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HUMAN RETINA EXPRESSES BOTH CONSTITUTIVE AND INDUCIBLE ISOFORMS OF NITRIC OXIDE SYNTHASE mRNA

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SUMMARY: The present study provided evidence for the presence of two forms of nitric oxide synthase(NOS) gene in the human retina. Expression of retinal constitutive type(rbNOS) and inducible type(riNOS) of NOS was detected in human retinal poly A^+RNA by reverse transcriptase polymerase chain reaction(RT-PCR) method. The deduced amino acid sequence of the human retinal rbNOS showed more than 99% homology with human brain bNOS and that of riNOS was identical to the chondrocytes inducible iNOS with the exception for one amino acid. These differences in amino acid sequences of rbNOS and riNOS,with their counterparts in human brain and human chondrocytes sequences,were only in the non-cofactor binding sites. Northern blot analysis of the human retinal poly A^+ RNA and total RNA, using the PCR-amplified riNOS probe revealed the existence of riNOS message with the appearance of the band with the expected size of 4.4kb, while the message for rbNOS was not detectable. This was the first report of the deduced nucleotide sequence identification of two NOS genes from a human tissue, while there had been earlier reports from culture cells.

Nitric oxide synthase(NOS) catalyzes the synthesis of nitric oxide(NO), a potent biological mediator, which is involved in the smooth muscle relaxation and plays diverse roles in various cells and tissues (1). The calcium/calmodulin dependent isoform of NOS is constitutively expressed in endothelial cells (2) and brain (3, 4). The inducible isoform of NOS(iNOS) is calcium independent, with the exception of the recently cloned inducible form of NOS from human hepatocytes (5). The iNOS was first shown to be present in rodent macrophage (6) and was shown to be induced by exotoxins and/or cytokines. The brain type bNOS(or cNOS) has also been shown to be localized in the rat retina where the retina makes contact with the choroid and the pigmented epithelium (7)

In the retina, guanylate cylase plays a key role in the phototransduction by maintaining high levels of cGMP (8). NO activates soluble guanylate cyclase in brain (9) and is also implicated in the activation of particulate guanylate cyclase of bovine rod outer segments (10). It is interesting that in

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bovine retina, NO is synthesized by a constitutive calcium dependent NOS (10, 11), while the retinal pigmented epithelial cells express the macrophage type inducible NOS, upon stimulation by various cytokines and lipipolysaccharide(LPS) (12, 13). Such induction of NOS has also been demonstrated in rat glial cells (14) and in human retinal pigmented epithelial cells (15). Calcium independent LPS-inducible NOS has also been demonstrated in astrocytes, microglial, and C6 glioma cells (16). So far there has been no report demonstrating the NOS mRNA expression and cDNA sequence in retina. Earlier studies from this laboratory have demonstrated the expression of different forms of guanylate cyclases in retina. In view of the increased interest in the understanding of NO signal pathway being linked to guanylate cyclases, we have investigated the presence of NOS in retina at molecular level.

In the present study, we report that both the constitutive brain type and the inducible chondrocytes type forms of NOS are expressed in human retina. We adopted the approach of reverse transcriptase polymerase chain reaction (RT-PCR) and indentified the full length sequences of these two forms of NOS in human retina.

MATERIALS AND METHODS

Source of human poly A^+ RNA and total RNA

Poly A^+ RNA used was made from a pool of 22 normal Caucasian male/female(16-70 yrs. old) retinal tissue specimens by Clonetech laboratories using a modified guanidinium thiocyanate method followed by poly $(A)^+$ RNA selection with two rounds of oligo(dT)-cellulose columns. And total RNA was collected by Clontech laboratories from a pool of 26 male/female caucasian(16-75 yrs. old) retinal tissue samples. Causes of death of patients from whom the specimens were obtained were of sudden death and traumatic natures for poly $(A)^+$RNA and total RNA, respectively.

