Signaling and distribution of NPR-Bi, the human splice form of the natriuretic peptide receptor type B

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Hirsch, Jochen R., Nikola Skutta, and Eberhard Schlatter. Signaling and distribution of NPR-Bi, the human splice form of the natriuretic peptide receptor type B. Am J Physiol Renal Physiol 285: F370–F374, 2003.—Recently, we described a splice variant of the human natriuretic peptide receptor type B (NPR-Bi) in human proximal tubule cells [immortalized human kidney epithelial cells (IHKE-1) that lacks a functional guanylate cyclase domain (Hirsch JR, Meyer M, Mägert HJ, Forssmann WG, Mollerup S, Herter P, Weber G, Cermak R, Ankorina-Stark I, Schlatter E, and Kruhøffer M. J Am Soc Nephrol 10: 472–480, 1999). Its signaling pathway does not include cGMP, cAMP, or Ca2+, but leads to inhibition of K+ channels. In patch-clamp experiments, effects of tyrosine kinase receptor blockers on C-type natriuretic peptide (CNP)-mediated depolarizations of membrane voltages (Vm) of IHKE-1 cells were tested. The epidermal growth factor (EGF) receptor blocker genistein (10 μM) abolished the effect of CNP (0.2 ± 0.4 mV, n = 7), and comparable results were obtained with 10 μM daidzein (n = 8). Aminogenistein (10 μM, n = 5) and tyrphostin AG1295 (10 μM, n = 5) had no significant effects. EGF (1 nM) hyperpolarized cells by −5.3 ± 0.8 mV (n = 5). This effect was completely blocked by genistein or daidzein. The Cl−-channel blocker NPPB (10 μM, n = 5) inhibited the EGF-mediated hyperpolarization. mRNA expression of NPR-B and NPR-Bi shows reversed patterns along the human nephron. NPR-B is highly expressed in glomeruli and proximal tubules, whereas NPR-Bi shows strong signals in the distal nephron. Expression of NPR-Bi in the cortical collecting duct was also confirmed with immunohistochemistry. In other human tissues, NPR-B shows strongest expression in pancreas and lung, whereas in the heart and liver NPR-B is the dominating receptor. In conclusion, CNP inhibits an apical K+ channel in IHKE-1 cells independently of cGMP and so far this effect can only be blocked by genistein and daidzein. Tyrosine phosphorylation might be the missing link in the signaling pathway of CNP/NPR-Bi.

C-type natriuretic peptide; signal transduction; tyrosine kinase; patch clamp analysis; proximal tubule; kidney

Natriuretic peptides are structurally related peptides that bind to different receptors and display a variety of biological actions (6, 16). Although quite a lot is known about the actions of ANP and the related urodilatin that binds, similar to BNP, to the natriuretic peptide receptor (NPR)-A (GC-A), little is known about C-type natriuretic peptide (CNP) (1, 11, 12). CNP, which is found in the plasma and urine as a 53- and 22-amino acid ring-forming peptide, binds to the NPR-B (GC-B) receptor, which leads to an increase in intracellular cGMP (4). Recently, a splice variant of the NPR-B receptor was detected and described as NPR-Bi. This receptor carries a 71-bp insert on the mRNA level, leading to a frameshift and truncated protein when translated (8). Due to the generation of an early stop codon, the receptor lacks a functional guanylate cyclase domain and does not increase intracellular cGMP when activated by CNP (8). cGMP-independent signaling for natriuretic peptides has so far only been described for the NPR-C or “clearance” receptor that can influence the adenylate cyclase activity through G proteins (3, 13). We speculated that tyrosine phosphorylation might be the signaling pathway for the NPR-Bi receptor (7, 8).

It is known that phosphorylation processes regulate the activity of guanylate cyclase receptors (15). Recently, it could be shown for the guanylate cyclase receptor GC-C, which is bound by guanylin and uroguanylin, that not only serine/threonine-specific phosphorylation steps regulate guanylate cyclase receptors but also tyrosine phosphorylation (5).

In this study, we demonstrate that CNP inhibits K+ conductance independently of cGMP and that this effect can be blocked only by genistein and daidzein but not by the structurally related aminogenistein or another tyrosin kinase receptor blocker tyrphostin AG1295. Furthermore, we show the expression pattern of NPR-Bi in human tissues, especially in various segments of the human kidney.

