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CYSTATIN S: MOLECULAR BIOLOGY AND SYMPATHETIC II\INERVATION

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SUMMARY

• *The ratsubmandibular glands (SMG) are innervated by both the sympathetic and the parasympathetic branches of the autonomic nervous system which, in turn,regulate the secretory functions of the glands. Parasympathetic innervation of rat SMG is present at birth, whereas sympathetic nerve fibers reach the glands by postnatal day 5. Isoproterenol (IPR), a fi-adrenoceptor agonist, induces hypertrophic and hyperplastic enlargements of rat salivary glands, and induces the expression of a number of genes, including cystatin S, a member of family 2 of the cysteine proteinase inhibitor superfamily. Cystatin S gene expression is tissue- and cell type-specific, occurs transiently during development, and is not observed in adult animals. Sympathetic denervation of adult SMG, achieved by removing the superior cervical ganglion, reduces the induction of the cystatin S gene by a single injection of IPR. In addition, recent data indicate that the j3-receptors present early in the developing SMG are functional and are capable of responding to IPR by increasing the ex pression of the cystatin S gene, even when sympathetic nerve fibers have not reached the gland. The level of I PR-induced cystatin S mRNA remains constant until day 8, at which time a dramatic induction of cystatin S mRNA is observed. This statistically significant increase in cystatin S mRNA at day 8 was diminished, but not completely suppressed, upon sympathectomy of one day old animals. These data indicate that an intact sympathetic innervation is not requisite for induction of cystatin S gene expression by IPR in developing SMG. However, sympathetic innervation is required for the full IPR response of the cystatin S gene in these glands. Collectively, these experiments suggest that factor(s) derived from the sympathetic nervous system participate in IP R-induced expression of the cystatin S gene in the rat SMG.* ***(Biomed Rev 1998; 9:33-46)***

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INTRODUCTION

• Proteolysis is a common mechanism of activation or inactivation of polypeptides that perform important physiolo gical functions in all living creatures. There are four major classes of enzymes responsible for these activities in eukaryotes. They are classified on the basis of the amino acid at their active sites or their dependence upon metal ions, that is, cysteine-, serine- and aspartylproteases, and metalloproteases. Cystatins are naturally occurring cysteine proteinase inhibitors present in a variety of tissues and body fluids. A11 known mammalian cys-tatins are members of an evolutionarily-related superfamily of proteinase inhibitors consisting of three major families, family 1 (the stefms), family 2 (the cystatins), family 3 (kininogens), as well as other cystatin-related proteins. These families are distinguished on the basis of their polypeptide chain complexity and their cellular location (1,2). The cystatins no doubt play a major role in mediating normal physiological processes in which cysteine proteinases are involved, e. g.

intracellular catabolism of polypeptides and proteins (3), processing of proenzymes and prohormones (4), extracellular degradation of collagen (5), bone resorption (6), and apoptosis (7,8). They are also involved in pathophysiological processes such as prevention of penetration and destruction of tissues by bacteria(9,10) and viruses (1 l,12),periodontal, lung, andjoint inflammatory diseases (13), regulation and assembly of many viral proteins including those of HI V-1(12), and cancer progression (14). Thus, impairment of cystatin function can lead to uncontrolled proteolysis and therefore to devastating and irreversible damage.

CYSTATINS IN DISEASES

• The importance of cystatins is further emphasized by diseases that are directly caused by genetic alterations of cystatins, and diseases that are associated with decreased quantities of cystatins. Cystatin C, found in highest concentra tions in mother's milk, semen, and spinal fluid, is unusually low in the cerebrospinal fluid of patients with multiple sclerosis (15). A defective cystatin has been identified in the skeletal muscle of mice with hereditary muscular dystrophy (16). Icelandic pa tients with familial amyloid angiopathy, or hereditary cerebral hemorrhagia, have an autosomal dominant form of amyloidosis which results in massive cerebral hemorrhages andthrombotic strokes leading to death. This disease is a direct result of an autosomal dominant variant form of cystatin C that has a point mutation in the second exon of the cystatin C gene - C AG instead of CTG, a glutamine is substituted for a leucine residue (17-20). Significantly, two mutations (3-'splice site mutation and stop codon mutation) in the cystatin B gene have been found to be directly responsible for the disease progressive myoclonus epi lepsy, an autosomal recessive inherited form of epilepsy (21).

SALIVARY CYSTATI MS

• Cystatins are widely distributed in human tissues and body fluids. They are present at high levels in saliva, cerebrospi nal fluid, milk, tears and amniotic fluid where they constitute an important barrier against pathogens. Salivary glands of mam mals are specialized to produce secretions that are important in early digestive processes and that bathe and protect the oral cavity, and the secreted cystatins are thought to play a major role in this process. The salivary cystatins, first identified in human saliva as cysteine-containing phosphoproteins (22,23), were purified by several investigators, and a salivary gland-specific cystatin, cystatin S, was found to be synthesized in the acinar eel Is of submandibular and parotid glands (24-26); cysta tin S was absent fronxbronchial mucosal glands and pancreas. Isemura *et al* (24,25) demonstrated that whole human saliva contains at least eight cystatins (S 1 to S8), differing only in their N-terminal region. Additional cystatins have been isolated from whole human saliva (SA, SN) and human submaxillary/sublingual saliva (SA-1). These cystatins differ in their isoelectric points, inhibitory specificities, and length of their polypeptide chains. Three genes encoding cystatins are produced inhuman salivary glands; one of these is a pseudogene, and the other two encode cystatins S and SN (27). Genomic Southern blots indicate that cystatin genes expressed in human salivary glands form a multigene family with at least seven members. Subsequently, a rat salivary cysteine proteinase inhibitor gene, cystatin S, was identified (28). It was demonstrated that rat cystatin S is specific for cysteine proteinases. The expression of the rat cystatin S gene is tissue- and cell type-specific, and can be precociously induced in both the SMG and the parotid glands by the (3-adrenergic agonist isoproterenol (IPR) (29). The rat cystatin S gene is also unique in that it is expressed early during development of rat SMG, and that it can be induced, by (3-adrenoceptor-mediated mechanisms, to express in adult glands where it is normally turned off.