cDNA cloning and sequencing

The first-strand cDNA was synthesized in a reaction volume of 20 μl containing 1μg of poly $(A)^+$ (Clontech), 1μg of random hexamer primer, and 0.2 μg of oligo (dT) primer with an RT-PCR kit according to manufactuer's instructions (Invitrogen, San Diego, Ca). Primers were designed from the cofactor binding site of several NOS cDNA sequences (Fig. 1). Deoxyinosine was inserted at ambiguous codon positions. The sense oligonucleotide, 5'-CCICGCTICTACTCCATCAGCTCCTC-3' (F/26S), was derived from PRY(F)YSISSS, an amino acid sequence conserved in enzymes that bind FAD-Isoalloxazine. The antisense oligonucleotide, 5'-AAIGGIGCIATICCIGTICCIGGICC-3' (N/26A), encodes GPGTGIA(V)PF, an amino acid sequence conserved in the NADPH-ribose binding site. RT-PCR product(254 bp)was analyzed by 0.8% agarose gel electrophoresis (Fig. 2). Isolated PCR product was subcloned into the pCRII TA cloning vector(Invitrogene, San Diego, Ca). DNA sequences of the cloned PCR product were determined by dideoxynucleotide chain termination using Sequenase Version 2.0(U. S. Biochemical Corp.) and analysed by macDNASIS softwere (Hitachi, San Bruno, Ca).

RT-PCR for full cDNA construction

Two different sequences were generated from the same RT-PCR product. It was found that one of these sequences(254bp) was identical with the constitutive cNOS of human brain(bNOS), another one was identical with the inducible form of human chondrocyte type(iNOS). To obtain the full length of cDNA from the retina poly A^+ RNA, gene specific primers for RT-PCR were designed from the both bNOS and iNOS cDNA. PCR products of suspected size showed clear or faint bands and was isolated by PCR prep column(Promega). Fragments subcloned were sequenced by same method as above.

Northern blotting

Northern blot analyses of poly A^+ RNA and total RNA were carried out using 1.2% agarose gel containing 6% formaldehyde and Hybond N^+ nylon membranes (Amersham, Aylesbury, UK).

Three different fragments for bNOS and two for iNOS from RT-PCR products were used as probes. Double-stranded cDNA fragments were labelled with [α-^{32}P]dCTP using a DNA megalabelling Kit (Amersham, Aylesbury, UK). Filter was hybridized at 48°C for 16h in hybrid solution I containing 50% formamide(Onchor, Gaithersburg, Md.). Blot was then exposed to Kodak XAR film at -80°C for 5 days. Also different membrane(only total RNA) was exposed to Phosphoimager(Molecular dynamics, Sunnyvale, Ca.) for 16 hrs.

RESULTS

In an attempt to screen the human retinal cDNA library, we generated a probe from human retinal poly A$^+$ RNA using RT-PCR technique. Primers for this reaction were designed from the highly conserved cofactor binding site region of the cDNA sequences published for bNOS and iNOS. Two primers as mentioned in materials and method were synthesized with deoxyinosine insertion at ambiguous codon positions. The sense primer was derived from an amino acid sequence that bind FAD-Isoalloxazine and the antisense primer was derived from the conserved amino acid sequence in NADPH-ribose binding site (Fig. 1). The PCR reaction amplified the expected size product (254bp), which was visualized by the ethidium bromide staining of the gel electrophoresed PCR product (Fig. 2). The amplified product was purified and subcloned into pCRII TA cloning vector. Several positive subclones were sequenced for the identification of the amplified product. Interestingly, these subclones from same band yielded two different sequences, revealing the PCR amplification of two different gene products of identical size (254bp). The sequence comparison identified one of the amplified products to be 100% identical to the corresponding region of the reported cDNA sequence of the human constitutive brain NOS(bNOS), while the other product showed 100% homology with that for the human chondrocyte inducible NOS(iNOS).

Attempts to isolate full length sequences of these two forms from human retinal cDNA library by using the above PCR products as probes were not successful. Therefore, we have employed the sensitive PCR technique to obtain full length sequences of both the forms of NOS from the reverse transcribed human retinal cDNAs. Five and four sets of gene specific primers(data not shown) were designed from the reported cDNA sequences of the human brain bNOS and the human chondrocyte iNOS, respectively. These primers were employed to amplify the human retinal cDNAs. Each time the PCR products were subcloned and sequenced. Figure 3 illustrates the PCR amplified regions of both retinal brain type(rbNOS) and chondrocyte inducible type(riNOS) forms

Fig. 1. Comparison of the amino acid sequences of the reported four different NOS forms. The underlined region was selected for the designing of primers for the first round of PCR reaction. Chon: human chondrocyte, Hepa: human hepatocyte, MuMCL: murine macrophage cell line, Brain: human brain, iNOS: inducible NOS, bNOS: consitutive brain NOS.

