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ORIGINAL ARTICLE

# Expression of nitric oxide synthases in parathyroid gland adenoma and parathyroid gland hyperplasia

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## Abstract

**OBJECTIVE:** The aim of the presented study was to perform the immunohistochemical detection of endothelial (eNOS) and inducible (iNOS) isoform of nitric oxide synthase in the adenomatous and hyperplastic parathyroid gland in relation to the apoptotic process.

**DESIGN AND SETTING:** Tissue samples from 12 patients with parathyroid gland adenoma (PGA) and 10 patients with secondary parathyroid gland hyperplasia (PGH) were collected during surgery at the Department of Otorhinolaryngology and Head and Neck Surgery of The First Faculty of Medicine in Prague.

**METHODS:** Three-step immunoperoxidase reaction on acetone-fixed cryostat sections was performed using both polyclonal and monoclonal antibodies against eNOS and iNOS. The detection of apoptotic cells was done using antibody against cleaved caspase-3 as an apoptotic marker.

**RESULTS:** The immunoreactivity to eNOS antibody was observed in the endothelial lining of vessels in PGA, PGH and in the rim of normal parathyroid gland adjacent to PGA sample. Variable expression of eNOS was confirmed in arteries, arterioles, capillaries and veins in the glandular parenchyma as well as in the surrounding connective tissue. There was no iNOS immunoreactive cell detected in any examined sample. No apoptotic cells were detected.

**MAIN RESULT:** Our findings confirm that eNOS is regularly expressed in the vasculature of PGA and PGH.

**CONCLUSION:** eNOS observed in the vasculature of the enlarged parathyroid glands can serve as a factor that contributes to the viability of hypertrophic pathologic tissue. The lack of stimulating signals may be a reason for negative iNOS detection and negligible apoptotic rate.

**Abbreviations:**

| | |
|---|---|
| eNOS | endothelial nitric oxide synthase |
| iNOS | inducible nitric oxide synthase |
| nNOS | neuronal nitric oxide synthase |
| PGA | parathyroid gland adenoma |
| PGH | parathyroid gland hyperplasia |
| NO | nitric oxide |
| PG | parathyroid gland |
| PBS | phosphate buffered saline |
| NGS | normal goat serum |
| IgG | immunoglobulin G |
| DAB | diaminobenzidine |

## Introduction

The main function of the parathyroid gland (PG) is the regulation of calcium ion homeostasis via parathormone secretion. There are several pathological states, however, in which this homeostatic role is severely disturbed. These two types of disorders are primary and secondary hyperparathyrodism. The most frequent cause of primary hyperparathyroidism is PGA. It is a benign tumor that develops as a result of certain not yet fully elucidated genetic mutations. The secondary hyperparathyroidism manifests itself by hyperplasia and is associated with the renal disease. The initial hyperplasia is stimulated by the depletion of calcium serum level and the decrease of vitamin D production – both being a consequence of the renal disease. The parathyroid gland reacts by increased parathormone production and parenchymal cell hyperplasia as well. Different cytokines play a role in pathological processes affecting the parathyroid gland having either growth promoting or inhibitory effects mediated by their direct influence of the cell cycle [2]. Similar functions to those exerted by cytokines were described in cardiovascular, immune and nervous system for the unique signal molecule – nitric oxide (NO). This endogenously produced gas is formed by three specific isoforms of nitric oxide synthase. Endothelial (eNOS) and neuronal (nNOS) isoforms constitutively produce smaller amounts of NO, while inducible (iNOS) isoform is expressed as a response to various stimuli and produces usually great amount of NO. Apart from its multiple roles in almost any organ or tissue, this endogenously produced free radical is believed to be a modulator of cell proliferation and cell death. NO has apparently a bifunctional role in the regulation of cell growth. It was repeatedly demonstrated that NO could either stimulate or inhibit cell proliferation. Direct influence of cell cycle regulating protein expression was documented for instance during the inhibition of vascular smooth muscle cell proliferation [8]. Stimulatory effect on cell proliferation, on the other hand, was documented in liver. Primary rat hepatocytes that are normally in a quiescent state were primed by NO to respond to mitogenic signals conveyed by appropriate cytokines [5]. Its effect on apoptosis varies also according to the particular situation [9, 3, 4]. Both apoptosis and cell proliferation are important variables in the equation, which determines the size of an organ. PGA and PGH represent two disorders that are manifested by an enlargement of this endocrine organ. It is possible to consider whether NO, knowing its effects on cell proliferation and cell death, is involved in the etiopathogenesis of both diseases. However, the literature data on NOS expression in parathyroid gland are scarce. Thus, the objective of this study was to detect two different isoforms of nitric oxide synthase – iNOS and eNOS in normal PG, PGA and PGH. In addition, the frequency of apoptotic process was evaluated using immunohistochemical detection of cleaved caspase-3 as an apoptotic marker