MATERIALS AND METHODS

Cell culture. Immortalized human kidney epithelial (IHKE-1) cells (derived from human embryonic kidneys) were cultured as described previously (18). In short, IHKE-1 cells (passages 162-168) were grown in 50-ml tissue culture flasks (Greiner, Frickenhausen, Germany) in Dulbecco’s modified Eagle’s/F-12 medium (1:1) containing 15 mM HEPES, pH 7.3, 1.6 nM EGF, 100 nM hydrocortisone, 83 μM transferrin/insulin, 29 nM Na2SeO3, 10 mM NaHCO3, 20 mM l-glutamine, 1,000 U/l penicillin/streptomycin, and 1% fetal calf serum. Cells were incubated at 37°C in an atmosphere of 5% CO2-95% air. After 7 days, the confluent mono-
layers were trypsinized with Ca²⁺- and Mg²⁺-free phosphate-buffered saline and 0.05% trypsin-EDTA (Biochrom, Berlin, Germany). Cells grew polarized on glass coverslips, with the apical surface facing upward.

**Patch-clamp studies.** Coverslips with confluent IHKE-1 monolayers were mounted at the bottom of a perfusion chamber on an inverted microscope (Axiovert 10, Zeiss, Oberkochen, Germany). The perfusion chamber was continuously perfused at a rate of 10–20 ml/min at 37°C with a standard solution containing (in mM) 145 NaCl, 1.6 K₂HPO₄, 0.4 KH₂PO₄, 5 d-glucose, 1 MgCl₂, and 1.3 Ca²⁺-gluconate, pH 7.4. Cells were rinsed for at least 20 min before the electrophysiological measurements.

Membrane voltages (V_m) were measured with the slow whole cell patch-clamp technique. For this method, pipettes were filled with a solution containing (in mM) 95 K⁺-gluconate, 30 KCl, 4.8 Na₂HPO₄, 1.2 NaH₂PO₄, 5 d-glucose, 0.73 Ca²⁺-gluconate, 1 EGTA, 1.03 MgCl₂, and 1 ATP, pH 7.2. To this solution 162 μM nystatin was added before use. Patch pipettes had an input resistance of 2.5–12.5 MΩ. V_m was measured in the current clamp mode of a patch-clamp amplifier (WeKaGraph WK-250R, WKK, Kaltbrunn, Switzerland) and recorded continuously on a pen recorder (WeKaGraph WK-250R, WKK, Kaltbrunn, Switzerland).

**RT-PCR analysis.** Total RNA was isolated using the RNeasy-kit (Qiagen, Hilden, Germany). Isolated total RNA was incubated with 10 U DNase I (Promega, Heidelberg, Germany) at 37°C for 1 h to digest isolated traces of genomic DNA. RNA and DNase I were then separated by an additional cleanup step using a new RNeasy column. cDNA first-strand synthesis was performed in a total reaction volume of 30 μl containing 5 μg total RNA, 10 nM dNTP-Mix (Biometra, Göttingen, Germany), 1 nM p(dT)₁₀ nucleotide primer (Boehringer, Mannheim, Germany), and 200 U molony murine leukemia virus reverse transcriptase (Promega). Of each cDNA first-strand reaction mixture, 1/6 was then subjected to a 50-μl PCR reaction in a UNO II thermo cycler (Biometra) using 20 pmol of each primer and 1 U of Taq DNA polymerase (Qiagen). Reaction conditions were as follows: 3 min at 94°C, 30 s at 59°C, and 1 min at 72°C, 1 cycle; 30 s at 94°C, 30 s at the optimal annealing temperature (OAT), and 1 min at 72°C, 30 cycles; and 30 s at 94°C, 30 s at OAT, and 10 min at 72°C, 1 cycle. PCR reaction products were analyzed by agarose gel electrophoresis. Positive signals obtained from PCR experiments were sequenced by SeqLab (Göttingen, Germany). The following PCR primers were used (listed in 5’- to 3’-direction). The sequence is followed by the expected fragment length for the respective sense and antisense primer: NPR-Bi sense: GAC TCT CAC TCG TCA AGC CCT AGT CTC, NPR-Bi antisense: TTC AGC GCT GTA CCA TTA GAC TCC, fragment length: 169 bp, OAT = 60°C; NPR-B sense: GAG ACG ATT GGG GAT GTA AGT TA, NPR-B antisense: TTC AGC GCT GTA CCA TTA GAC TCC; fragment length: 277 bp, OAT = 60°C; GAPDH sense: CTG CCC CCT CTG CGT ATG, GAPDH antisense: GTC CAC CAC CCT GGT GCT GT, fragment length: 614 bp, OAT = 61°C.