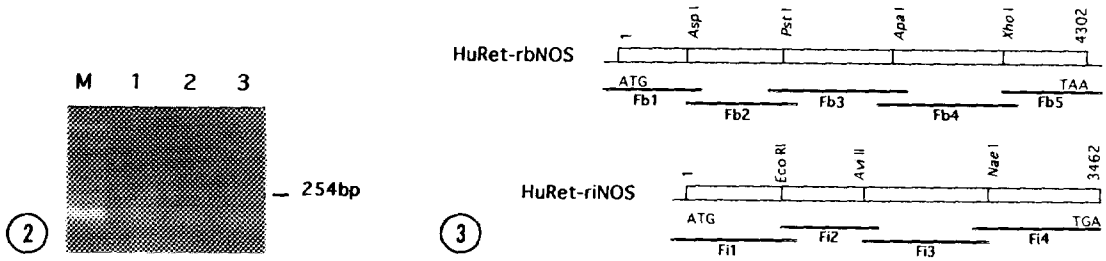

Fig. 2. RT-PCR products of human retinal NOS from poly A+ RNA. Amplified products using the deoxyinosine incorporated primers were 254bp in length. Each band(1, 2, 3) contains two different sequences. M: 100bp ladder size marker.

Fig. 3. Full constructs and RP-PCR products of human rbNOS (Fb) and riNOS (Fi). These RT-PCR products(Fb1-5, Fi1-4) were made with gene specific primers designed from human brain bNOS and chondrocyte iNOS. The common restriction enzyme sites from the overlapping sequences were selected to obtain full construct of the reading frame.

of NOS from human retinal cDNA. The common restriction enzyme sites were selected in making the complete reading frame constructs of both the forms. Full amino acid sequence of the rbNOS showed differences in several positions when compared with that of the human bNOS, while the riNOS sequence showed complete homology with the human iNOS, except for one amino acid at position 608 (Fig. 4). These differences in amino acd sequences of rbNOS and riNOS, with their counterparts of human bNOS and iNOS sequences were only in the non-cofactor binding sites. The comparison of the amino acid sequence in different positions beween rbNOS and human bNOS and between riNOS and human iNOS was also shown in figure 4.

Northern blot analysis of the the human retinal poly A+ RNA and total RNA was performed using both the rbNOS and riNOS RT-PCR product probes of different sites. The data indicated a prominent band at 4.4kb similar in size with the reported iNOS from the human chondrocytes (Fig. 5). However, the message for rbNOS (assumed size ~10kb) could not be detected on the blot.

DISCUSSION

NO synthase plays an important role in the synthesis of NO which is involved in a variety of biological processes. The role of NOS in the signal transduction and the involvement in the phototransduction has been reported by showing the presence of the NOS in retinal tissues of rat (7), bovine (10, 12), and human (15). In these studies, NOS has been identified in the retinal tisues or cells using histochemical or biochemical techniques. In addition, Goureau et al. has shown that the inducible NOS was expressed by treatment of cytokine or endotoxin in retinal pigmented epithelial and glial cells (14, 15). However, cDNA sequence of retina NOS has not been reported. The present study demonstrates that at least two types (retinal brain type, rbNOS and inducible type, riNOS) of NOS are present in human retina and that rbNOS is similar to both human and rat brain NOS(more than 99% homology) and retinal inducible type NOS(riNOS) is identical to human chondrocyte inducible type with the exception for one site. The presence of similar forms of respective human constitutive NOS and human inducible NOS may be the results of posttranslational modification of the same gene, although artifact in the process of RT-PCR can not

