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Short communication

Synthesis and structure–activity of antisense peptides corresponding to the region for CaM-binding domain of the inducible nitric oxide synthase

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Abstract – Nitric oxide synthase (NOS) catalyses the conversion of L-arginine to nitric oxide (NO) which plays an important role in the regulation of cellular functions and intracellular communications. Three distinct isoforms of NOS have so far been identified, two constitutive and one inducible. All three mammalian isoforms of NOS contain putative CaM-binding domains with the canonical composition. In this paper we report the synthesis and the inhibitory activity on rat neuronal and lung inducible NOS of antisense peptides corresponding to the antisense strand read in 3′ to 5′ (CALM 1) or 5′ to 3′ (CALM 2) direction of the region encoding for the CaM-binding domain of the inducible NOS isoform (residues 503–522). CALM 1 inhibited, at all the concentrations tested (0.01–1 mM), both the inducible and constitutive NOS (IC_{50} 98 μM and 56 μM, respectively), while CALM 2 (0.01–1 mM) was ineffective on both isoforms. The acetylation of CALM 1 at its amino terminal (CALM 8) completely abolished its inhibitory activity. We also synthesized and analysed the activity of amino terminal truncated analogues (CALM 3–7) of CALM 1, which selectively inhibited the inducible isoform, although less potently than the parent compound. The pentapeptides (CALM A–D) deriving from the cleavage of CALM 1 were ineffective, except the pentapeptide CALM C corresponding to the residues 513–517, which was as potent as the parent compound (IC_{50} 65 μM). © 2000 Éditions scientifiques et médicales Elsevier SAS

calmodulin / antisense peptides / nitric oxide synthase

1. Introduction

Nitric oxide (NO) is a messenger molecule mediating several biological functions such as vasodilation, neurotransmission, antitumour and antimicrobial activity [2]. NO is produced by nitric oxide synthase (EC 1.14.13.39) that catalyses the conversion of L-arginine to NO and citrulline [3]. Three isoforms of NO synthase (NOS) have so far been identified [4]. Two, NOS1 and NOS3, are constitutively expressed and activated by agonist-induced elevation of Ca^{2+} and subsequent binding to calmodulin (CaM) [5–7]. Type 1 NOS is the neuronal, while type 3 is the endothelial isoform. The small amount of NO generated by the constitutive isoforms acts homeostatically as an intracellular signal [2]. Type 2 NOS is induced in macrophages as well as in other cell types and tissues in response to cytokines and bacterial endotoxins [8–11]. NOS2 is Ca^{2+}-independent but contains a CaM-binding domain and CaM functions as a subunit of the enzyme remains tightly bound even in the absence of elevated intracellular calcium [12]. The large amount of NO synthesized by inducible NO synthase plays a key role in host defence mechanisms as a cytotoxic molecule for invading micro-organism and tumour cells is involved in pathological vasodilation and tissue damage, and plays an important role in inflammation [2]. Thus the search for potent and selective inhibitors of the inducible NOS might have an important therapeutic relevance.

High affinity CaM-binding domains for both constitutive and inducible NOS have been identified. The residues 725–747 (754) of the rat cerebellar sequence have been identified as the CaM-binding region for this enzyme [13–15] and the residues 503–522 (532) as the CaM-binding region of the mouse sequence of the inducible enzyme [15, 16].

