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Inhibition of calcium-calmodulin kinase restores nitric oxide production and signaling in submandibular glands of a mouse model of salivary dysfunction

[1]Florencia Rosignoli, [1]Valeria Roca, [2]Roberto Meiss, [1]Nicolás Pregi & *,[1]Claudia Pérez Leirós

[1]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires – CONICET, Argentina and [2]Academia Nacional de Medicina, Buenos Aires, Argentina

1 Nitric oxide is an intracellular and diffusible messenger of neurotransmitters involved in salivary secretion, as well as an inflammatory mediator in salivary gland diseases. It is synthesized by three different isoforms of nitric oxide synthase (NOS), each subject to a fine transcriptional, post-transcriptional and/or post-translational regulation. Our purpose was to study the possible mechanisms leading to NOS downregulation in submandibular glands of normal mice and in the nonobese diabetic (NOD) mouse model of salivary dysfunction with lower NOS activity.

2 NOS activity and cGMP accumulation were determined by radioassays in submandibular glands of both mice in the presence of the protein kinase inhibitors KN-93 and bisindolylmaleimide. NOS I mRNA and protein expression and localization were assessed by RT–PCR, Western blot and immunohistochemistry.

3 A downregulatory effect of calcium–calmodulin kinase II (CaMK II) on NOS activity in submandibular glands of both NOD and BALB/c mice was observed. Our results are consistent with a physiological regulation of NOS activity by this kinase but not by PKC in normal BALB/c mice. They are also supportive of a role for CaMK II in the lack of detectable NOS activity in submandibular glands of NOD mice. KN-93 also restored cGMP accumulation in NOD submandibular glands.

4 The downregulation of NOS in NOD mice seems to be mainly mediated by this kinase rather than the result of a lower expression or different cellular localization of the enzyme. It was not related to different substrate or cofactors availability either.
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Keywords: NOS regulation; CaM kinase inhibition; KN-93; salivary dysfunction; NOD mice

Abbreviations: CaM kinase, calmodulin kinase; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KRB, Krebs–Ringer bicarbonate; L-NMMA, L-N^G-monomethyl arginine; NADPH, nicotinamide adenin-dinucleotide phosphate; PBS, phosphate-buffered saline; VIP, vasoactive intestinal peptide

Introduction

Among the various signaling pathways involved in the nervous control of saliva flow, nitric oxide might be assigned a critical role based on its dual behavior as an intracellular and diffusible messenger of neurotransmitter receptors that promote salivary flow, as well as an inflammatory mediator in a growing variety of diseases (Nathan, 1997; Alderton *et al*., 2001; Cirinol *et al*., 2003). Nitric oxide, shown to promote saliva secretion in various species (Lomniczi *et al*., 1998; Looms *et al*., 2001; Rosignoli & Perez Leiros, 2002a), is synthesized from L-arginine by a family of nitric oxide synthase (NOS) enzymes that occur in three different isoforms in mammalian tissues, each subject to a fine transcriptional, post-transcriptional and/or post-translational regulation that includes substrate and cofactor availability and phosphorylation among other mechanisms (Nathan & Xie, 1994; Förstermann *et al*., 1998). Phosphorylation of NOS I by PKC or by calcium-calmodulin (CaM) kinase II (CaMK II) downregulates NOS activity in most tissues (Nathan, 1997). We have reported that PKC strongly downregulates NOS activity and NO-mediated signaling in rat submandibular and parotid glands that results in a reduced amylase secretion in parotid glands (Perez Leiros *et al*., 2000; Rosignoli & Perez Leiros, 2002a). Evidence supporting the regulation of NOS I by CaMK II in neural cells has been provided. The phosphorylation of the residue Ser^{847} on NOS I by this kinase-attenuated NOS activity by inhibiting the binding of CaM to NOS (Hayashi *et al*., 1999).

Sjögren's syndrome is an autoimmune rheumatic disease characterized by a severe dryness of the mouth and the eyes (Strand & Talal, 1980; Jacobsson & Manthorpe, 1995; Fox & Michelson, 2000). The pathogenic mechanisms of the disease are presently unknown and the observation that the mild glandular infiltration cannot fully account for the marked impairment of saliva flow strongly suggests that neural regulatory pathways may have a role in the pathogenesis of *sicca syndrome*. The nonobese diabetic (NOD) mouse model is chosen among other models to study Sjögren's syndrome because of its unique characteristic of developing a deep

*Author for correspondence at: Biological Chemistry, University of Buenos Aires, Ciudad Universitaria, Pab. II, 4° piso, Buenos Aires 1428, Argentina; E-mail: cpleiros@qb.fcen.uba.ar