**Immunocytochemistry.** Kryo slices of a human kidney were blocked for 15 min at room temperature with 1% blocking agent (Roche, Mannheim, Germany). The primary polyclonal antibody against NPR-Bi (Immundiagnostik, Bensheim, Germany) was incubated overnight at 4°C in a wet chamber. After three washing steps in PBS, the secondary antibody (goat-anti-rabbit, Vector, Burlingame, CA) was incubated for 45 min followed by three more washing steps in PBS. The kryo slices were then incubated for 45 min in streptavidin, Alexa Fluor 594 Conjugate (MobiTec, Göttingen, Germany) followed by five washing steps in PBS. Finally, the slices were covered with mounting media containing DAPI (Vector). Statistical analysis. Data are presented as means ± SE, with the number of experiments given in brackets. For statistical analyses, Student’s paired and unpaired two-sided t-tests were used. For paired comparisons, each effect was compared with its own averaged pre- and postexperiment controls. A P value <0.05 was considered significant and is indicated by an asterisk.

**RESULTS**

To verify if the cGMP-independent CNP-induced depolarization in the human proximal tubulus cell line IHKE-1 is specifically blocked by genistein or generally blocked by a variety of tyrosine kinase receptor blockers, we tested the epidural growth factor (EGF) receptor blocker genistein, its “inactive” form daidzein, the structurally related aminogenistein that is also an inhibitor of the p56lck tyrosine kinase, and the PDGF receptor blocker tyrphostin AG1295. In 57 patch-clamp experiments, the membrane voltage (V_m) of IHKE-1 cells was −42 ± 1 mV. In seven paired experiments, CNP (10 nM) depolarized V_m by 2.8 ± 0.4 mV. In the presence of genistein (10 μM), the CNP-induced depolarization was abolished (0.2 ± 0.4 mV). After the washout of genistein, CNP depolarized V_m again by
As a negative control, we used daidzein, the inactive form of genistein. To our surprise, daidzein (10 μM) reversed the CNP-induced depolarization (n = 8). Aminogenistein (10 μM, n = 5) and tyrphostin AG1295 (10 μM, n = 5) had no significant effect on the CNP-induced depolarization (Fig. 1). To verify the specificity of genistein and daidzein, both were tested on their inhibitory effect of the EGF-mediated signaling pathway. In five paired experiments, EGF (1 nM) hyperpolarized Vm by −5.3 ± 0.8 mV (Fig. 2). In the presence of genistein, this effect was fully inhibited to 1.2 ± 1.1 mV and after the washout of genistein also fully reversible (−4.1 ± 0.9 mV). Daidzein again mimicked the effect of genistein, clearly showing that this substance is not inactive in the human proximal tubule cell line IHKE-1 (Fig. 2). Although the CNP-induced depolarization is due to the inhibition of a K+ channel (7–9), the EGF-induced hyperpolarization is due to the inhibition of a Ca2+-dependent Cl− channel since the Cl− channel blocker NPPB (10 μM) blocked the EGF effect on Vm (Fig. 2). Other Cl− channel blockers, such as DIDS and SITS, and Na+ channel blockers, such as amiloride, had no significant effect on the EGF-mediated hyperpolarization (data not shown). EGF also did not interact with CNP as there was no significant effect of EGF on the CNP-induced depolarization (2.6 ± 0.4 vs. 2.7 ± 0.6 mV, n = 6). This demonstrates that EGF and CNP act through independent pathways as shown in a simplified cell model in Fig. 3.

