Differential gene expression of the three natriuretic peptides and natriuretic peptide receptor subtypes in human liver

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Abstract

Background—Various effects of atrial natriuretic peptide (ANP) on the liver have been observed. However, there is limited information about the types of receptors for natriuretic peptides expressed by the human liver.

Aim—To investigate gene expression of the three NP receptor types (NPR) as well as of the NP in human liver.

Methods—Presence of mRNA coding for all three NPR and for ANP, brain and C-type natriuretic peptide (BNP, CNP) was investigated by reverse transcription-polymerase chain reaction (RT-PCR). Human liver tissues and hepatocellular carcinoma tissues were examined.

Results—Specific PCR products for all three NPR, namely NPR-A, B, and C, could be detected. Moreover, ANP and CNP, but not BNP mRNA was detectable. The concentration of ANP transcripts was up to fivefold higher in hepatocellular carcinoma compared with non-tumorous liver tissue of the same subjects. No difference in the expression of NP receptors relative to GAPDH mRNA of tumorous and non-tumorous tissue was observed except of slightly increased NPR-A transcripts.

Conclusion—These data show that NPR transcripts are coexpressed with ANP and CNP mRNA in the human liver. This provides evidence for a local NP system in the human liver.

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Keywords: atrial natriuretic peptide, liver, gene expression.

Since the discovery of atrial natriuretic peptide (ANP) most of the studies with the cardiac hormone have focused on its renal and cardiovascular effects.^{1 2} However, there is increasing evidence for further biological effects of ANP.³ For instance ANP also seems to affect liver functions. A cytoprotective effect of the peptide on perfused liver against ischaemia and reperfusion injury has been demonstrated.⁴ Furthermore, ANP has been reported to prevent damage of isolated hepatocytes by oxygen radicals^{5 6} as well as to inhibit the growth of hepatoblastoma (HEP G2) cells.⁷ These effects of ANP on the liver seem to be mediated by specific receptors as suggested by increased particular guanylate cyclase activity in liver membranes after exposure to ANP.⁸ Further-

more, binding studies using radiolabelled ANP showed specific binding sites for ANP on rat liver membranes.⁹

Meanwhile two further natriuretic peptides have been identified, namely brain natriuretic peptide (BNP)¹⁰ and C-type natriuretic peptide (CNP).¹¹ Whereas ANP and BNP show similar pharmacological profiles,² CNP differs. It elicits mainly vasodilatory rather than diuretic effects.¹² This difference in action may be explained by the existence of distinct receptor subtypes for NP and a different pattern of their distribution in the body.^{13 14} The natriuretic peptide receptor-A (NPR-A) is a membrane bound guanylate cyclase coupled receptor responding to both ANP and BNP, but not to CNP.^{13 15} A second guanylate cyclase coupled receptor, called NPR-B has been identified and seems to specifically bind CNP.¹⁶ The third receptor termed ANPR-C, is not linked to guanylyl cyclase and recognises all three NP.^{13 17}

Up to now no information existed about possible effects of BNP and CNP on the liver, nor about the types of receptors for NP being expressed by the human liver. Therefore the aim of this study was to examine the presence of mRNAs coding for the NP receptors type A, B as well as C and the expression of the specific ligands within the liver, namely ANP, BNP, and CNP. Reverse transcription-polymerase chain reaction (RT-PCR) was used for this purpose.

In addition, as expression of ANP receptors has been demonstrated in a hepatoblastoma cells line,⁷ tissue of hepatocellular carcinoma was included in the study. Differences in expression of NP receptors were examined by PCR. Quantification of mRNA levels for NP could be performed by means of competitive PCR using genomic DNA as competitor DNA.¹⁸

Methods

Tissues

The study was conducted according to the ethical guidelines of the declaration of Helsinki. Tumorous and non-tumorous liver tissue was obtained at surgery and immediately frozen in liquid nitrogen. Routine histology was performed on each specimen taken. Resection of hepatocellular carcinoma was performed in patient 1 (male, 67 years, no evidence for liver disease) and in patient 2 (male, 57 years, mild alcoholic cirrhosis (Child-Pugh grade A)).

