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AUTONOMIC RECEPTOR REGULATION OF RELEASE AND PRODUCTION OF NITRIC OXIDE IN HUMAN UROTHELIAL CELLS

Hypothesis / aims of study

Over the past decade and a half, nitric oxide (NO) has been recognized as one of the signalling molecules in the urinary bladder. However, despite numerous data from studies in various species and disease states, many of the exact actions of NO are still unclear. It has been shown that activation of autonomic receptors, both muscarinic and β-adrenergic, can cause release of NO from urothelial cells and alterations in the expression of NO synthase. However, the quantitative details over time for this have not been fully outlined, neither has the actual intracellular production of NO upon receptor activation previously been visualized. 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. However, this requires the precise mechanisms of expression and release of NO to be unraveled. This study aimed to quantitatively and time dependently link activation of autonomic receptors to alterations in expression of NO synthase, intracellular production of NO and subsequent release of NO in two human urothelial cell lines. A pharmacological approach was utilized in order to determine the impact of the different autonomic receptors.

Study design, materials and methods

Two human urothelial cell lines, one immortalized normal (UROtsa) and one malignant (T24), were cultured and used for all experiments. These were treated for 4, 24 or 72 hours with either the non-selective muscarinic agonist methacholine, α-adrenoceptor agonist phenylephrine, β-adrenoceptor agonists dobutamine (β1/2) or isoproterenol (non-selective), non-selective muscarinic antagonist atropine or β-adrenoceptor antagonists propranolol (β1/2) or L-748,337 (β3). Supernatant samples for measurement of the amount of released NO were collected during the cultivation time period and after 0, 4, 24 or 72 hours the cells were either fixed for immunocytochemical staining or homogenized for western blot experiments.
Two principally different methods were used to quantitatively measure the release of NO – colorimetric assays utilizing the Griess reaction and a novel custom built amperometric nitrite selective sensor. Immunocytochemical staining was performed to observe expression patterns for endothelial and inducible NO synthase as well as for all five subtypes of muscarinic receptors (M1-5) and all five subtypes of adrenoceptors (α1/2, β1/2/3). Further, 1,2-diaminoanthraquinone (DAQ), a molecule that reacts with NO and forms a fluorophore that can be visualized with a rhodamine filter under a fluorescent microscope, was used to immunocytochemically visualize intracellular production of NO. Western blot was employed to detect expression of endothelial and inducible NO synthase as well as β-adrenoceptors (β1/2/3).

Results

Measurements with the nitrite sensor showed a significant increase in the release of NO in both the UROtsa and T24 cell lines upon treatment with dobutamine (at 24 h, from 24.24±0.86 to 44.02±5.14 and 30.05±3.47 to 59.51±7.12 nA in UROtsa and T24, respectively; n=6; $p<0,001$ in both groups). This increase remained constant during the duration of the experiments (at 72 h) and was confirmed by data from the colorimetric assays. However, neither methacholine nor the studied antagonists affected the release of NO from the urothelial cells.
When utilizing DAQ to study the production and distribution of NO, it seemed that untreated cells demonstrated a higher production of NO in close vicinity of the nucleus, as compared to the rest of the cytosol. Upon treatment with dobutamine, the production of NO seemed to first (at 4h) redistribute evenly throughout the cytosol and then (at 24 h) increase. This observation is in concordance with the NO release data. Surprisingly, treatment with methacholine caused a similar redistribution of NO. This, however, did not cause an increase in the release of NO. Neither phenylephrine nor any of the antagonists affected the distribution or production of NO.

The immunocytochemical stainings showed expression of endothelial NO synthase, inducible NO synthase, all five muscarinic receptors (M1-5) and all five subtypes of adrenoceptors (α1/2, β1/2/3) in both cell types. Interestingly, in untreated cells, the expression of endothelial and inducible NO synthase seemed to be concentrated to the area in close vicinity of the nucleus. This pattern was altered by treatment with dobutamine for 24 or 72 hours, which seemed to induce a more even distribution of the enzymes throughout the cytosol.
While the expression of all receptor subtypes remained unchanged by all drugs during the 72 hour treatment period, Western blot experiments showed that dobutamine treatment during 72 hours, but not 24 hours, caused an increase in the expression of endothelial NO synthase and a tendency towards a decrease in the expression of inducible NO synthase in UROtsa cells.

Interpretation of results

Our data support previous findings that activation of β-adrenoceptors causes alterations in the expression of NO synthase and the release of NO. In this study we could link these alterations to changes in the pattern of production of NO as well as quantify the release of NO over time. Interestingly, the amount of released NO remained constant during the entire studied time period, despite alterations in the expression pattern of NO and NO synthase.
Even though we could see an effect of muscarinic receptor activation on the pattern of production of NO, this did not affect the amount of released NO. This finding is interesting when compared to data in the literature which show that muscarinic receptor activation can lead to prominent release of NO during certain disease states.
Since none of the antagonists showed any effects on expression or release, one can draw the conclusion that the endogenous (however unstimulated) release of acetylcholine and noradrenaline is not sufficient to affect the NO pathways in urothelial cells.

Concluding message

Activation of β-adrenoceptors on both healthy and malignant human urothelial cells causes alterations in the pattern of expression of NO synthase and, subsequently, the pattern of production of NO. Further, it increases the level of expression of endothelial NO synthase, the rate of production of NO and the amount of released NO. Despite alterations in the pattern of production of NO, activation of muscarinic receptors does not seem to alter the release of NO from healthy or malignant urothelial cells.

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