REGULATION OF GYSTATIN S GEME EXPRESSION

• Little is known about the regulation of cystatin genes. Our laboratory was the first to demonstrate |3-adrenergic regu lation of expression of a cystatin gene, rat cystatin S, and is currently investigating the neural mechanisms regulating sali vary cystatin genes, using the rat cystatin S gene as a prototype. The rat cystatin S gene is expressed postnatally in the develo ping SMG; the concentration of cystatin S mRNA reaches peak levels at 28 days after birth. Cystatin S mRNA subsequently declines to barely detectable levels at 32 days and to undetect-able levels at 35 days following birth (29). Regulation of the cystatin S gene by the autonomic nervous system is suggested by its inducibility in the SMG, as well as the parotid gland, by the p-adrenoreceptor agonist IPR(28,30). IPR induces changes in salivary glands that mimic normal development of the glands. It has profound effects on the growth and function of SMG and parotid gland of rats and mice, stimulating DNA synthesis, hy~ perplastic/hypertrophic enlargements of the glands, and syn thesis/release of secretory proteins (31,32). In addition, IPR accelerates differentiation of acinar cells in developing SMG, and induces the precocious expression of the cystatin S gene (28,29). Upon IPR induction, cystatin S mRNA is approximately 20-fold more abundant in the rat SMG than in the partoid gland, and cystatin S mRNA is undetectable in the sublingual gland, suggesting that tissue-specific factors participate in its regula tion. Our earlier data suggested that sex hormones might also play a role in the regulation of the cystatin S gene, since its res ponse to IPR was 4-fold higher in adult females than in adult males (30). In order to examine regulation of cystatin S gene expression,wehavesequenced the gene(33),and 1911 bpofS'-flanking DNA. The nucleotide sequence of the gene encoding the precursor form of rat cystatin S shows that the organization of the rat cystatin S gene is simi lar to the human genes encoding the salivary cystatins S, SN, SA, and cystatin C, in that they all

have the same number of introns and exons, and similar exon-intron structure. A comparison of the DNA and protein sequence of these human cystatins and the rat cystatin S gene shows significant identity (50% for protein, 60% for DNA). Sequence of the 5 '-flanking region of the rat cystatin S gene (Fig. 1) revealed CAAT (-48) and TATA (-28) boxes upstream of the initiation codon, as well as several possible regulatory sequences that resemble those identified for other hormonally responsive eukaryotic genes. The sequence 5 '-TGACATCA-3' located at position -1777 in the 5' -flanking region of the cystatin S gene is a potential CREB/AP-1 binding site (34-37). Two potential identical glucocorticoid receptor binding sites are located atpositions -1487 and -187 (5 '-TGTGAT-3') (3 8); there isapotentialAP-2bindingsite(-194,TGGGGA)(39)adjacentto the putative glucocorticoid receptor binding site located at position -187. In addition, another potential glucocorticoid/ progesterone receptor responsive element, 5'-TGTTCT-3', is located at position -753 (40,41). Interestingly, the 5'-flanking region of the gene contains three regions that are common to all known sequenced salivary gland-specific genes (Table 1). In the rat Cys S gene they are: I, -711 TTTCCTACC; II, -865 AGTGACCCTA; III, -947 TGAGGGAGTG. The 5 '-flanking region of the rat cystatin S gene also contains a GT-rich region of 27 GT repeats that are located between domains II and III; this GT repeat region is flanked by TTT. The first intron sequence also contains another consensus CRE highly homologous to one found in the IPR-inducible mouse and hamster proline-rich-protein (PRP) encoding genes (42).

The expression of the cystatin S gene induced by IPR in the SMG of adult rats seems to be regulated through the (31-adrenergic receptor-cAMP-PKA pathway, since the effect of IPR is blocked by specific (31-, but not by p2-adrenergic antagonists (43). The effects of (3 1 -adrenergic receptor stimulation are mediated by the adenylatecyclase-cAMPpathway. Administration of IPR leads to a rapid increase of intracellular cAMP (44,45). Transcription of a large number of eukaryotic genes including the proto-oncogene *c-fos,* somatostatin, tyrosine hydroxylase, proenkephalin.vasoactive intestinal peptide(V!P),a-chorionic gonadotropin, and phosphoenolpyruvate carboxykinase is activated in response to increased intracellular levels of cAMP. Each of these genes has a common and well characterized short

Table 1. *Common elements in human, macaque, rat, and mouse salivary gland-specific genes*

Gene	I	II	III
hAMY1C	-849 TTTCCTACC	-802 AGAGTCCCTG	-784 TGAGGGATGC
rCystatin S	-711	-865 ..T.A....A	-947 ..... GTG
hCST1	-48 . C....G..	-173 C-.....CA	-245 AA...ACG.A * -178 --.....GAG
hCST2	-48 .C....G.A	NS	NS
hCSTP1	-48 .C.....AT	-173 ....-....C.	-245 A.....CGG
hPRP1	-212 .A.....	-183 .....AA	-167 .....A.
hPRPII	-212 CA....C..	-183 .....A.	-167 .....A.
macPRP	-936 .....G.	-96 ...A-...A.	-129 .A.....T.
mPRP	-400 .A.....T	-15 ..T.G.....	-173 .....T.A.G *
mPsp	-697 .G.....	-16 ...A....A.	-671 .....ATG