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secretory dysfunction that correlates poorly with the sparse lymphomononuclear infiltrates in submandibular glands or the complete absence of infiltrates in parotid glands (van Blokland & Versnel, 2002). This observation is consistent with the hypothesis that defects in some functional or signaling regulatory pathways within the target organ might increase susceptibility to an inflammatory response as reported in other autoimmune models (Perez Leiros *et al*., 1994; Roitt *et al*., 1998) and could account for the progressive loss of secretory function in NOD mice. In line with this, submandibular glands from NOD mice showed increased expression of cysteine proteases and matrix metalloproteases (Robinson *et al*., 1997; Yamachika *et al*., 1998), an abnormal expression of β-adrenergic receptors in both submandibular and parotid glands (Hu *et al*., 1994), and we have recently shown a lower activity of NOS in both submandibular and parotid glands at early stages of disease, in the absence of mononuclear infiltrates in the glands (Rosignoli *et al*., 2001). The loss of NOS activity was associated to a differential expression of NOS I by Western blot assays, with no changes of the other two isoforms of the enzyme, together with a lower response to neurotransmitter receptor-mediated signaling through nitric oxide in these mice (Rosignoli *et al*., 2001; Rosignoli & Perez Leiros, 2002b). Based on the assumption that early alterations in intracellular regulatory pathways might contribute to the above observations, thus predisposing the tissue to an inflammatory response, we studied the possible mechanisms leading to NOS downregulation in NOD submandibular glands. We present evidence of a downregulatory effect of CaM kinase II on NOS activity in normal BALB/c mice glands. We also show a post-translational regulation of NOS activity by CaMK II that occurs in the absence of infiltrating leukocytes in the glands of prediabetic NOD mice. Additional evidence presented here also indicates that NOS regulation does not involve changes in mRNA levels or in cellular localization of NOS I, and it is not related to substrate or cofactors availability.

Methods

Animals

NOD and BALB/c female mice were bred and maintained in the Central Animal Care facility at the University of Buenos Aires (Ciudad Universitaria, Buenos Aires). NOD mice (15 weeks old) were fasted overnight with water *ad libitum* before being used. They were routinely tested for blood glucose levels using the glucose oxidase method in 20 μl samples of NOD and control sera (Wiener Lab., Rosario, Argentina). NOD mice used throughout were considered prediabetic, as their values of serum glucose (133 ± 9 mg dl^{-1}, $n = 5$) did not significantly differ from those of control mice (119 ± 7, $n = 5$). Salivary glands from NOD mice showed no signs of mononuclear cell infiltration as observed on slices from glands embedded in paraffin and stained with hematoxylin–eosin as reported previously (Rosignoli & Perez Leiros, 2002b). All studies were conducted according to standard protocols of the Animal Care and Use Committee of the School of Exact and Natural Sciences, University of Buenos Aires.

NOS activity

NOS activity was measured in submandibular glands from fasted mice using L-[U-^{14}C]arginine as substrate as described earlier (Perez Leiros *et al*., 2000; Rosignoli & Perez Leiros, 2002b). Whole glands were incubated with 0.2 μCi L-[U-^{14}C]arginine (Amersham Pharmacia Biotech, Buckinghamshire, England, about 300 mCi mmol^{-1}), except for the kinetic studies where the incubation was performed with different L-arginine concentrations ranging from 0.14 to 10 μM in 500 μl of Krebs–Ringer bicarbonate (KRB) solution pH 7.4 gassed with 5% CO_2 in O_2 at 37°C for 30 min. Then, tissues were homogenized in 20 mM HEPES (pH 5.5) with 0.5 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM EGTA. NOS activity was calculated as total activity minus that measured in the presence of 500 μM L-N^G-monomethyl arginine (L-NMMA) (Sigma, MO, U.S.A.), and the activity of constitutive isoforms (calcium dependent) was assessed by incubating the tissues in KRB without calcium and with 5 mM EGTA. When used, inhibitors of kinases were included from the beginning of the incubation time at the final concentrations indicated. To analyze the availability of substrates and cofactors of NOS, we used a modification of the method of Bredt & Snyder (1990) for the assay of NOS activity. Salivary glands were homogenized, centrifuged at 10,000 × g 10 min and the supernatants were incubated at 37°C in a buffer containing 20 mM HEPES (pH 7.4), 0.2 μCi L-[U-^{14}C]arginine, 1 μM L-arginine, 1 mM DTT, 1.2 mM $CaCl_2$, 0.1 mM tetrahydrobiopterine and 1 mM nicotinamide adenin-dinucleotide phosphate (NADPH) for 30 min. Again the values of NOS activity shown are total minus L-NMMA and were expressed as fmol [^{14}C]citrulline-min^{-1} mg of protein^{-1}.