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Histological examination showed a hepatocellular carcinoma with a low (patient 1) and with a medium degree of differentiation (patient 2), respectively.

mRNA extraction

Total cytoplasmatic RNA of human liver tissue was extracted by the guanidinium-thiocyanate/cesiumchloride method.¹⁹ The integrity of RNA preparations was confirmed by electrophoresis on agarose gels (2.5%). mRNA was isolated by adsorption of total RNA to oligo dT magnetic beads (PolyAtract Isolation System, Promega, Heidelberg, Germany) and quantified by ultraviolet adsorption.

cDNA synthesis

One µg mRNA was mixed with p(dT) 12-18 (0.5 mg), 20 units of RNA ribonuclease inhibitor, 1 mM desoxyribonucleotides (dNTP), 5 mM MgCl₂ in 10 µl transcription buffer (10 mM TRIS-HCl, 50 mM KCl, 0.1% Triton ×100) before adding 15 U avian myeloblastosis virus reverse transcriptase (Reverse Transcription System Kit, Promega, Heidelberg, Germany) and incubating for 20 minutes at 42°C. As previously described²⁰ efficiency of transcription was estimated by incubating an aliquot (4.5 µl) of the reaction mix with 1 µCi of (α-³²P) desoxycytosinetriphosphate (dCTP) (3000 Ci/mmol). Thereafter samples were heated (95°C, 5 min) and then quickly chilled on ice. The amount of synthesised cDNA was estimated by the amount of (α-³²P)dCTP incorporated, which was determined after separation of free dCTP. Separation was achieved by precipitation with trichloroacetic acid (TCA) (5%) and filtration (glass fibre filters, GF/B, Whatman, Maidstone, UK). Efficiency of cDNA synthesis varied between 50% and 85%. cDNA content of samples was equalised to 10 ng/ml in PCR buffer (20 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin) and stored at -20°C.

Oligonucleotides used for PCR amplification

Oligonucleotides purified by high performance liquid chromatography (HPLC) (MWG, Ebersberg, Germany) had the following sequences:

ANP (human gene²¹) sense primer: 5'-CAGCATGAGCTCCTTCTCCA-3'; antisense primer: 5'-TCCGCTCTGGGCTCCAATC-CT-3' according to.²²

BNP (human gene²³) sense primer: 5'-TTCCTGGGAGGTCGTTCCAC-3'; antisense primer: 5'-CATCTTCCTCCCAAAG-CAGCC-3'.²²

CNP (human gene²⁴) sense primer: 5'-CG-CACCATGCATCTCTCCCAGCTGCT-3'; antisense primer: 5'-CGCCGCACTAACAT-CCCAGGCCGC-3' according to.²⁵

NPR-A (human gene¹⁵) sense primer: 5'-GG-AGCGGACCCAGGCATACCTGGAGG-3'; antisense primer: 5'-AGGTCAGCCTCGGG-TGCTACTC-3' modified after.²⁶

NPR-B (human gene²⁷) sense primer: 5'-GG-TGGCACCAGCATATTGGACAAC-3'; anti-

sense primer: 5'-TACAGGAGTCCAGGAG-GTCCTT-3' modified after.²⁶

NPR-C (human gene¹⁷) sense primer: 5'-GTGGCCCGGCTTGCATCGCACTGG-3'; antisense primer: 5'-TCCGGATGGTGTCA-CTGCTC-3'.²⁸

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (human gene²⁹: 5'-TCC-CTCAAGATTGTCAGCAA-3' and antisense 5'-AGATCCACAACGGATACATT-3' modified after.³⁰

Amplification method

PCR was performed in PCR buffer (100 µl) to which the following components (final concentration) were added: 50 µM dNTPs, 1 nM BSA, 0.1 mM of each primer, 1 µCi α-³²P-dCTP, two units of Taq DNA polymerase (Promega, Heidelberg, Germany), and various amounts of template DNA. The mixture was overlaid with mineral oil and subjected to 30 cycles for the NP and tubulin sequences and to 37 cycles for the NP receptor sequences. The amplification profile for the NP and GAPDH consisted of denaturation at 94°C for three minutes, primer annealing (at 59°C for NP and at 57°C for GAPDH) for one minute, and extension at 74°C for three minutes (for six minutes after the last cycle).³¹ For the NP receptor mRNA amplification primer annealing was performed at 55°C for NPR-A and NPR-B, and of 57°C for NPR-C. In all experiments the presence of possible contaminants was tested by reactions in which either cDNA was omitted or mRNA was added instead of cDNA. To ensure that amplification of templates was exponential under the experimental conditions used, optimal conditions were elaborated for each template – that is, PCR with increasing amounts of initial templates as well as with increasing number of cycles were done.³¹