Nineteen hundred and eleven base pairs (1911 bp) of 5'-flanking sequence of the rat cystatin S (r Cystatin S) gene were compared with 251 bp of the human salivary amylase gene (hAMY 1C; Ref 118), 1 kb each of the human proline-rich proteins (hPRPs I and II; Ref 119), macaque proline rich protein gene (macPRP, MnP4; Ref 120), mouse proline-rich protein gene (mPRP, Ref 121), and the parotid secretory protein gene (mPsp, Ref 122). Approximately 360 bp of the 5 '-flanking region of the human salivary cystatin genes CST1 and CSTP1, and 100 bp of the human CST2 gene were compared (Ref 27). NS, not sequenced; *, reverse orientation; .... identity.

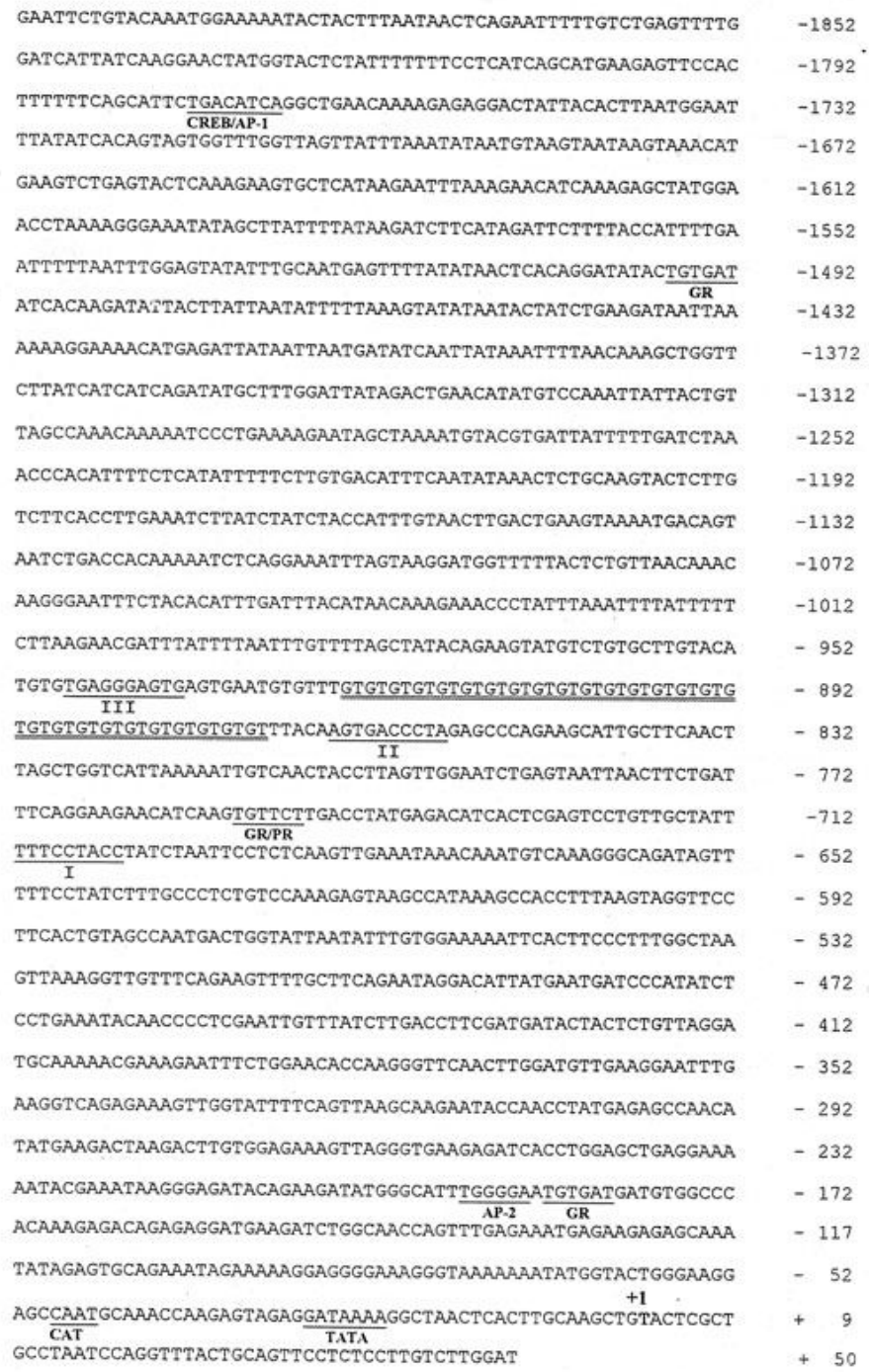


Figure 1. *Sequence of 5 '-flanking region of the rat cystatin Sgene. The DNA sequence includes 1911 bp 5' of the transcription start-site and 50 bp past the transcription start-site. The CCAA T box is located at position -48; the TA TA box is located at -28. Three potential glucocorticoid responsive elements are located at position -1487, -753, and -187, and an adjacent AP-2 binding site is located at-194. A potential CREB/AP-1 binding site is located at position -1777. Three salivary gland-specific domains are located at -711. -865. and -947. A GT-rich region is identified beginning at -924.*

palindromic core motif 5-TGACGTCA-3, the cAMP response element, in their promoter sequence (46-50). The effect of cAMP on gene transcription is usually rapid and does not require newly synthesized protein(s), suggesting that transcriptional modulation bye AMP involves the covalent modification rather than *de novo* synthesis of nuclear factors (49,51). If an increase of intracellular cAMP is sufficient for the induction of cystatin S gene expression in acinar cells, one would expect the gene to be expressed *in vivo* in response to IPR even in the presence of protein synthesis inhibitors. Results presented here, however, show that IPR-induced expression of the cystatin S gene in rat SMG is dependent on newly synthesized proteins, since its expression is completely blocked by simultaneous treatment of the animals with cycloheximide (Fig. 2).