Guanosine 3′,5′-cyclic monophosphate (cGMP) determination

cGMP accumulation was determined in submandibular glands from fasted mice by means of a radioimmunoassay with anti-3′,5′-cGMP antisera from Chemicon Int. and [^{125}I]cGMP (> 2200 Ci mmol^{-1}) labeled by Dr Omar Pignataro from the IBYME (Buenos Aires, Argentina). Samples were prepared by incubating whole submandibular glands for 30 min in 1 ml KRB with 100 μM 3-isobutyl-1-methyl xanthine gassed with 5% CO_2 in O_2, vasoactive intestinal peptide (VIP) was added in the last 15 min at the final concentrations indicated and, when used, 500 μM L-NMMA and/or 3 μM KN-93 (KN) was included from the beginning of the incubation time. Submandibular glands were homogenized in ethanol and after being evaporated, residues were dissolved in 50 mM sodium-acetate buffer (pH 6.2) for subsequent cGMP determination and results were expressed in fmol mg^{-1} tissue wet weight.

Immunohistochemistry

Submandibular glands from NOD and BALB/c mice were fixed in 4% paraformaldehyde overnight at 4°C. The tissues were embedded in paraffin wax and sections of 4 μm were cut and placed on silanized glass slides. The immunoperoxidase staining kit Ldab2 (DAKO) was used according to the protocol recommended by the manufacturer. Briefly, tissue sections were deparaffinized, rehydrated in buffer and endogenous peroxidase activity of the tissues was quenched

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with 0.1% hydrogen peroxide in methanol for 30 min. The sections were incubated overnight at room temperature with the primary antibodies diluted 1 : 100 in phosphate-buffered saline (PBS). The primary antibodies were anti-NOS I monoclonal (BD, Transduction Laboratories, U.S.A.). Control sections without primary antibody were made. Biotinylated secondary anti-mouse antibodies were added and the sections incubated for 1 h. The sections were then incubated with streptavidin–biotin–streptavidin peroxidase complex for 30 min between each step, and sections were washed for 5 min in PBS. Finally, diaminobenzidine (DAKO) was used as the peroxidase substrate, and the tissue sections were counterstained with hematoxylin.

Immunoblotting

Submandibular glands were excised from fasted mice and immediately homogenized at 4°C in 50 mM Tris-HCl buffer (pH 7.5) with 0.15% Triton X-100 and protease inhibitors as reported previously (Rosignoli *et al*., 2001). Once centrifuged at $5000 \times g$ 10 min at 4°C, supernatants were frozen at −80°C until used and an aliquot of each sample was separated for protein determination. Extracts (100 μg protein/lane), positive controls for NOS I and NOS III (BD, Transduction Labs, KY, U.S.A.) and molecular weight standards (Amersham Pharmacia Biotech Inc., NJ, U.S.A.) were subjected to 7.5% SDS–PAGE, transferred to nitrocellulose membranes and revealed with specific monoclonal antibodies against NOS I or NOS III (BD, Transduction Labs, KY, U.S.A.) using an alkaline phosphatase conjugate (Sigma Chem Co, MO, U.S.A.) as we have described earlier (Rosignoli *et al*., 2001).

Arginase assay

The activity of arginase was determined from the urea production using a colorimetric kit (Wiener Lab., Rosario, Argentina). Whole glands were incubated with either 1 or 10 μM L-arginine in 500 μl of KRB solution gassed with 5% CO_2 in O_2 at 37°C for 30 min. Then, the salivary glands were homogenized in 20 mM buffer HEPES (pH 5.5). The homogenate was centrifuged at $18{,}000 \times g$ for 10 min and urea concentration was determined spectrophotometrically in the supernatants. The rate of urea production was used as an index for arginase activity.

RNA extraction and cDNA synthesis

Total RNA was extracted from isolated submandibular glands and brain using Trizol RNA Isolation System (Invitrogen, U.S.A.) according to the manufacturer's instructions. Total RNA (5 μg) was added to a reverse transcription mixture (3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT, 0.5 mM 2′-deoxynucleoside-5′triphosphate, 200 U murine Moloney leukemia virus reverse transcriptase and 2 μg oligo-(dT)18. The resulting mixture was incubated for 1 h at 37°C and then the sample was heated for 5 min at 60°C and maintained at −20°C until PCR amplification.