## Methods

Tissue samples from 12 patients with PGA and 10 patients with PGH were collected during surgery at the Department of Otorhinolaryngology and Head and Neck Surgery of The First Faculty of Medicine in Prague. All patients signed informed consent and the local ethical committee approved the study. Immediately after the removal, samples were frozen in liquid nitrogen and kept in freezer at –80 °C temperature until sectioning. Cryostat sections (7 μm) were cut and kept again at –80 °C until the immunohistochemical processing. After thawing at room temperature for 5 min, the sections were fixed for 8 min in acetone at 4 °C and then washed in PBS. Endogenous peroxidase was blocked by incubating samples with 0.3% $H_2O_2$ in PBS for 20 min. Non-specific antibody binding was blocked by incuba-

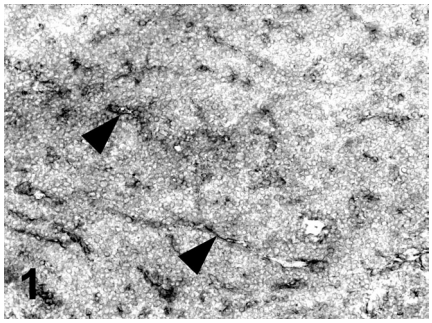


**Figure 1:** Sample from parathyroid gland hyperplasia showing the positive eNOS labeling (▶) in the capillaries and smaller vessels within the parenchyma. The number of positive cells depended on the vascular density. Orig. mag. obj. 20X.

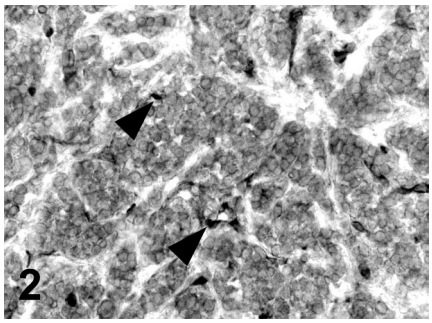


**Figure 2:** Sample from parathyroid gland adenoma showing the positive eNOS labeling (▶) in capillaries and smaller vessels within the parenchyma. The number of positive cells depended on the vascular density. Orig. mag. obj. 20X.

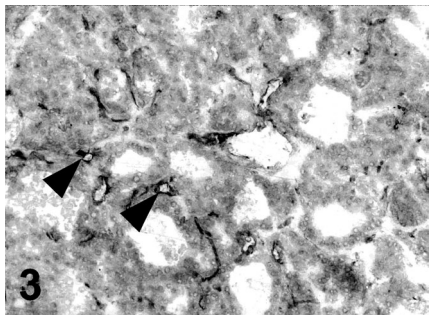


**Figure 3:** Representative photograph of a section from adenoma showing the positive eNOS labeling (▶) in the capillaries surrounding the follicular structures found in some adenomatous glands. Orig. mag. obj. 20X.

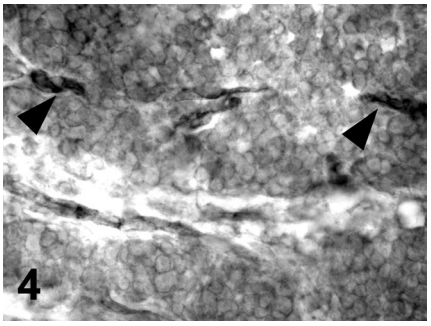

**Figure 4:** Endothelial cells in capillaries displayed both cytoplasmic and membrane localization of the reaction product after immunostaining for eNOS (▶). Orig. mag. obj. 63X.