Based on our previous findings that IPR-induced levels of steady-state cystatin S mRNA in SMG is greater in adult female than in adult male animals (29), we hypothesized that gonadal steroids and glucocorticoids participated in IPR-regulation of the cystatin S gene. Thus, we examined the *in vivo* regulation of the cystatin S gene by gonadal steroids and/or glucocorticoids. We quantitated the steady-state levels of cystatin mRNA in intact and/or adrenalectomized (adx) and castrated/ovariectomized (cx/ovx) animals in order to determine if gonadal hormones or glucocorticoids participate in the regulation of cystatin S gene expression. The data indicated that the differential expression of the cystatin S gene in female *vs* male SMG is not only regulated by the p-adrenoreceptor-mediated pathway, but other factors and/or steroid hormones also play a part in its regulation. Furthermore, dexamethasone appears to be acting in a gonadal hormone-dependent fashion in its participation in regulating the cystatin S gene, it negatively regulates the cystatin S gene in females (52).

AUTONOMIC NERVOUS SYSTEM AND CYSTATIN S GENE EXPRESSION

• Both branches of the autonomic nervous system innervate the major salivary glands of the rat, and regulate the volume and composition of saliva (53). In adult animals, stimu lation of cholinergic and cc-adrenoreceptors elicits the formation of moderate amounts of saliva rich in electrolytes, whereas stimulation of (3-adrenoreceptors stimulates secretion of small amounts of salivarich in proteins (54-57). It has long been known that the size of salivary glands is influenced by the autonomic nervous system, and that in adult rats prolonged electrical stimulation of the sympathetic branch of the autonomic nervous system *via* the superior cervical ganglion (SCG) causes enlar gement of salivary glands by increasing both cell size and cell number (59,60). Denervation of adrenergic target tissues leads to compensatory upregulation of a- and p-adrenergic recep tors, and to supersensitive responses to adrenergic agonists. This supersensitivity has been documented, for example, in rat cerebral cortex(61), raturinarybladder(62), ratpineal gland (63), rat parotid gland (64), and in smooth muscles of rabbit iris (65). Surgical or chemical sympathetic denervation in adult rats results in supersensitivity of the SMG to the p-adrenergic sialogogic effects of norepinephrine and IPR (66-71). Catecholamine stores in sympathetically innervated organs are depleted rapidly after a single administration of reserpine, and the depletion is almost complete (>95%) within the first day (72). As a

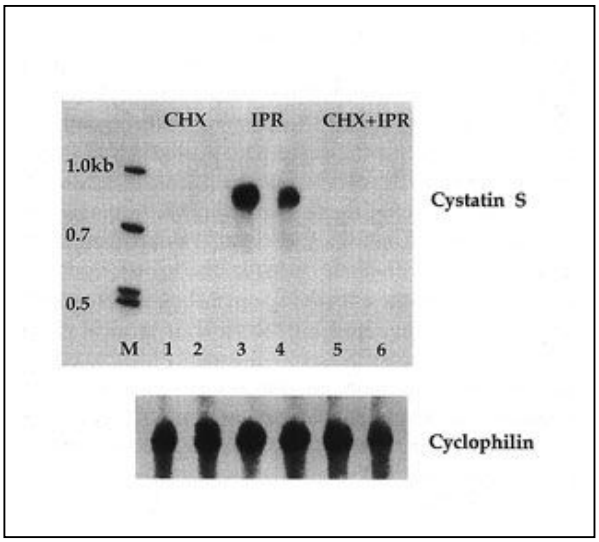


***Figure** 2. Northern blot analyses of the effect of cycloheximide on [PR-induced cystatin S gene expression. Five micro-grams of total RNA extracted from submandibular glands was electrophoresedinpolyacrylamide-nreagels. The RNA, dissolved in sterile distilled water containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), was mixed with 10 fjlof'loadingbuffer (80%formamide, WmMEDTA, O.Smg/mlxylenecyanol, 0.5 mg/mlbromophenol blue) andelectrophoresed at 250 volts for 1 hr in 4% polyacrylamide-8 M urea gels in IX TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA), using a minigel system from Hoefer Scientific Instruments. As molecular weight markers, 5000 c.p.m. ofa^{32}P-labeledDNA ladder (UnitedStates Biochemical Corporation) were used. The RNA was electrotransferred for 1 hr at 250 mamps in 0.5X TBE onto Gene Screen Plus membranes. The RNA blots were hybridized overnight at 65 °C with the ^{i2}P-labeled cystatin S riboprobe and a cyclophilin riboprobe, washed according to Shaw et al (28), and analyzed in the Phosphorlmager (Molecular Dynamics). Lanes 1 and 2 are from two adult female rats treatedwith cycloheximide alone, lanes 3 and4 arefrom two female rats treated with IPR. Lanes 5 and 6 arefrom two female rats treated with cycloheximide and IPR simultaneously.*