PCR amplification of cDNA

The cDNA was then amplified using the specific primers for NOS I with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers serving as an internal control. The NOS I sense and antisense primers used for the amplification of the NOS I cDNA fragment were chosen from Bartlett *et al*. (1999). Q10 Their sequence were, respectively, from 5′ to 3′ TGTGTGGGCAGGATCCAGTG and GGGACAGGCGCTGAACTCCA (amplified fragment of 507 bp). The primers used for GAPDH amplification were as follows: sense, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; and primer antisense, 5′-TCCTTGGAGGCCATGTAGGCCAT-3′. Reverse-transcribed cDNA (3 μl) were amplified in PCR mixture (total volume 25 μl) containing 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.2 mM dNTP, *Taq* polymerase 1 U and 800 nM of sense and antisense primers for NOS I amplification. The mixture was subjected to the PCR conditions: denaturation at 94°C for 5 min followed by 35 repeated cycles of denaturation at 94°C for 40 s, primer annealing al 55°C for 40 s and elongation at 72°C for 2 min, followed by a final extension cycle of 72°C for 10 min on an AMPLITRON II thermal Cycler. PCR products were size fractionated on 2% agarose gels and visualized by staining with ethidium bromide using a size molecular marker.

Drugs

KN-93 and bisindolylmaleimide I were from Calbiochem (U.S.A.), primers were synthesized by Invitrogen (U.S.A.), murine Moloney leukemia virus reverse transcriptase and oligo-(dT)18 were from Amersham Biotech (U.S.A.), *Taq* polymerase was from Invitrogen (U.S.A.), NADPH, HEPES, L-NMMA, DTT, Triton X-100, tetrahydrobiopterine, alkaline phosphatase conjugate and cGMP were from Sigma (U.S.A.). NOS antibodies were from BD (U.S.A.) and secondary antibodies with biotin and streptavidin–peroxidase from DAKO. All other chemicals used were of analytical grade.

Statistical analysis

Statistical significance of differences was determined by the two-tailed *t*-test for independent populations. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. Differences between means were considered significant at $P < 0.05$.

Results

NOS activity, expression and localization in submandibular glands of NOD and BALB/c mice

Figure 1a shows the lower activity of NOS in submandibular glands of NOD mice compared to BALB/c mice of the same age. This value also differed from NOS activity in glands of younger NOD mice (160 ± 40 fmol mg^{-1} in 8-week-old NOD mice, $n = 5$, $P < 0.05$). The calcium dependency of NOS activity confirms the constitutive nature of the isoforms present in normal mice glands. To address whether expressional regulation of NOS in submandibular glands of NOD mice could account for the lower activity observed, and provided the fact that NOS I is the major isoform in salivary glands and the only isoform affected in NOD mice as reported previously (Rosignoli *et al*., 2001), each mouse was studied for NOS activity, protein expression, immunohistochemical localization

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and mRNA levels of NOS I. Figure 1b shows the altered electrophoretic pattern of NOS I in the contralateral gland of each mouse used for NOS activity in Figure 1a. Finally, no change in mRNA levels or the localization of the enzyme in NOD compared with BALB/c mice was observed as shown in Figure 1c and d. Immunohistochemical studies indicated a periacinar and periductal localization of this isoform and the signal did not change in intensity or distribution between glands from NOD and control mice. The lower activity of the NOS was measured in the absence of infiltrating leukocytes. As stated previously (Rosignoli *et al*., 2001), NOS II was undetectable in murine submandibular glands, while NOS III expression did not change in NOD with respect to BALB/c glands (data not shown).

Among the regulatory pathways known to modify the biosynthesis of nitric oxide at a post-transcriptional level, the availability of substrate and cofactors plays an important role (Alderton *et al*., 2001). To further analyze this possibility, we assayed NOS activity in an extract of the submandibular glands where cofactors were exogenously added, on the one hand, and also we assayed the activity of arginase, an enzyme that binds L-arginine to produce urea, on the assumption that an increased arginase activity could reduce the substrate availability for the NOS in NOD mice. Table 1 shows the complete lack of specific activity of the NOS in submandibular gland extracts of NOD mice even in the presence of exogenously added cofactors. Also, there were no differences in the activity of arginase at both substrate concentrations tested ruling out that an increased arginase activity could deprive the NOS of substrate (Table 1).