Quantification of PCR products

For relative quantitation of NP PCR products we used the method of competitive PCR using genomic DNA as previously described in detail.^{20, 31} Briefly, a constant amount of cDNA (ANP 10 ng, BNP 100 ng, CNP 150 ng, respectively) and serial dilutions of competitor human genomic DNA (ANP 0.63-5 ng; BNP 0.63-10 ng; CNP 12.5-100 ng) were amplified. Aliquots of PCR products (8 µl) were submitted to gel electrophoresis (6% acrylamide) and the DNA bands were identified by silver nitrate staining followed by exposure to x ray films (-70°C). The bands were cut into vials, dissolved in 30% H₂O₂, and counted in a β counter in the presence of scintillation fluid. PCR amplification of each sample was performed at least in duplicates and each experiment was repeated twice.

For comparison of NPR mRNA concentrations dose curves were performed to assess appropriate amounts of template for each of the three NPR-PCR as well as for the amplification of the GAPDH sequences. Similar efficiency of amplification of cDNA from

normal and tumour tissue was shown by parallelism of respective dose curves. mRNA expression of the NPRs were evaluated by comparing PCR signals of tumorous and non-tumorous tissues. To ensure similar amounts of initial cDNA mRNA of the house keeping gene GAPDH was amplified in a separate PCR. As lower initial concentrations of cDNA were mandatory for linear amplification of GAPDH transcripts compared with NPR mRNAs, GAPDH and NPR transcripts could not be coamplified. NPR signals were corrected referring to PCR signals for GAPDH sequences in both tissues. Samples were processed as described above. PCR amplification was performed in duplicate or triplicate and each experiment repeated twice.

Results

Detection of mRNAs coding for the NP receptor types

A representative autoradiogram (Fig 1A) shows the presence of mRNA transcripts for all three NP receptors in the human liver. Various amounts of liver cDNA were amplified in the presence of the corresponding pairs of primers resulting in a 692, 768, and 378 bp signal for the A, B, and C receptor specific sequences, respectively. As control for possible contaminations, mRNA instead of cDNA was amplified in parallel and no DNA bands were visible after electrophoresis. cDNA from cells (rat C6-glioma) transfected with either human A or B receptor cDNA (provided by Prof Dr Gerzer, Köln) served as positive control.

Figure 1: Representative PCR of cDNA sequences of NP receptors (A) and natriuretic peptides (B) in normal human liver tissue (patient 2). Corresponding amplification products were size fractionated by polyacrylamid gel electrophoresis and exposed to x ray films. cDNA were generated from normal human liver (Li). cDNA from transfected cells (C6) served as positive control and mRNA from liver (mRNA) was used as negative control. (A) NPR-A transcripts (692 bp); lane 1: liver cDNA (10 ng); lane 2: cDNA of NPR-A transfected cells (0.1 ng); lane 3: mRNA (10 ng) from human liver. NPR-B transcripts (768 bp); lane 1: liver cDNA (2 ng); lane 2: cDNA of cells (C6) transfected with NPR-B cDNA (0.1 ng); lane 3: liver mRNA (2 ng); NPR-C transcript (378 bp); lane 1: liver cDNA (80 ng); lane 2: mRNA (80 ng). (B) cDNA (10 ng, 50 ng) of human liver contains ANP transcripts (430 bp) as well as CNP specific sequences (394 bp), but no BNP transcript (291 bp) even at 100 ng initial cDNA input. Not reverse-transcribed mRNA served as control for contamination and did not show any PCR products.

Demonstration of mRNA transcripts coding for the different NP

As shown in Figure 1(B) the specific ligands for the A and B type of NP receptors – that is, ANP and CNP were expressed in human liver tissue. In contrast, mRNA coding for BNP was not detectable. Amplification of cDNA (10 and 50 ng each) using specific primers yielded a clear single band of 430 bp for the ANP transcripts and a 394 bp comprising DNA fragment for CNP. No band was visible for BNP specific amplification (291 bp) even when higher amounts of cDNA (up to 100 ng) were used. When the PCR procedure was carried out in the absence of reverse transcriptase the corresponding bands were not seen, nor any other DNA fragments. This shows that the PCR products originate from mRNA and not from genomic DNA or another contamination. As the primers were designed to recognise sequences localised to different exons of the peptide genes, PCR transcripts from gDNA would be detectable by their different size (ANP: 533 bp; BNP: 522 bp; CNP: 838 bp).