result, there is a decreased concentration of norepinephrine at the synapse, a marked increase in adrenergic receptors, (73), and also supersensitivity to IPR in salivary glands (58,74). Binding studies have demonstrated that the increase in the [3-adrener-gic response in sympathectomized rat SMG is paralleled by an increase in the density of the (31 -adrenergic receptors in membranes prepared from the glands, with no change in binding affinity (70,72,75). The supersensitivity of the SMG is also illustrated by the increase in intracellular cAMP in response to IPR in cells from SMG of rats previously treated with reserpine as compared with cells from glands of control animals (74). Basal c AMP levels were similar between control (1 *A+l-* 0.3 pmol/mg) andreserpine-treated glands (2.0+/-0.6pmol/mg). However,the increase in intracellular c AMP in response to IPR was markedly accentuated in glands from reserpine treated rats (79-fold increase) compared with control glands (13-fold increase; 74). It has also been demonstrated that the SMG remains super-sensitive to norepinephrine 6 weeks after sympathetomy (76).

We have analyzed the effect of sympathectomy of the SMG of adult female rats, by removing the superior cervical ganglion (bilaterally or unilaterally), or by chemical sympathectomy by treatment with reserpine, on IPR-induced expression of the cystatin S gene. The amount of cystatin S mRNA. as determined by quantitation of Northern blots, in the SMG of bilaterally sympathectomized, IPR-treated animals was reduced to 47 % that of SMG of sham operated, IPR-treated animals. In unilaterally sympathectomized, IPR-treated rats, the amount of cystatin S mRNA in the sympathectomized SMG was 44 % that of intact glands. The amount of cystatin S mRNA in the glands of adult rats treated with a single dose of reserpine for each of 2 days 24 hr before a single IPR injection was 30 % that of the non-reserpine, IPR-treated glands (see Table 2; 77). These results were somewhat unexpected. If, cystatin S gene expression is regulated directly by the p 1 -adrenergic receptor pathway, one would have expected one of the following results: (*l*) overexpression of the cystatin S gene in response to IPR in sympathectomized animals since P1 -adrenoreceptors are upregulated upon surgical sympathectomy or reserpine treatment and SMG become super-sensitive to p-adrenergic agonists, and *(if)* alternatively, if the intracellular concentration of cAMP reached in the SMG of nondenervated rats upon treatment with IPR is sufficient to produce the maximal level of expression of the cystatin S gene, it would be expected that in the denervated glands (which are supersensitive to IPR), a further increase in the intracellular concentration of cAMP in response to IPR would not produce any significant change in the levels of cystatin S gene expression. However, both bilateral and unilateral sympathetic de-nervation reduced the levels of cystatin S gene expression in response to IPR, and a comparable effect was observed in rats previously treated with reserpine. In other words, the expression of the rat cystatin S gene mediated by pi-adrenergic receptor stimulation was partially blocked by sympathetic denervation, indicating there is clearly a lack of correlation between cAMP accumulation and cystatin S gene expression .

There are several precedents in the literature for similar phenomena. It has been found that the ability of IPR to stimulate ornithine decarboxylase (ODC), a growth related enzyme, in heart, lung and kidney is reduced by neonatal sympathectomy (78). P-adrenergic receptors are not coupled directly to ODC gene expression, but rather receptor stimulation initiates the synthesis of new ODC molecules through a cAMP-dependent process mediated by the protooncogene *c-fos.* Sympathetic denervation by 6-hydroxydopamine produces a loss of the ability of stimulated p-adrenoceptors to induce expression of the protooncogene *c-fos,* resulting in reduced expression of ODC in the rat cerebellum (79). There are also interesting examples in the rat parotid gland resembling the reduction of cystatin S gene expression in response to IPR in the SMG of sympathectomized rats. The sympathetic and parasympathetic nervous systems have a regulatory role on PRP synthesis in parotid glands, since there are significant decreases in the synthesis of PRP in the denervated parotid glands of unilaterally sympathectomized (14 % reduced), parasympathectomized (reduced by approximately 16 %), and double denervated rats (sympathectomized and parasympathectomized; 26 % reduced). The effect is additive as double denervated glands show changes

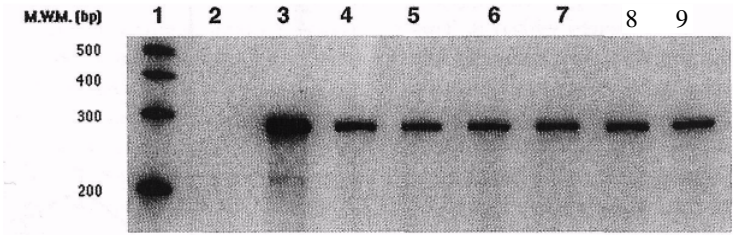
Table 2. Effect of aittonomic denervation on IPR-induced expression of the cystatin S gene in female rat submandibiilar glands

Animal	*Denervation*	*Cystatin S gene expression (% of the expression in control non-denervated glands)*	*Significance (p value)*
Adult	Bilateral Sx [a] (n=4)	47 %	0.029
	Unilateral Sx (n=8)	44 %	0.005
	Chemical Sx (Reserpine; n=5)	30 %	0.024
8 day-old	Unilateral Sx [b] (n=4)	62 %	0.023

Sx. sympathectomy bv removing the superior cervical ganglion (a). Animals unilaterally sympathectomized at one day of age (b).

