the  $\beta_3$ -selective adrenoceptor antagonist L-748,337. In UROtsa cells (A), propranolol and L-748,337 completely blocked the dobutamine-induced NO release at a concentration of 10<sup>-6</sup> M and 10<sup>-5</sup> M, respectively. Similarly, in T24 (B) cells, propranolol completely blocked dobutamine-induced NO release at a concentration of 10<sup>-6</sup> M (after 24 h) or 10<sup>-5</sup> M (after 72 hr). However, L-748,337 only partly blocks the dobutamine-induced NO release in T24 cells. \* denotes a significant increase compared to the corresponding 0-hr time-point; & denotes a significant difference from dobutamine treatment at the respective time-points.  $n = 6$  in all groups. Two-way ANOVAs followed by a Tukey post-hoc test, A: Treatment:  $F(7,120)=115$  and Time:  $F(2,120)=182$ , both  $p<0.0001$ ; B: Treatment:  $F(7,120)=204$  and Time:  $F(2,120)=667$ , both  $p<0.0001$ ).