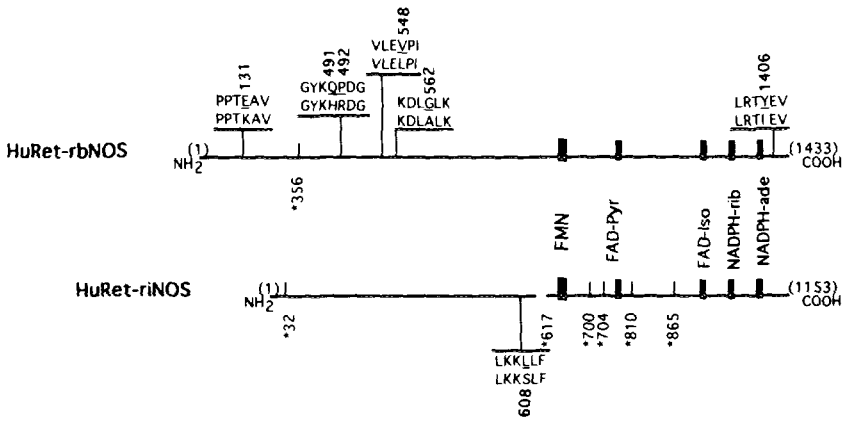

Fig. 4. Alignment of the predicted amino acid sequences of human retinal brain type NOS(HuRet-rbNOS) and inducible type NOS(HuRet-riNOS) with human brain bNOS and chondrocyte inducible iNOS forms. The numbers with asterisk markers represent the gap of each sequence. The bold numbers represent the amino acid sites(underline) that are different in rbNOS and riNOS(upper sequences) from human bNOS and chondrocyte iNOS(lower sequences), respectively.

be excluded. However, we believe that the expression of rbNOS is specific to human retinal tissue, and is a new addition to NOS gene family.

It has been suggested that NOS responsible for the release of NO from the bovine retina might be the constitutive brain type NOS in biochemical study (10). Our results indicate that in human retina, NOS involved in L-arginine:NO pathway may be also constituitive form because mRNA message detected with RT-PCR technique. Unfortunately, we have not been able to demonstrate the expression of mRNA using northern blot analysis. These may indicate the low level expression of the rbNOS in human retina. The presence of constitutive brain type isoform of NOS in human retina may have important implications in understanding the NO signal pathway.

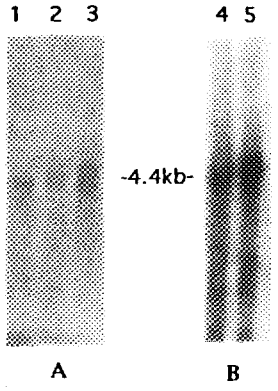

Fig. 5. Northern blot hybridization of human retinal poly A^+ RNA and total RNA using the RT-PCR products of the riNOS cDNA as probe. Total RNA(lanes 1, 2 and 5: 20μg, 4: 10μg) and poly A^+ RNA(lane 3: 2μg) were used to identify the expression of riNOS mRNA(4.4 kb). Letters A and B indicate results exposed with X-ray film(5 days) and phosphoimage(16 hrs.), respectively.

In general, the constitutive endothelial and brain forms are calcium dependent (7, 17), while the inducible NOS is calcium-independent (1). However, the evidence in the literatures suggest that calcium-dependency can not be duduced from amino acid sequence only. For example, Geller et al. has shown that putative calmodulin binding site exist in the amino acid sequence of human hepatocyte form (5). This enzyme is inducible form but calcium-dependent differently from other inducible forms, while human chondrocyte form is calcium-independent in spite of the amino acid sequences of calcium-calmodulin site are identical between two sequences (18). Therefore, the fact that cDNA sequence of the riNOS is identical to that of human chondrocyte form except for one site does not necessarily mean that this form is calcium dependent.

NO produced by inducible NOS has been considered one of the toxic factors in viral infection and other pathiological conditions, such as cardiovascular diseases (20), colorectal adenocarcinoma tumor cell line (19) or injury of central nervous system(CNS) (21, 22), leading to cell damage. Dighiero et al. have recently demonstrated high levels of NO production in the human retina under a pathological condition(AIDS) (23). Chondrocyte form of NOS has been expressed in a pathological conditions, such as in human tumor cells (19). However, expression of inducible NOS in human tissues and cells has been more difficult to demonstrate. In spite of that, we have shown that the mRNA of riNOS cDNA is expressed in human retina and identical to the inducible NOS of human chondroytes. To our knowledge, this is the first report demonstrating a direct evidence of the existence of an inducible NOS isoform in the human tissue using DNA sequencies without treatment of any cytokines or endotoxin. It would be a useful tool to examine the relationship between NOS and guanylate cyclase as well as further applications, once these genes are expressed in mammalian cells.

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