Regulation of NOS activity by calcium-CaM kinase in NOD submandibular glands

In order to assess if PKC-mediated phosphorylation could have a role in the observed reduction of NOS activity as we had reported in normal rat submandibular and parotid glands (Perez Leiros *et al*., 2000; Rosignoli & Perez Leiros, 2002a), we assayed NOS activity in the presence of a specific PKC inhibitor, bisindolylmaleimide. Table 2 shows that the incubation of these glands with bisindolylmaleimide had no effect on NOS activity in BALB/c or NOD mice. PKC inhibition did not alter NOS activity at 0.3 and 3 μM L-arginine either (data not shown). In contrast, the inhibition of calcium-CaM kinase with KN-93 resulted in a stimulation of NOS activity that was specifically inhibited by the NOS inhibitor L-NMMA in NOD submandibular glands. BALB/c glands also showed an increase in NOS activity in the presence of KN-93, suggesting

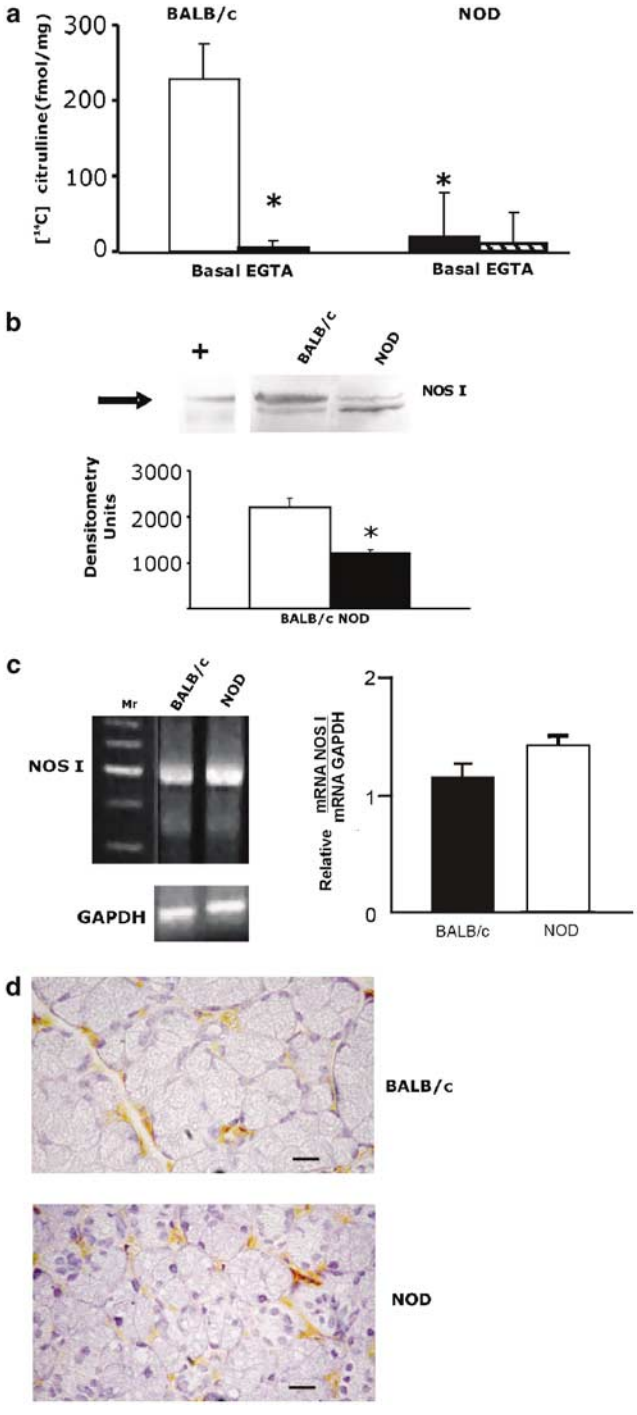

Figure 1 NOS activity and expression in mouse glands. (a) NOS activity in submandibular glands of BALB/c and NOD mice. NOS activity (BASAL) was measured with L-[U-^{14}C]arginine (0.7 μM) as described in Methods, and constitutive calcium dependency of NOS activity (EGTA) was determined in the presence of 5 mM EGTA. Incubations were run in the presence or absence of L-NMMA 500 μM. Results are the mean ± s.e.m. of at least five experiments, $^*P<0.05$ vs basal NOS activity in BALB/c mice. (b) NOS I expression in BALB/c and NOD glands as revealed by Western blot assays measured in the contralateral gland of each one of the mice used in (a) for basal NOS activity measurement. The blot shown is representative of five others run similarly where densitometric analysis of the corresponding band was performed with Array Gauge and Image Gauge software (Fuji Film, Japan). (+) Positive control of NOS I. Results are the mean ± s.e.m. of five experiments, $^*P<0.05$ vs BALB/c. (c) NOS I mRNA expression evaluated by RT–PCR in submandibular glands of BALB/c or NOD mice. On the left, there is a representative agarose gel with NOS I-amplified fragment of 507 bp as indicated by size molecular markers (Mr) and GAPDH. On the right, there is the relative intensity of NOS I vs GAPDH bands from glands of BALB/c and NOD mice quantified with Array Gauge and Image Gauge software (Fuji Film, Japan). Results are means ± s.e.m. of five separate experiments. (d) Immunohistochemistry of NOS I in submandibular slices of BALB/c and NOD mice counterstained with hematoxylin. Sections shown are representative of four other glands analyzed similarly (bar = 10 μm).