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Symbols and abbreviations are in accord with recommendations from [1]

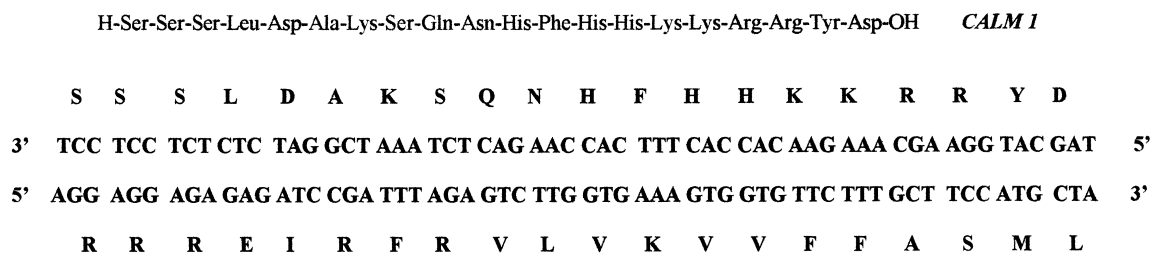

Figure 1. Oligonucleotide sequence of iNOS identified as CaM binding region.

Antisense peptides are synthesized upon antisense oligonucleotide sequence and may act as antagonist to the peptide or protein encoded by the sense strand [17]. We have synthesized antisense peptides named CALM 1 and CALM 2 corresponding to the antisense strand read, respectively, in 3′ to 5′ (CALM 1) or 5′ to 3′ (CALM 2) direction of the region coding for the CaM-binding site on inducible (residues 503–522) NOS isoform [15] (*figure 1*) and evaluated their inhibitory action on both rat neuronal constitutive and lung inducible NOS. Besides, we have synthesized amino terminal truncated analogues of CALM 1 (CALM 3–7) and divided the CALM 1 into four pentapeptides (named CALM A–D) to better understand the minimal sequence able to interact with the NOS enzymes (*figure 2* and *table I*).

2. Chemistry

Peptides were assembled by Fmoc solid phase method peptide synthesis on a MilliGen 9050 continuous flow synthesizer using commercially available Wang resin (loading 0.7 mmol/g). Repetitive Fmoc-cleavage was accomplished with 20% of piperidine-DMF solution. Peptide couplings were carried out using three equivalents of each Fmoc-amino acid and DIPCDI as the condensing reagent in the presence of DIEA for 1 h. After completion of the synthesis the protected peptides were cleaved from the resin, and the amino acid side chains were simultaneously deprotected in 3 h by treatment with a TFA/H_2O/Et_3SiH (88:5:7) mixture. No particular base-induced side reactions during Fmoc deblocking of peptides were

Name	Structure of Peptides
CALM 1	H-Ser-Ser-Ser-Leu-Asp-Ala-Lys-Ser-Gln-Asn-His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 2	H-Pro-Pro-Ser-Leu-Asp-Ser-Phe-Ser-Asp-Gln-His-Phe-His-His-Glu-Lys-Ser-Gly-His-Asp-OH
CALM 3	H-Ser-Ser-Leu-Asp-Ala-Lys-Ser-Gln-Asn- His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 4	H-Ser-Leu-Asp-Ala-Lys-Ser-Gln-Asn- His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 5	H-Leu-Asp-Ala-Lys-Ser-Gln-Asn- His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 6	H-Asp-Ala-Lys-Ser-Gln-Asn- His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 7	H-Ala-Lys-Ser-Gln-Asn- His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM 8	Ac -Ser-Ser-Ser-Leu-Asp-Ala-Lys-Ser-Gln-Asn-His-Phe-His-His-Lys-Lys-Arg-Arg-Tyr-Asp-OH
CALM A	H-Ser-Ser-Ser-Leu-Asp-OH
CALM B	H-Ala-Lys-Ser-Gln-Asn-OH
CALM C	H-His-Phe-His-His-Lys-OH
CALM D	H-Lys-Arg-Arg-Tyr-Asp-OH

Figure 2. Sequence of antisense peptides CALM 1–8 and CALM A–D.

Table I. Physical constants of the investigated peptides.