approximately equal to the combined individual changes of sympathectomyandparasympathectomy(80). Maintenance of rats exclusively on a liquid diet results in a reduction in the size of the parotid gland 4 days after introduction of the liquid diet (81). However, reintroduction of a solid diet restores the gland size and also induces a burst in cell proliferation that peaks after 2 days ofthe dietary change (82). This dietary change is accompanied by increased masticatory activity, and growth responses appear to be mediated by the autonomic nervous system (83). A marked increase in the activity and cell surface levels of the enzyme pi-4 galactosyltransferase is also induced in rat parotid glands when autonomically mediated activity of the gland is increased by a dietary change from all liquid to solid food (84). The same effect is observed following increased acinar cell proliferation ofthe parotid gland that occurs after chronic exposure ofthe rats to IPR (85). However, removal of either parasympathetic or sympathetic innervation to the parotid gland prior to the dietary change results in a partial inhibition ofthe increase in p 1 -4 galactosyltransferase enzymatic activity: in parasympathectomized glands the increase in enzyme activity was 51 % that ofthe innervated glands, and with sympathec-tomy, it was 41 % (86). This picture resembles the observed reduction of IPR-induced expression of the cystatin S gene in sympath-ectomized SMG, and strongly suggests that the expression ofthe cystatin S gene in the SMG, p1-4 galactosyltransferase and proline-rich proteins in the parotid gland, and probably other genes regulated through the p-adrenergic pathway (like ODC), require the participation of additional factors from the sympathetic and/or parasympathetic nervous system. Tissue slices of SMG respond to norepinephrine and IPR *in vitro,* as determined by an increase in the levels of cAMP (73, 74). Isolated rat SMG acini are responsive to a-and P-adrenergic agonists as indicated by increased secretion of mucin in response to norepinephrine and IPR (45,87-89), dibutyryl-cAMP (90), and forskolin (45,87). Responsiveness ofthe SMG to IPR *in vitro* has also been documented by the function of stimulatory G proteins (Gs), increased adenylate cyclase activity (91), and increased levels of intracellular cAMP (45). However, cystatin S gene expression is not induced *in vitro* in primary cultures of SMG cells in response to IPR. This lack of inducibility of cystatin S gene expression has also been observed in primary cultures or tissue slices of SMG treated with norepinephrine, forskolin, dibutyryl-cAMP, p-estradiol, ordexamethasone. No induction of cystatin S gene expression has been detected by RT-PCR analyses in primary cultures under different conditions of (0 serum concentration (from 0-10% PCS), *(if)* surface where the cells were grown (plastic, collagen, laminin), *(Hi)* time of exposure to the agonists, and (/v) varying concentration of IPR (Fig. 3).

Treatment of adult rats with 6-hydroxydopamine, which depletes norepinephrine stores, destroys the ability of nerves to take up exogenous norepinephrine, selectively destroys the sympathetic nerve terminals (92-94), and greatly reduces DNA synthesis in SMG of IPR-treated animals. A single dose of 6-hydroxydopamine given one hr prior to the administration of IPR completely eliminated the DNA synthesis induced by IPR in rat SMG (95).



B +c SUBMANDIBULAR GLAND CELLS *(ii*

IPR + - - - + + +

Figure 3. *Reverse-transcriptase analysis of cystatin S gene expression in control and IPR-treated cells from primary cultures of rat siibmandibular glands. Cystatin S mRNA was amplified using a GenAmp kit (Perkin Elmer Cetus, Branchbwg, NJ) following the manufacturers instructions. The PCR products were radioactively labeled using 0.1 /jM[12]P-ATP in each reaction tube during the last 5 cycles of amplification. As a template, 250 ng of total RNA were used; as a positive control, 100 ng of total RNA from siibmandibular glands of adult female rats treated with IPR were used. The upstream primer 5 '-GATATCATGAACCACGAAAGA-3' that primes at postion +152 to +172, and the downstream primer 5'-CTAGGTCACTTTCTGGGTGGC-3 ', priming at position +5024 to +5044 ofthe genomic DNA sequence, amplify a 296 bp DNA fragment from the cDNA. This distinguishes the PCR product from any potential amplification from the genomic DNA, that would produce a 4892 bp fragment. The amplification products were analyzed in 5%polyacrylamide-wea denaturing gels, transfered onto GeneScreen Plus membranes and analyzed in the Phosphorlmager.*

Furthermore, the increase in DNA synthesis induced by IPR *in vivo* could not be reproduced *in vitro.* However, if rats are treated with IPR before the SMG are removed and cultured *in vitro,* DNA synthesis proceeds normally (Barka, personal communication), indicating that the DNA synthesis machinery ofthe SMG in culture remains functional. This suggests that the SMG require exposure *in vivo* to other sympathetic factor(s) in addition to IPR in order to be able to initiate the series of events leading successfully to initiation of DNA synthesis and replication. Again, experiments analyzing the increase in size ofthe rat parotid gland induced by a dietary change from all liquid to solid food, as discussed previously, provides additional support to this hypothesis. This change in diet is followed by an average increase of 200% in 3 H-thymidine uptake into the parotid gland. However, removal of either parasympathetic or sympathetic in-nervation to the parotid gland prior to the dietary change resulted in a partial inhibition ofthe increase; values for the parasym-pathectomized gland were 51 % ofthose ofthe innervated gland, and the values for the sympathectomized parotid gland were 42% ofthose ofthe innervated gland. Removal of both autonomic nerves resulted in complete inhibition of 3H-thymidine uptake into the parotid glands (86). Altogether these data suggest that at least some ofthe effects of pl-adrenergic receptor stimulation *in vivo,* e.g. induction of DNA synthesis, and expression of specific set of genes, are not regulated directly *via* c AMP, and that additional factor(s) from sympathetic and/or parasympathetic nerve terminals are required. In addition, IPR-induced expression ofthe cystatin S gene in SMG is dependent on newly synthesized proteins, and cystatin S gene expression does not increase in the glands of sympathectomized rats treated with IPR, which are supersensitive to P-adrenergic agonists, compared to the nondenervated glands similarly treated with IPR. This indicates that the cystatin S gene is not directly regulated by the P-adrenergic receptor-c AMP pathway. However, pl-adrenergic specific antagonists completely block the ability of IPR to induce cystatin S gene expression (87). Collectively, these results indicate that the pl-adrenergic receptor pathway is necessary, but is not sufficient for the full IPR induction of cystatin S gene expression.