Because we were interested in the distribution of NPR-Bi and NPR-B in the human kidney, we performed RT-PCR on isolated glomeruli and tubules to check for their expression pattern along the nephron. Figure 4 shows the interesting result. Although NPR-B is mainly and strongly expressed in glomeruli and proximal tubules, NPR-Bi is weakly expressed in proximal tubules, stronger in thick ascending limbs, and predominantly expressed in collecting ducts. Because the PCR was performed with the same mRNA/cDNA

![Image](http://ajprenal.physiology.org/)

**Fig. 3.** Simplified cell model of IHKE-1 cell. The basolateral membrane contains the Na+/K+ATPase that provides the driving force for the Na+-driven transport systems of the luminal membrane. Furthermore, a housekeeping K+ channel allows K+ recycling across the basolateral membrane. The exit pathways for the substrates are left out. The luminal membrane contains, besides the already mentioned Na+-coupled transport systems, an hIK-like K+ channel that is most likely responsible for the repolarization of these cells, thus establishing the driving force for the Na+-coupled transport systems. This Ba2+-inhibitable K+ channel is inhibited by CNP cGMP independently through the NPR-Bi receptor. This inhibition can be reversed by genistein. Genistein also blocks the EGF-mediated inhibition of a Cl− channel in the same membrane. Because this Cl− channel is not blocked by SITS or DIDS but NPPB, it is most likely a Ca2+-dependent intermediate-conductance outwardly rectifying Cl− channel (ICOR).

**Fig. 4.** mRNA expression patterns of natriuretic peptide receptor (NPR)-Bi and NPR-B in the human nephron amplified by RT-PCR. The intensity of the NPR-Bi signal increases toward the distal part of the human nephron, being strongest in the collecting duct, whereas the intensity of the NPR-B signals showed the opposite distribution, being strongest in the glomeruli. As a positive control, GAPDH was amplified for each segment (displayed is only one sample because the intensities of the GAPDH signal did not vary). As negative controls, all tests were performed in parallel with either no cDNA or no Taq-polymerase.

**Fig. 2.** Effects of genistein (n = 5), daidzein (n = 5), and NPPB (n = 5) on the EGF-mediated hyperpolarization of Vm of IHKE-1 cells. In paired experiments, EGF (1 nM) hyperpolarized Vm of IHKE-1 cells by 3–5 mV. This hyperpolarization was inhibited by the EGF receptor blocker genistein and its “inactive” form, daidzein, and the Ca2+-dependent Cl− channel blocker NPPB. *P < 0.05.
batches for NPR-B and NPR-Bi and each sample had its own GAPDH control, the data can be seen semi-quantitatively, meaning expression of NPR-B is increasing toward the proximal part of the nephron, whereas that of NPR-Bi is increasing toward the distal parts of the human nephron (Fig. 4). This result is also confirmed by immunocytochemistry, which shows a clear signal of the antibody against NPR-Bi in the luminal membrane of the collecting duct (Fig. 5). Due to the interesting reversed distribution pattern of NPR-B and NPR-Bi in the nephron, we performed RT-PCR with different human tissue samples to monitor the distribution of the two human receptors in other tissues. Two signals of NPR-Bi clearly stood out compared with NPR-B. The expression in lung and pancreas was dominated by NPR-Bi (Fig. 6). NPR-B expression was strongest in the heart and liver. Keeping in mind that lung, pancreas, and colon are the three tissues mostly affected in cystic fibrosis, we also performed PCR experiments with human colon tissues, confirming that both receptors are equally well expressed (data not shown).

DISCUSSION

CNP is known to bind the natriuretic peptide B (NPR-B/GC-B) receptor and increase intracellular cGMP. It is known to display biological actions in the cardiorenal system, bronchial tree and pulmonary vasculature, and the endocrine, gastrointestinal, and neuronal systems (4). Recently, it was shown that CNP can also act independently of cGMP by inhibiting a Ca\(^{2+}\)-dependent, intermediate-conductance, hIK1-like K\(^+\) channel in human IHKE-1 cells derived from the human renal proximal tubule (8). Besides the well-known NPR-B receptor, a splice variant (NPR-Bi) was found that is the dominant pathway for CNP in these cells. The NPR-Bi receptor lacks a functional guanylate cyclase domain, thus inhibiting the hIK1-like K\(^+\) channel cGMP independently. Furthermore, we were able to

NPR-Bi mRNA expression patterns of NPR-Bi and NPR-B in different human tissues amplified by RT-PCR. Although both receptors are equally well expressed in the heart, brain, placenta, and liver tissue, expression of NPR-Bi is clearly dominant in the lung and pancreas.