Comparison of NP gene expression in tumorous and non-tumorous liver tissues

For the purpose of a relative quantification of mRNAs coding for NP competitive PCR analysis was performed. Figure 2(A) shows a representative experiment for amplification of

Figure 2: (A) Representative competitive PCR amplifying ANP specific templates of cDNA (10 ng) of non-tumorous (NT1) and tumorous liver tissue (T1). Decreasing amounts of gDNA were coamplified (NT: 5.0–63 ng; T: 10.0–63 ng). (B) Representative titration curves of cDNA from non-tumorous (■) tumorous (+) liver tissue for amplification of ANP sequences. Data were obtained by cutting the corresponding bands of PAGE gels visualised with silver nitrate (A) and counting the radioactivity incorporated in the PCR products. The ratio of gDNA/cDNA related radioactivity was plotted on a log-log scale against the amount of gDNA.

Figure 3: Relative ANP and CNP mRNA levels in tumorous (T) and non-tumorous (NT) liver tissue of patient 1 and 2. Values (means) are derived from titration curves of two experiments performed in duplicate.

ANP sequences. A constant amount of cDNA is amplified together with serially decreasing amounts of standard gDNA. As the gDNA concentration decreases, the signal of the amplification product (533 bp) becomes less intense, and intensity of the target cDNA (430 bp) increases. Bands were cut, quantified by their amounts of radioactivity, and the PCR product ratios (gDNA/cDNA) were plotted as a function of the initial amount of gDNA (Fig 2(B)). Based on the equivalence region, where the PCR product ratio was around 1, the shift towards the right of the curve obtained with cDNA from tumour tissue indicates an increased amount of PCR product. The principal set up for amplification of CNP sequences was the same as for ANP. Figure 3 summarises the results of competitive PCR analysis. Tumour tissue of patient 1 expressed 5.5-fold and fourfold higher mRNA concentrations of ANP and CNP, respectively, compared with non-tumorous tissue. Patient 2 showed also increased NP mRNA expression (threefold for ANP mRNA and twofold for CNP mRNA).

Relative quantification of NP receptor transcripts

Competitive PCR using gDNA as standard could not be used for quantification of NPR transcripts. NPR sequences deducing from gDNA are much longer than the transcripts originating from cDNA. Thus, efficiency of amplification of gDNA and cDNA cannot be expected to be similar, which is a prerequisite for reliable quantification of PCR transcripts. Quantification of NPR transcripts has been

achieved as follows: equal amounts of cDNA extracted from tumorous and non-tumorous tissue, which had been proved in previous experiments to be amplified in a linear mode, were subjected to PCR. The amount of amplified NP receptor fragments was evaluated by the amount of radioactive dCTP incorporated during the PCR procedure. In addition, expression of GAPDH mRNA was examined. GAPDH expression in normal liver was not significantly different from tumorous liver (range 90–120%). In contrast, mRNA concentrations for tubulin, another 'house keeping gene', were increased up to twofold in the tumorous liver (data not shown). Therefore, the amounts of NPR transcripts were corrected for the respective GAPDH mRNA expression. Figure 4 shows a representative autoradiogram of receptor transcripts in tumorous liver and normal tissue. The Table summarises the quantification of the NPR-A, -B, and -C mRNA in control and tumour tissue corrected relative to GAPDH expression. An increase of mRNA expression was present for the A-receptor (twofold) in patient 1. mRNA coding for the B-type and C-type receptor seemed not to be significantly changed in tumour tissue.

Discussion

Our data show the presence of specific mRNAs coding for the A-type as well as for the C-type of NP receptors in human liver. These findings extend previous reports of specific binding sites for ANP in the liver.⁵⁻⁹ Moreover, we detected expression of the B-type receptor in the liver, a novel finding. With regard to the relative level of expression of the three NP receptors, the NPR-B receptor seems to be highly expressed when comparing the low initial amounts of liver cDNA necessary to obtain a signal. An exact analysis of the degree of NP expression, however, requires absolute quantification of PCR products, which cannot be achieved by our PCR protocol.