In the rat SMG, the sympathetic nerves reach the gland by postnatal day 5, and activate secretion beginning on postnatal day 6 (97,98). p-adrenergic receptors are found on the surface of secretory cells as early as day 1 after birth (73), and they are functional since they are able to induce peroxidase secretion occurs in response to IPRstimulation (98,99). Our experiments are consistent with these reports and indicate that p-adrenergic receptors, present early in the developing rat SMG, are functional in that they are capable of responding to IPR stimulation by inducing the expression ofthe cystatin S gene even at 3 and 4 days of age, when sympathetic nerve fibers have not reached the gland (Fig. 4). This indicates that sympathetic innervation is not requisite for IPR-induced expression ofthe cystatin S gene. However, once the SMG is innervated by sympathetic nerve terminals, a more robust response to p-receptor stimulation is evident; the level of expression of cystatin S mRNA induced by IPR on postnatal day 8 is significantly higher than the levels during previous days (days 3-6) (Fig. 4). Morphological analyses of developing rat SMG show a continuous change in the composition of acinar and proacinar cell populations at 3,5, and 8 days of age (100). The levels of cystatin mRNA in the SMG of IPR-treated rats do not parallel the changes in acinar and proacinar cell populations, and are not significantly different between 3 and 6 days of age, indicatingthat IPR-induced expression ofthe rat cystatin S gene during early development ofthe SMG is not j ust a function ofthe degree of differentiation ofthe acinar cells. Between days 4 and 6 after birth, the number of P-adrenergic receptors increases four-fold in the rat SMG. This increase in the number of (3-receptors coincides with the appearance of adult-type stimulus-secretion coupling in the gland (98). However, increase in the number of P-adrenergic receptors is not paralleled by a similar increase in the level of expression ofthe cystatin S gene induced by IPR. As in the sympathectomized adult SMG, an increase in the number of p -adrenergic receptors was not accompanied by an increase in the expression ofthe cystatin S gene upon IPRtreatment. Membrane-associated adenylate cyclase activity from the SMG of 1 - and 4-day old rats

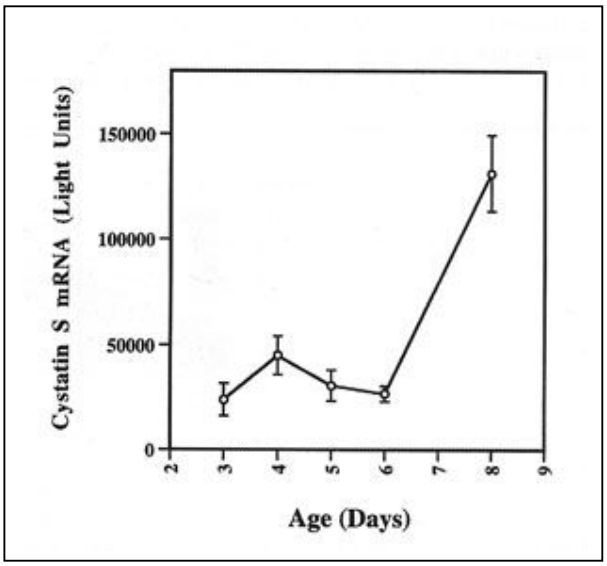


Figure 4. *Quantitative analyses of IPR-induced cystatin S gene expression during early development of the rat sub-mandibnlar gland. Northern blots were qitantitated using the Phosphorlmager. The values are expressed as cystatin S mRNA light units (mean LU +/- SEM) and the data were analyzed by one wayANO VA and the post-hoc analysis was performed by the Tukey-HSD multiple range test with the significance level set at 0.05. Each data point represents the mean of 10 (9 for day 4)vales*

is stimulated reproducibly 25-40 % by IPR, and membranes from glands of 6-day old animals show activation of adenylate cyc-laseby IPR, similar to the 2.5 to 3.5-fold activation seen in the membranes of glands of adult rats (98). This indicates that by postnatal day 6, all the components of the (3 -adrenergic receptor signaling pathway are functionally coupled. Again, this increase in adenylate cyclase activation by IPR was not paralleled by a corresponding increase in the level of expression of cystatin S gene in the SMG of 6-day old rats. In summary, the development of SMG during the first postnatal week is a continuous process that is not reflected in a similar increase in the expression of the cystatin S gene induced by stimulation of the p -adreno-ceptors by an agonist. It was only after 6 days of age that a significant increase in cystatin S gene expression was observed in response to IPR, indicating that expression of the rat cystatin S gene is not entirely dependent on the p-receptor/cAMP path way, and, again, that additional factor(s), probably from the sympathetic nervous system, are required for its full response to IPR.