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Table 1 NOS and arginase activity in submandibular glands

	NOS activity (fmol (mg prot. min)$^{-1}$)		*Arginase activity* (μg urea (mg prot. min)$^{-1}$)	
	BALB/c	*NOD*	*BALB/c*	*NOD*
Basal	39.9±4.9	0.58±5.8[a]	16.9±2.7	13.8±1.5

Homogenates of submandibular glands were incubated for 30 min with L-arginine (1 μM) in the corresponding medium for NOS and arginase activity assays as indicated in Methods. For NOS activity assays, the incubations were run in the absence and presence of L-NMMA (500 μM) to calculate nonspecific and it was included from the beginning of the incubation time. Results shown are the means±s.e.m. of at least four different experiments run in duplicate.
[a]Significantly different from basal value in BALB/c mice ($P<0.05$).

Table 2 Effect of PKC and CaM kinase inhibitors on NOS activity

	NOS activity (fmol mg^{-1})	
	BALB/c	*NOD*
Basal	228±25	20±25[a]
Bisindolylmaleimide (Bis)	221±26	12±38
KN-93	376±31[a]	148±23[b]

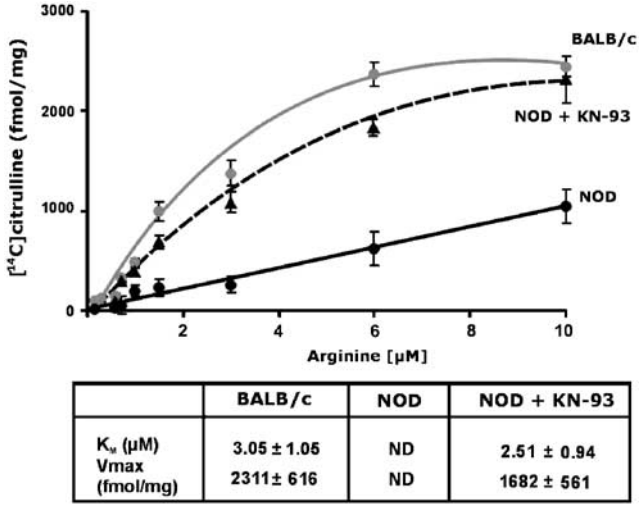
Submandibular glands were excised and incubated for 30 min in the corresponding medium as indicated for NOS activity in Methods. Bisindolylmaleimide (50 nM) and KN-93 (3 μM) were added from the beginning. Incubations were run in the presence or absence of L-NMMA (500 μM). Results shown are the means±s.e.m. of at least four different experiments.
[a]Significantly different from basal value in BALB/c mice ($P<0.05$).
[b]Significantly different from basal and bisindolylmaleimide value ($P<0.05$).

a physiological regulation of NOS I by this kinase in murine submandibular glands.

To further characterize the inhibitory effect, we assayed NOS activity at different L-arginine concentrations in the absence and presence of the inhibitor KN-93 to calculate NOS kinetic parameters. The curves depicted in Figure 2 and the corresponding kinetic data shown in the inset indicate the apparent absence of NOS activity detectable in NOD glands (no saturable activity) in contrast with the activity measured in BALB/c glands. When KN-93 was included in the incubation media, the presence of the CaMK II inhibitor restored the activity of the NOS in NOD submandibular glands and the kinetic parameters were similar to those calculated for control BALB/c mice.

Effect of KN-93 on cGMP levels in submandibular glands of NOD mice

We have already reported that VIP can stimulate NOS activity in submandibular glands of BALB/c mice but not in NOD glands (Rosignoli *et al*., 2001). In order to investigate whether the restoration of NOS activity could also influence cGMP accumulation in NOD mice in response to the VIP, submandibular glands were incubated with CaM kinase inhibitor KN-93 for cGMP determination. Figure 3 shows that KN-93 induced an increase in cGMP accumulation stimulated by VIP in NOD submandibular glands comparable to that observed in BALB/c glands. L-NMMA inhibited this effect in both NOD and BALB/c mice, indicating that the restoration of cGMP stimulation observed was dependent on nitric oxide production.



	BALB/c	NOD	NOD + KN-93
K_M (μM)	3.05 ± 1.05	ND	2.51 ± 0.94
Vmax (fmol/mg)	2311± 616	ND	1682 ± 561

Figure 2 Kinetic studies of NOS activity measured in submandibular glands of BALB/c and NOD mice incubated in the absence (NOD) or presence (NOD + KN-93) of 3 μM CaM kinase inhibitor KN-93 and increasing L-arginine concentrations ranging from 0.14 to 10 μM in 500 μl of KRB as stated in Methods. Experiments were run in the absence or presence of 500 μM L-NMMA to subtract nonspecific and values plotted are the means±s.d. of at least three different glands for each point. K_M and V_{max} in the inset were calculated with Microcal Origin Software. ND: not determined.