![Fig. 6. mRNA expression patterns of NPR-Bi and NPR-B in different human tissues amplified by RT-PCR. Although both receptors are equally well expressed in the heart, brain, placenta, and liver tissue, expression of NPR-Bi is clearly dominant in the lung and pancreas.](image)

![Fig. 7. Simplified cell model of IHKE-1 cell demonstrating the different cGMP-dependent and -independent pathways used by natriuretic peptides to block the hIK1-like K\(^+\) channel of the apical membrane. The basolateral membrane contains the Na\(^+\)-K\(^+\)-ATPase that provides the driving force for the Na\(^+\)-driven transport systems of the apical membrane. Furthermore, a housekeeping K\(^+\) channel in the basolateral membrane provides the stoichiometry. The exit pathways for the substrates are left out. The luminal membrane contains, besides the already mentioned Na\(^+\)-coupled transport systems, an hIK1-like K\(^+\) channel that is most likely responsible for the repolarization of these cells, thus establishing the driving force for the Na\(^+\)-coupled transport systems. This K\(^+\) channel is blocked directly by cGMP generated by ANP, BNP, or urodilatin through the NPR-A/GC-A receptor, by guanylin, uroguanylin, or STa through the GC-C receptor. Extracellular cGMP also inhibits this K\(^+\) channel. cGMP is released into the tubular lumen either by glomerular cells or by a newly described pump in proximal tubule cells. CNP blocks this K\(^+\) channel cGMP independently through NPR-Bi most likely by tyrosine phosphorylation, which can be blocked by genistein.](image)
show that neither Ca\(^{2+}\) nor cAMP played any role in the regulation of this K\(^{+}\) conductance (8). Due to the structure of the receptor and a recent report that demonstrated a key role for tyrosine phosphorylation in the activity of the related GC-C receptor (5), we tested various tyrosine kinase blockers. From these inhibitors, only genistein, a known inhibitor of the EGF receptor (2), and its inactive form, daidzein, were able to block the CNP-induced depolarization of membrane voltage. Neither the structurally related amino-genistein nor the PDGF receptor blocker tyrphostin AG1295 had any significant effects. Although genistein and daidzein also blocked the EGF-mediated hyperpolarization, they displayed no direct effect on the hIK1-like K\(^{+}\) channel itself (8). A direct interaction independent of tyrosine phosphorylation had been reported for some K\(^{+}\) channels, a Cl\(^{-}\) channel, and the Na\(^{+}\)-2Cl\(^{-}\)-K\(^{+}\) cotransporter (14, 19, 20). In IHKE-1 cells, daidzein was also not an inactive substance but acted like genistein. So far, these two substances are the only blockers capable of inhibiting the CNP-mediated signaling through NPR-Bi, which is most likely related to tyrosine phosphorylation. The hIK1-like K\(^{+}\) channel apparently plays an important role in these human proximal tubule cells because it can be blocked by ANP, BNP, and urodilatin cGMP independently through NPR-A/GC-A (9), by CNP cGMP independently through NPR-Bi (8), and by guanylin, uroguanylin, and STa cGMP dependently through GC-C (17). Furthermore, cGMP inhibits this K\(^{+}\) channel directly from the extracellular as well as the intracellular surface (10) as displayed in Fig. 7. The diverse expression pattern of NPR-B and NPR-Bi in the human nephron clearly indicates different tasks for CNP through these receptors. The cGMP-independent pathway seems to play an important role in the human collecting duct, where NPR-B is not expressed at all and NPR-Bi showed its strongest signal on the mRNA and protein level. When the mRNA expression pattern of NPR-Bi in various human tissues is viewed, it is striking that signals from lung and pancreas stick out. The fact that NPR-B and NPR-Bi are also strongly expressed in the human colon indicates a role for NPR-Bi in cystic fibrosis-related diseases. Regulation of hIK1 in the basolateral membrane of lung or colon cells by tyrosine phosphorylation might be a key element in the regulation of Cl\(^{-}\} secretion in these cells. Further investigations are needed to clarify the role of NPR-Bi and its interaction with hIK1 and CFTR in human cell types such as Calu-3 (lung), T84 (colon), and CF-PAC (pancreas).

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DISCLOSURES

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