In SMG of neonatal rats, chemical sympathectomy does not affect the time of appearance or the number of p-adrenergic receptors (101), indicating that the rat SMG does not become supersensitive after chemical sympathectomy during early development. In addition, sympathetic innervation of the rat SMG is not fully developed in newborn animals, and catechol-ainine-containing neurons are not seen before day 5 of age (97, 98). Such a non-innervated SMG offers an excellent experimental model to test the hypothesis that an intact sympathetic innervation is required for IPR-induced expression of the cystatin S gene. We tested this hypothesis by analyzing IPR-induced expression of the cystatin S gene in the rat SMG during early posnatal development, before and after the sympathetic nerve terminals reach the gland (as described above), and in sym-pathectomized and nondenervated SMG of 4 and 8 days of age that have been unilaterally sympathectomized by removing the superior cervical ganglion on postnatal day 1. Since sympathetic fibers only reach the rat SMG by postnatal day 5, unilateral sympathectomy on day 1 allows the analyses in the same animal, of the effect of IPR on cystatin S gene expression in partially or completely innervated glands, and glands that have never received sympathetic innervation. A comparison of the levels of cystatin S mRNA induced by IPR in innervated versus sympathectomized (sympathectomy at day 1) glands of 8-day old animals indicates that sympathetic factor(s) are required for the full IPR-induction of cystatin S gene expression (Table 2). Moreover, these results are similar to those of the sympathetic denervation experiments in adult rats, and show that the reduction in the level of cystatin S gene expression induced by IPR is the result of depriving the denervated gland of sympathetic factor(s), independently of the condition of P-adrenergic agonist supersensitivity in the SMG after sympathectomy, i.e. no supersensitivity in the newborns or super-sensitivitv in adults.

A growing list of neuropeptides has been identified that appear to play an important role in the regulation of the SMG metabolism (102). Secretin and VIP enhance norepinephrine (NE)- and substance P (SP)-mediated secretion of fluid and protein from rat SMG (103,104). Calcitonin gene-related peptide (CGRP), SP and VIP, are most likely involved in the non-adrenergic, non-cholinergic (NANC) secretion of saliva in response to stimulation of parasympathetic innervation (102,105,106). Galanin, another neurotransmitter, acts by inducing hyperpolarization of resting membrane potential in the rat SMG (107). There are limited physiological data on the effects of neuropeptide Y (NPY), the most abundant sympathetic neuropeptide in the salivary glands. Local, intraarterial infusion of NPY induces a dose-dependent vasoconstriction in the cat SMG (108), and NPY has also been proposed to be responsible for increased amylase secretion in parotid glands (109). Based on the distribution of NPY fibers, it has been suggested that in addition to its vascular effect, NPY may also have a functional role in the regulation of secretion from the parotid, sublingual and, possibly, the SMG of the rat (110). Interestingly, the rat SMG and parotid gland become supersensitive not only to P-adrenergic agonists but also to sympathetic neuropeptides as a result of autonomic denervation (111).

In addition to their role in synaptic communication, neurotran-smitters are now recognized to play an important trophic role in cell differentiation within both the central nervous system and peripheral neural target tissues (79). Rats denervated at birth by 6-hydroxy dopamine treatment showed an impaired response to vasopressin or angiotensin in stimulating cardiac ODC activity. Responsiveness of the kidney was affected only for vasopressin, and in the lung denervation had only transient effects on hormonal responses. This confirmed that sympathetic input is required for proper development of some, but not all, hormonal responses, and indicates that the role of neuronal factors is tissue specific (112). There is increasing evidence that neuropeptides exert receptor-mediated effects on gene expression. VIP, secretin, and peptide histidine isoleucine increased the expression of tyrosine hydroxylase gene, the rate-limiting enzyme in the catecholamine biosynthetic pathway, and this effect is mediated by the cAMP second messenger pathway (113). VIP also stimulated *c-fos* and *c-myc* gene expression in several breast cancer cell lines (114). VIP andNE induced massive gly-cogen synthesis in mouse cortical astrocytes that was mediated by specific receptors coupled to the cAMP signal transduc-tion cascade in a protein synthesis-dependent fashion (115). More recently, it has been shown that glycogen synthesis induced by VIP, pituitary adenylate cyclase-activating peptide, and NE was mediated by induced expression of the transcription factors CC AAT/enhancer binding protein (C/EBP)-b and C/EBP-d(l 16). The fact that several neuropeptides regulate the expression of different transcription factors reinforces the notion that they play an important role in the physiology of their

peripheral target tissues, and suggests that they may establish complex interactions in the regulation of gene expression in those tissues.

CONCLUSION

• Since the expression of the cystatin S gene in respon se to IPR in surgically, bilateral and unilateral, sympathectomized and chemically, reserpine, denervated SMG is reduced to about the same extent and not totally suppressed, suggests that other factor(s) in addition to the p-adrenergic pathway partici pate in its regulation. In addition to norepinephrine, the major neurotransmitter in the sympathetic nerve terminals, other neurotransmitters or neuropeptides are also depleted by sympathectomy. It is possible that one or more of these factors parti cipate in the induction of the cystatin S gene by IPR. Interest ingly, the concentrations of VIP, CGRP and SP are increasing during development of the SMG when the cystatin S gene is maximally expressed (117). Furthermore, since the two branches of the autonomic nervous system act in parallel in the SMG, the parasympathetic nerve terminals may also provide factor(s) that are candidates for playing a role in IPR-induced expression of the cystatin S gene. Collectively, the evidence suggests that the regulation of the cystatin S gene is orchestrated by a complex set of neural, hormonal, and developmental factors.

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