Compounds	FAB-MS m/z		HPLC[a, b] rt (min)	Yield (%)[c]
	Calculated	Found		
CALM 1	2 441.67	2 442	8.55	65
CALM 2	2 314.18	2 315	9.11	58
CALM 3	2 354.59	2 355	6.91	75
CALM 4	2 267.51	2 268	7.08	58
CALM 5	2 180.43	2 181	7.22	67
CALM 6	2 067.27	2 067	6.58	58
CALM 7	1 952.43	1 952	7.31	71
CALM 8	2 483.67	2 484	9.33	71
CALM A	507.50	508	6.41	93
CALM B	546.58	546	6.21	87
CALM C	704.79	705	6.33	91
CALM D	736.85	737	6.35	88

[a] Eluents: A (0.1% TFA in CH_3CN) and B (0.1% TFA in H_2O); analytical HPLC on a μ-Bondapak C_{18} silica column (125 Å, 15–20 μm, 30 × 300 mm); linear gradient from 5% A/95% B to 80% A/20% B over 25 min, UV detection at 220 nm, flow rate 1 mL/min. [b] The final HPLC purity of the peptides was always > 98%. [c] Yields of purified peptides were calculated as percentage of the theoretical yield, based on the starting material.

observed. Purification of target peptides was achieved by preparative HPLC and the homogeneity of the purified products was assessed by analytical HPLC. Structure verification was achieved by amino acid analysis and mass spectrometry.

3. Results and discussion

In our system the activity of constitutive NOS from rat cerebella was 15.1 ± 0.6 pmol/min/mg of protein (n = 10), while the activity of inducible isoform from lungs of lipopolysaccharide-treated rats was 18.8 ± 0.9 pmol/min/mg of protein (n = 10).

CALM 1 (0.01–1 mM) inhibited in a concentration-dependent manner both isoforms of NOS (*table II*). The inhibition was significant at all the concentrations tested ($P < 0.01$). The IC_{50} was 98 μM and 46 μM for lung inducible and neuronal constitutive isoforms, respectively. The lack of selectivity towards the inducible isoform could be due to a certain homology of the amino acid sequence of the respective CaM-binding domains [16], since they possess a number of common structural features such as the presence of basic and hydrophobic amino acids [18]. Moreover it has been reported that additional binding site(s) within the region comprised by amino acids 484–726 appear to contribute as well to the property of the inducible NOS CaM-binding domain [16]. Thus the antisense peptide 503–522 seems to correspond to a necessary, but not sufficient, amino acid sequence able to selectively inhibit the inducible NOS. This concept seems to be supported by the fact that the inhibitory activities are quite low (mM range). On the contrary to what was observed for CALM 1, CALM 2 (0.01–1 mM) did not inhibit, at all the tested concentrations, either the neuronal constitutive or the lung inducible isoform (*table II*). Thus the antisense strand read in 5′ to 3′ direction seems to codify for an antisense peptide devoid of any activity towards both isoforms of NOS.

Thus we focused our attention on CALM 1. The acetylation of this peptide at its amino terminal resulted in a peptide (CALM 8) totally inactive on both NOS isoforms (*table III*). This seems to be a common feature for antisense peptides since it has been reported that modification at the carboxy- and amino-termini can drastically reduce their activity [19].

The next step was to perform further structural modification and characterization of the CALM 1 peptide, synthesizing amino terminal truncated analogues (CALM

Table II. Inhibitory effect (% inhibition) of CALM 1 and CALM 2 on lung inducible (iNOS) and neuronal constitutive (nNOS) enzyme.*

(mM)	iNOS		nNOS	
	CALM 1	CALM 2	CALM 1	CALM 2
0.01	17 ± 2.5	n.i.	22 ± 1.5	n.i.
0.03	28 ± 3.1	n.i.	46 ± 3.0	n.i.
0.10	56 ± 2.6	n.i.	71 ± 2.1	n.i.
0.30	68 ± 3.0	n.i.	78 ± 3.1	n.i.
1.00	81 ± 2.8	n.i.	86 ± 2.7	n.i.

* NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to citrulline. Each result represents the mean ± SE of three separate experiments. n.i. = no inhibition.