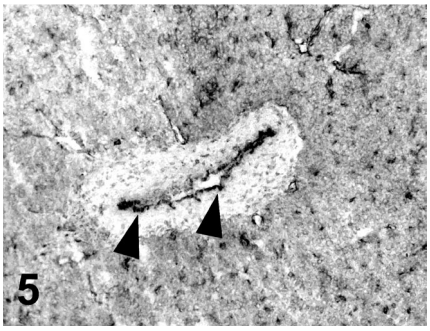

**Figure 5:** Photograph of greater vessel within the parenchyma of the adenomatous gland showing eNOS immunoreactivity in the endothelium (▶). Orig. mag. obj. 20X

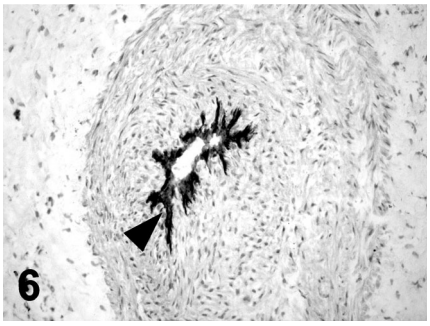

**Figure 6**: Photograph of a vessel within the connective tissue adjacent to the adenomatous gland showing strong eNOS immunoreactivity in the endothelium (▶). Orig. mag. obj. 20X.

tion with 10% normal goat serum (NGS) for 60 min and endogenous avidin and biotin were blocked by the Endogenous Avidin/Biotin blocking kit (Zymed, USA) according to the protocol of the manufacturer. Sections were incubated with anti-iNOS and anti-eNOS rabbit polyclonal antibodies 1:100 in PBS + 1.5% (NGS) for 60 min at room temperature. To verify the results obtained by polyclonal antisera, some samples were incubated with anti-eNOS monoclonal antibody (BD Transduction Laboratories, USA) 1:50 or anti-iNOS monoclonal antibody (Santa Cruz, USA) 1:100 in the same diluent and under the same conditions. Biotinylated goat anti-rabbit IgG (Santa Cruz, USA) diluted 1:100 or goat anti-mouse IgG (Stressgen, Canada) diluted 1:400 in PBS + 1.5% NGS was applied for another 30 min. Detection was performed by Vectastain – ABC Elite staining kit (Vector, USA). For the peroxidase detection DAB+ chromogenic substrate (DAKO, Denmark) was applied. Harris's hematoxylin was used for counterstaining and sections were mounted in the aqueous medium.

For the detection of cleaved caspase-3 the cryosections were fixed and pretreated as in NOS detection protocol. Primary rabbit polyclonal antibody (#9661; Cell Signaling Technology, Beverly, MA, USA) was applied diluted 1:800 in PBS + 5% NGS overnight at 4 °C. The subsequent visualization of the bound antibody was performed as above. The specifity of apoptotic cell labeling with this antibody was previously verified on primary rat hepatocyte culture and on samples of human tonsil.

## Results

The positivity of eNOS immunostaining was detected in all samples of PGA, PGH and in the rim of normal parathyroid tissue adjacent to one PGA sample, as well. Expression of eNOS revealed its localization regularly in endothelial lining of arteries, arterioles, capillaries and veins in the connective tissue and within the glandular parenchyma (*Fig. 1–4*). The most prominent labeling was observed in greater vessels supplying the gland (*Fig. 5, 6*). The level of eNOS expression in the capillary endothelium, although not precisely quantified, differed slightly among individual samples. However, this variation in the labeling intensity was similar in PGA or PGH. No epithelial cells displayed immunoreactivity to eNOS. In general, the overall intensity of eNOS immunoreactivity corresponded more to the abundance of vessels rather then to the specific pathological category. The immunohistochemical detection of iNOS expression in PGA and PGH did not confirm any immunopositive cells within examined samples. The immunostaining performed on some samples using monoclonal primary antibodies against both NOS isoforms gave identical results. When apoptosis detection was performed using cleaved caspase-3 as an apoptotic marker there were no immunoreactive cells found in any sample.