The presence of the B-type receptor shows that the liver represents a target organ for CNP believed to be the specific ligand for NPR-B. Initially, this type of NP receptor together with its specific ligand CNP was thought to be restricted to the central nervous system, representing a local central natriuretic peptide system.^{11 16 32} By means of a more sensitive technique such as PCR, mRNA for the NPR-B could also be demonstrated in peripheral organs – that is, the kidney and the heart.^{28 33} The high sensitivity of PCR may also explain why we could demonstrate gene expression of

Figure 4: Comparison of NP receptor- and GAPDH transcripts in tumorous (T) and non-tumorous (NT) liver tissue of two patients (1, 2). NPR-A (692 bp), -B (768 bp), and -C (378 bp) specific PCR products as well as GAPDH specific transcripts (303 bp) were separated by PAGE and further processed as described in Methods. For the amplification of NPR-A, -B, -C, and GAPDH sequences 10, 2, 80, and 0.05 ng cDNA; respectively were used for amplification.

mRNA of different NPRs in hepatocellular carcinoma

	T1	T2
NPR-A	215 (26)	136 (18)
NPR-B	93 (22)	128 (23)
NPR-C	114 (10)	80 (22)

mRNA concentration of NPRs in tumour tissue of two patients (T1, T2) expressed in % of non-tumour tissue (NT) of the same livers. NPR-mRNA are corrected for GAPDH mRNA levels. Data shown as mean (SEM).

NP binding sites in the liver, whereas Wilcox *et al*¹⁴ using in situ hybridisation techniques did not observe NPR specific mRNAs in liver of primates.

It may be important to mention that some organs that exhibit NP receptors, express the specific NP ligands as well.^{29 33-37} Therefore, the existence of local NP systems was suggested. For instance, the heart known to be the major site of ANP and BNP production,¹⁻³ exhibits specific receptors for the NP.³³ Recent data showed that CNP as well as its receptor, NPR-B are coexpressed in various tissues, such as kidney, heart, and chondrocytes.^{30 33 35 36}

Endogenous NP are thought to elicit paracrine or autocrine effects, or both, in these organs. For instance, in rat chondrocytes an autocrine regulation of cell proliferation by CNP and its receptor NPR-B has been shown.³⁶

A similar hypothesis can be proposed for the liver based on our findings of ANP and CNP expression in addition to the presence of their specific NP receptors. The fact that BNP was not expressed in human liver deserves further attention. BNP exhibits similarities to ANP: both peptides share the heart as major site of synthesis as well as the pharmacological profile.^{1 2} In pathophysiological states, such as congestive heart failure or hypertension, however, BNP expression was affected to a different extent than ANP.^{34 38} The presence of ANP, but not of BNP expression in the liver could further support the contention of different roles of these two NP.

We found increased expression of ANP in hepatocellular carcinoma. Similar findings were made for other tumours, for example, of the reproductive system.²² In this regard it is of interest that ANP elicits antiproliferative effects in various tissues including a hepatoblastoma cell line.^{7 39 40} However, speculations about a local involvement of NP in tumour development clearly need further investigation. In this respect information on the type of liver cells expressing NPR and NP respectively, would be helpful. As NP receptors and NP have been described in vascular smooth muscle cells, epithelial and endothelial cells it has to be considered that transcripts originating from these cells might be detected using the highly sensitive PCR technique. However, there is evidence in the medical literature for the actual hepatocellular origin of NPR. Firstly, studies with isolated hepatocytes have shown that ANP elicits a dose dependent increase of cGMP indicating the presence of NPR-A receptors on hepatocytes.^{5 6} Secondly, binding experiments on whole liver tissue demonstrated the presence of functional NPR protein.⁹ Finally, studies on hepatoblastoma cells showed the presence of NPR-C.⁷ Thus, it seems reasonable to assume that the NPR-mRNAs found in our study relate to liver specific cells. However, PCR analysis of the different separated liver cell populations could be a promising approach for further studies.

In conclusion, we have shown the presence of transcripts of all three NP receptors as well as of mRNAs coding for ANP and CNP in

human liver. These data provide evidence for the existence of a local NP system in the human liver.

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