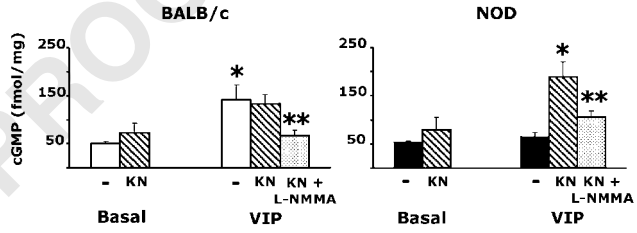


Figure 3 Effect of KN-93 on cGMP accumulation. Submandibular glands of BALB/c or NOD mice were incubated in the presence or absence of CaM kinase inhibitor KN-93 (3 μM) (KN) either in basal or in 10^{-7} M VIP-stimulated conditions. When used, L-NMMA (500 μM) was included from the beginning. Intracellular accumulated cGMP was determined by RIA as described in Methods. Values are the means±s.e.m. of four to six experiments. *$P<0.05$ vs basal of the same strain; **$P<0.05$ vs VIP + KN-93 in the same strain of mice.

Discussion

Here, we present evidence of a downregulatory effect of CaMK II on NOS activity in submandibular glands of both NOD and BALB/c mice. Our results are consistent with a physiological regulation of NOS activity by this kinase but not by PKC in normal BALB/c mice. They are also supportive of a

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role for CaMK II in the lack of detectable NOS activity in submandibular glands of NOD mice reported previously. The downregulation of NOS activity in NOD submandibular glands seems to be mainly mediated through phosphorylation by this kinase rather than the result of a lower expression or differential cellular localization of the enzyme. It is not related to different substrate or cofactor availability, either. The above conclusions are supported by three lines of evidence. First, based on the different electrophoretic pattern of NOS I obtained in Western blots of the contralateral gland of mice with altered NOS activity, we hypothesized that this alteration might reflect differences in the expression or stability of NOS I mRNA and/or different cellular localization of the protein. Glands presenting a lower activity of NOS analyzed in parallel by RT–PCR and immunohistochemistry showed no differences in either mRNA levels or enzyme localization between NOD and BALB/c glands, suggesting that the effect on activity seemed not to be due to a lower expression of the protein or its mRNA but to another regulatory pathway that, however, affected its electrophoretic mobility. The second line of evidence was provided by two additional enzymatic assays. The fact that no regulation was found at the expressional level in any of the glands studied, independently of the extent to which NOS activity was reduced, strongly suggested that other regulatory mechanisms might be responsible for the inhibition of the activity observed. In an attempt to investigate whether a different substrate or cofactors availability could have a role in this lower activity of the NOS in NOD mice, we performed two assays. First, as our experimental design for measuring NOS activity involves the entrance of the labeled arginine to the cell, we assayed NOS activity in lysates of the submandibular glands with the addition of all of the cofactors and the substrates exogenously. What we found is that the lack of specific activity in NOD mice glands remained the same as observed in whole glands ruling out that a lower transport of the substrate L-arginine into the cell or a reduced availability of cofactors were responsible of the effect. The second assay tested was arginase activity. On the knowledge that L-arginine is also a substrate for arginase though with higher K_M (mM for arginase against μM for the NOS), we assayed arginase activity in submandibular glands. No differences were observed between normal and NOD mice glands. The third line of evidence that supported the above conclusions is based on functional assays carried out in the presence of inhibitors of two protein kinases that normally downregulate NOS I. Based on previous reports from our laboratory referring to a strong downregulation of NOS in rat salivary glands by PKC, we first assayed a selective inhibitor of this kinase, but we could see no regulation by PKC in mice submandibular glands. In contrast, an inhibitor of calcium-CaMK II, KN-93, stimulated NOS activity in glands of control mice and restored NOS activity and nitric oxide-mediated signaling in NOD submandibular glands with kinetic parameters and cGMP values similar to those obtained in the control mice. In reference to cGMP production, the activation of soluble isoforms of guanylyl cyclase by the binding of nitric oxide to its heme moiety has been reported in various tissues (Alderton *et al*., 2001). Our results confirm and extend these observations to mice submandibular glands where cGMP accumulated upon VIP receptor activation appeared to come exclusively from this pathway, since L-NMMA completely blocked the cyclic nucleotide accumulation. Also, the presence of a phosphodiesterase inhibitor during the incubation time and the results obtained with L-NMMA are not supportive of a direct effect of KN-93 on phosphodiesterases and guanylyl cyclase, respectively.