Table III. Inhibitory effect (% inhibition) of amino terminal truncated CALM 1 analogues on lung inducible (iNOS) and neuronal constitutive (nNOS) enzyme*.

mM	CALM 3		CALM 4		CALM 5		CALM 6		CALM 7		CALM 8	
	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS
0.01	16 ± 2.2	n.i.	10 ± 2.5	n.i.	9.1 ± 1.1	n.i.	15 ± 1.3	n.i.	2.3 ± 0.8	n.i.	n.i.	n.i.
0.03	27 ± 2.5	n.i.	28 ± 2.2	n.i.	23 ± 2.7	n.i.	22 ±2.1	n.i.	12 ± 3.1	n.i.	n.i..	n.i.
0.10	41 ± 2.6	n.i.	41 ± 2.6	n.i.	39 ± 3.1	n.i.	38 ± 2.9	n.i..	31 ± 2.6	n.i.	n.i..	n.i.
0.30	48 ± 3.1	n.i.	47 ± 2.6	n.i.	47 ± 3.6	n.i.	48 ± 2.8	n.i..	48 ± 3.0	n.i.	n.i..	n.i.
1.00	55 ± 3.2	n.i.	55 ± 3.3	n.i.	53 ± 1.8	n.i.	54 ± 2.6	n.i.	53 ± 3.2	n.i.	n.i.	n.i.

* NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to citrulline. Each result represents the mean ± SE of three separate experiments. n.i. = no inhibition.

2–7, *figure 2*) with the aim to better investigate the role of this region in the inhibition of NOS enzymes. CALM 3 (504–522), which lacks a serine residue at the amino terminal position with respect to CALM 1, was less active than CALM 1 on lung inducible NOS, but totally inactive on the neuronal constitutive isoform (*table III*). We observed the same pattern for CALM 4 (505–522), lacking two serine residues, CALM 5 (506–522), which does not present any serine residues, CALM 6 (507–522), also lacking a leucine residue, and finally CALM 7 (508–522) which resulted from the deletion of the amino terminal pentapeptide of CALM 1 (*table III*). All these peptides reached, at the highest concentration tested (1 mM), an inhibition not exceeding 50–55%. These data suggest that the amino terminal position examined plays an important role in the inhibitory activity and selectivity on lung inducible NOS. In fact any manipulation in this region completely abolished the activity of CALM 1 on the neuronal enzyme, while only slightly decreased the inhibition on the inducible isoform (*table III*). Interestingly, as reported above, the acetylation of CALM 1 at its NH_2-terminal (CALM 8) resulted in a complete loss of its inhibitory activity. However these data do not exclude other sub-structural features crucial for the interaction with NOS enzymes.

Subsequently, we decided to cleave CALM 1 into four pentapeptides, named CALM A–D. First we evaluated the activity of the amino terminal pentapeptide (503–507, CALM A). Surprisingly it was completely inactive (*table IV*), not only on the neuronal constitutive but also on the lung inducible isoform, indicating that this amino acidic sequence did not correspond to the minimal sequence of the parent peptide able to inhibit the inducible isoform, although, as shown in *table III*, any manipulation in this region decreased the inhibitory activity of CALM 1. The carboxy terminal (518–522, CALM D) pentapeptide, as well as the pentapeptide (508–512, CALM B), close to the amino terminal pentapeptide were inactive on both isoforms. On the contrary the pentapeptide close to the carboxy terminal pentapeptide (513–517, CALM C) was a selective inhibitor of the lung inducible isoform. In fact it was devoid of any activity on the neuronal constitutive NOS and was as active (IC_{50} 65 μM) as CALM 1, the parent peptide (IC_{50} 98 μM), in inhibiting the lung inducible NOS. The inhibition was significant ($P < 0.01$) at all the tested concentrations (0.01–1 mM). These results suggest that this sequence is crucial for the inhibitory activity on the inducible isoform.