## Discussion

The present study provides evidence of different eNOS and iNOS expression in the pathological parathyroid gland. Endothelial NOS is an enzyme that is constitutively expressed in variable quantities in endothelial cells. NO produced by eNOS as a regulator of vascular tone can influence the level of blood supply in the pathologic tissue. This regulatory function of NO is important in various pathologic states accompanied by an excessive cell growth that is typical for tumor tissue. Here, eNOS was regularly expressed within both hyperplastic parathyroid gland and benign tumor – adenoma – of the parathyroid gland. Available data indicate NO involvement in different mechanisms of tumor promotion or tumor inhibition similar to the action of cytokines on the development of parathyroid gland disorders [16]. Indeed, NO was found to mediate many often opposing functions affecting the initiation, promotion and progression of tumors having either benign or malignant character. Although conflicting reports on the role of NO in tumor growth have been presented, the majority of studies support its tumor promoting effect. The anticancerogenic processes include tumor cytotoxic effects of immune cells that are often exerted by the induction of apoptosis in some malignancies [13, 14]. Mutagenic properties of NO and especially its reactive derivates like peroxynitrite and nitrosating species account for its potential of initiating malignant process. NO can damage DNA directly by the variety of different mechanisms including deamination and oxidation. Moreover, NO can negatively affect DNA repair mecha-

nisms since DNA repair enzymes can be inhibited by NO-mediated nitrosylation. NO may also under certain circumstances promote cancerogenesis by interaction with tumor suppressor gene p53 [1]. However, the interplay between NO and p53 is probably not relevant to the disturbed tissue homeostasis in parathyroid adenoma or parathyroid secondary hyperplasia since p53 is not expressed in parathyroid tissue [17]. NO causes the accumulation of p53 in affected cells and since this is not the case in PG lesions it may imply that the amount of NO does not reach the level that would cause p53 upregulation in the parathyroid tissue. Another important aspect is NO involvement in the apoptotic process. Again both positive and negative effects were described in studies using various tumor cell lines as a model. The response of tumor cells depends on their genetic constitution and the expression of factors that decide the fate of the cell in the environment with different NO levels. The above-mentioned interaction with p53 protein comes into play, but also p53 independent proapoptotic effect of NO on tumor cell was observed [11,12]. In the context of parathyroid disease, it is quite noteworthy the finding that cells lacking p53 were resistant to NO mediated apoptosis [6]. In the number of studies positive correlation was found between the expression either of iNOS or eNOS and the tumor growth, invasiveness and metastatic potential of the tumor. This tendency was confirmed in both human breast cancer and in the murine breast cancer model [7,15]. This would mean that the absence of iNOS expression and moderate eNOS expression observed in this study in the samples of PGA and PGH corresponds to the benign character of these states of increased cell number. In fact the study on differential expression of NOS in human prostate carcinoma compared to benign hyperplastic tissue revealed iNOS positivity in malignant epithelial cells but no immunostaining was observed in benign hyperplastic tissue. There was no difference in eNOS immunoreactivity between malignant and benign prostatic tissue [10]. Interesting would be then to compare observations presented in this study with the expression pattern of NOS in parathyroid carcinoma. However this affection is extremely rare among the pathologies behind the hyperparathyroidism so there was no case registered in the course of our study. One of the key prerequisite factors enhancing the proliferation of both benign and malignant tissue is a sufficient blood supply via the microvasculature. Indeed, the process of angiogenesis was attributed the significant role in tumor promotion. It was found that tumor-promoting properties of NO are related to this phenomenon. Much evidence suggests a stimulatory role of nitric oxide in angiogenesis in various conditions including tumor growth. Experimental studies with both eNOS and iNOS knock-out animals showed impaired angiogenesis whereas in the situation of NOS overexpression tumors tended to be highly angiogenic [11]. The viability of the pathologically enlarged gland, which is sustained by eNOS expressing vasculature, could be further illustrated by very low frequency of apoptotic processes. The absence of iNOS expression leads us to the conclusion that its induction is not responsible for excessive proliferation of PG cells either in PGA or PGH. This conclusion relates at least to the clinically manifested stage of disease, which is treated by the surgical removal of tissue.

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