Among various reported mechanisms of downregulation of NOS activity, availability of substrates and cofactors (Nathan & Xie, 1994) and phosphorylation by PKC and CaM kinase II have been assigned a critical role mainly for the neural isoform (Nakane *et al*., 1991; Bredt *et al*., 1992; Hayashi *et al*., 1999). On the basis of the negative results obtained when analyzing availability of substrates and cofactors clearly, indicating that these mechanisms were not limiting the ability of NOS to produce nitric oxide in NOD mice glands, we focused on kinase regulation of NOS. Particularly regarding NOS in salivary glands, we had previously shown that PKC strongly downregulated NOS in rat submandibular but not in sublingual glands (Perez Leiros *et al*., 2000), while it exerted Q13
a considerable inhibition on NOS activity in rat parotid glands (Rosignoli & Perez Leiros, 2002a). Even though our present Q14
results do not allow us to completely rule out the participation of PKC in NOS regulation of mouse glands, the lack of effect of a specific PKC inhibitor and the evident effect of KN-93 to stimulate NOS strongly support a role for CaM kinase rather than PKC in NOS regulation. These apparent differences between rat and mouse glands in their PKC-mediated regulation of the NOS could be ascribed to a differential parasympathetic tone of the glands reflected by the different response to carbachol observed in normal mouse (Rosignoli *et al*., 2001) compared to rat (Perez Leiros *et al*., 2000) Q15
submandibular glands. We have observed a substantial difference between species in the threshold for muscarinic-mediated stimulation of NOS activity in rat vs mouse glands. In fact, mouse glands showed no effect of 10^{-7} M carbachol and a deep inhibitory effect at higher concentrations of the agonist (Rosignoli *et al*., 2001). PKC is known to be activated by muscarinic acetylcholine receptor stimulation, and therefore it is conceivable that the intracellular milleu induced by this differential response to muscarinic receptor activation could condition the ability of PKC to downregulate NOS.

CaMK II activity is increased during apoptosis induced by various stimuli in human leukemia cell lines U937, HL-60 and KG1a (Wright *et al*., 1997). A similar role in apoptosis has been proposed for CaMK II in heart cells where sustained β_1 adrenergic receptor stimulation promotes myocyte apoptosis through this kinase independently of PKA signaling (Zhu *et al*., 2003). It is interesting to note that NOD mice glands, where the restoration of NOS activity and NO signaling by the CaM kinase inhibitor is supported by the present results, develop an apoptotic loss of acinar cells from the 8th week of age (Kong *et al*., 1998) with loss of acini and apoptotic images in submandibular epithelial cells shortly preceding the onset of NOS alterations (Rosignoli *et al*., 2003). The present results about similar mRNA levels and immunohistochemical expression of NOS I, although with a differential electrophoretic pattern, are supportive of a post-translational rather than a transcriptional regulation of the enzyme. Further evidence will be necessary to assess if the post-translational modification of NOS I by CaMKII shown here by pharmacological means could account for the differential electrophoretic pattern of NOS I observed in Figure 1 and in the previous work (Rosignoli *et al*., 2001). Based on the documented role of nitric oxide and cGMP in salivary secretion and the CaM kinase-

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impaired nitric oxide production reported here, it is tempting to speculate that this kinase, by regulating NOS, might have a role in the pathogenesis of the secretory dysfunction in Sjögren's syndrome. A sparse distribution of NOS I-containing nerve fibers localized around acini and ducts was reported in labial minor salivary glands of Sjögren's patients, although no functional correlate with NOS activity was provided (Kontinnen *et al*., 1997). Regarding CaM kinase-mediated regulation of NOS in disease, the phosphorylation of NOS I at Ser^{847} has been implicated in a model of transient forebrain ischemia (Osuka *et al*., 2002). Also, CaM has been related to Sjögren's syndrome based on the observation that the nuclear protein La/SSB, an autoantigen in Sjögren's patients, has CaM binding ability that could interfere its association with DNA (Castro *et al*., 1996). One of the most intriguing questions in inflammatory disorders is whether functional disabilities within the target organ might increase susceptibility to an inflammatory reaction. The possibility that the modification of structural or signaling molecules might activate detection systems that normally report injury should be further analyzed not only for seeking the origins of disease but also to develop more rational therapeutic approaches.

We conclude that CaM kinase but not PKC downregulates NOS activity in BALB/c and NOD submandibular glands and that the inhibition of CaMK II in NOD glands restores nitric oxide production and signaling.

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