4. Conclusions

CALM 1, the antisense peptide corresponding to the antisense strand read in the 3′ to 5′ direction of the region, coding for the CaM-binding site on the inducible (residues 503–522) NOS isoform, did not selectively inhibit this enzyme. In fact, as shown by the respective IC_{50}, it was equally active on the neuronal constitutive isoform. CALM 2, the antisense peptide corresponding to the antisense strand read in the 5′ to 3′ direction, did not exhibit any inhibitory activity on both isoforms.

The amino terminal region of CALM 1 seems to be important for the selectivity of this peptide on the lung inducible isoform. It is hard to understand the complete loss of activity towards the neuronal isoform of these truncated analogues, including CALM 3, lacking a single N-terminal residue. However, the experimental results show that any manipulation in this region (CALM 3–7) completely abolished the inhibitory activity of CALM 1 on the neuronal constitutive isoform, while only slightly decreased its inhibitory activity on the lung inducible isoform. Moreover when we tested the four pentapeptides (CALM A–D), deriving from the cleavage of CALM 1, none of them was active on the constitutive isoform. Thus

Table IV. Inhibitory effect (% inhibition) of pentapeptide analogues of CALM 1 on inducible (iNOS) and neuronal constitutive (nNOS) enzyme.*

mM	CALM A		CALM B		CALM C		CALM D	
	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS	iNOS	nNOS
0.01	n.i.	n.i.	n.i.	n.i.	20.2 ± 2	n.i.	n.i.	n.i.
0.03	n.i.	n.i.	n.i.	n.i.	39 ± 2.5	n.i.	n.i.	n.i.
0.10	n.i.	n.i.	n.i.	n.i.	62 ± 3.0	n.i.	n.i.	n.i.
0.30	n.i.	n.i.	n.i.	n.i.	72 ± 2.5	n.i.	n.i.	n.i.
1.00	n.i.	n.i.	n.i.	n.i.	89 ± 3.1	n.i.	n.i.	n.i.

* NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to citrulline. Each result represents the mean ± SE of three separate experiments. n.i. = no inhibition.

only the complete sequence coding for the CaM-binding domain of the inducible NOS was able to inhibit the neuronal enzyme.

The amino terminal pentapeptide (CALM A), although important for CALM 1 activity, did not correspond to the minimal sequence able to inhibit the inducible NOS, since it was devoid of any activity. The pentapeptide corresponding to the residues 513–517 (CALM C) was, instead, as potent as CALM 1 in inhibiting this isoform. Further studies are in progress in order to better clarify the SAR data observed with the tested compounds.

5. Experimental protocols

5.1. Chemistry

Solvents used for reactions were dried over 3 Å molecular sieves. Reversed-phase purification was routinely performed on a Waters Delta-Prep 4000 system equipped with a Waters 484 multi-wavelength detector on a μ-Bondapak C-18 silica (125 Å, 15–20 μm, 30 × 300 mm) high performance liquid chromatography (HPLC) column. The gradient used was identical to that of analytical determinations. The operational flow rate was 30 mL/min. Homogeneity and retention times (rt) of the purified products were assessed by analytical reverse phase (RP) HPLC with a μ-Bondapak C18–125 Å column 10 μm, 3.9 × 300 mm, spherical, with the following solvent system: A: 0.1% trifluoroacetic acid (TFA) in CH_3CN, B: 0.1% TFA in H_2O, linear gradient from 5% A/95% B to 80% A/20% B over 25 min, UV detection at 220 nm, flow rate 1 mL/min. The final HPLC purity of the peptides was always >98%. The yields of purified peptides were calculated as percentage of the theoretical yield, based on the starting material. All solvents were filtered and degassed prior to use. Reagent grade materials were purchased from Novabiochem and from Aldrich Chemical Co. and were used without further purification. Molecular weights of antisense peptides were determined by fast atom bombardment mass spectrometry (FAB/MS) on a ZAB 2 SE-FISONS. Dimethylformamide (DMF) was distilled immediately before use over CaH_2. Analytical data of all compounds were reported in *table I*.

5.2. Biological methods

5.2.1. Animals

Male Wistar rats 200–300 g (Charles River; Milan, Italy) were kept at controlled temperature (21–24 °C; 55 ± 10% humidity) and on a 12 h dark/light cycle and given free access to water and standard diet. A period of 7 days was allowed for acclimatization of the rats before any experimental manipulation. Rats for semi-crude preparations of the enzymes were divided in two groups: one for the constitutive and the other one for the inducible NOS.

5.2.2. Neuronal constitutive NO synthase

The constitutive isoform of NOS was prepared from rat cerebella. Animals were stunned by exposure to CO_2 gas and killed by bleeding. Cerebella were excised, partially cleaned and immediately frozen in liquid nitrogen.

5.2.3. Inducible NO synthase

The inducible isoform of NOS was prepared from lungs of rats treated with lipopolysaccharide from *Escherichia coli* (serotype No. 0127:B8; 6 mg/kg i.p., Difco, MI, USA). Six hours after treatment animals were anaesthetized with urethane (1.3 g/kg i.p.), the right ventricle was cannulated and the lung perfused through the pulmonary artery for 2–3 min with 0.9% NaCl (5 mL/min, outflow via the pulmonary vein), was explanted and immediately frozen in liquid nitrogen.

5.2.4. Semi-crude preparation of NO synthase

Cerebella or lungs were homogenized at 4 °C in four volumes of HEPES buffer 20 mM, pH 7.2, containing

320 mM sucrose, 1 mM DL-dithiotreitol, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 10 μg/mL leupeptin. The homogenates were centrifuged at 100 000 *g* (L8-70 ultracentrifuge, Beckman) for 30 min at 4 °C. The supernatants, i.e. the cytosolic fractions containing NOS activity, were stored at –70 °C until use and for not more than 7–10 days. Protein concentration in the cytosolic fractions was measured spectrophotometrically using bovine serum albumin as standard [20].

5.2.5. Assay of NO synthase activity

NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to citrulline, according to Salter et al. [21]. Briefly, an aliquot of the cytosolic fraction (100 μg of protein) was pre-incubated for 10 min at 37 °C in 50 mM potassium phosphate buffer pH 7.2 containing 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$ in the presence of compounds to be tested or vehicle. Compounds were tested at the following concentration: 0.01–1 mM. Samples were then incubated for 10 min at 37 °C with [U-^{14}C]arginine (150 000 dpm, specific activity 304 mCi/μmol, NEN Life Science, Cinisello Balsamo, Italy) and 20 μM L-arginine (Sigma Chemical Co., Milan, Italy).

The reaction was stopped by the addition of 1.0 mL of a mixture of H_2O/Dowex-50W 1:1 v/v (200–400, 8% cross-linked, H^+-form; Sigma Chemical Co., Milan, Italy). The Na^+-form of Dowex-50W was prepared by washing four times the H^+-form of resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11 000 *g* for 3 min) in a microfuge (Beckman, Microfuge 11) and an aliquot of the supernatant was taken for scintillation counting (4 mL Pico-Aqua; Packard 1500). The activity of constitutive Ca^{2+}/dependent NOS was determined from the difference between the labelled citrulline produced by control samples and samples containing 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA); the activity of inducible Ca^{+2}/independent enzyme was determined from the difference between the labelled citrulline produced by samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^G-monomethyl-L-arginine (L-NMMA). The activity of both isoforms was expressed as nmol/min/mg of protein.

5.2.6. Statistical analysis

Results are expressed as mean ± SE of *n* experiments. One way analysis of variance (ANOVA) followed by Bonferroni multiple choice test was used. $P < 0.05$ was considered as statistically significant.

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