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Aprotinin, the First Competitive Protein Inhibitor of NOS Activity

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Analogs of L-arginine represent the largest and potentially most useful class of NOS inhibitors. However, no competitive protein inhibitors of NOS activity are known so far. The effect of aprotinin (Kunitz inhibitor) on NOS activity is reported here, aprotinin being one of the most extensively studied globular proteins. Present data indicate that aprotinin, clinically used as a trypsin-like serine proteinase inhibitor, inhibits NOS-I and NOS-II with K_i values of 5.0×10^{-5} M and 7.8×10^{-5} M, respectively, at pH 7.5 and 37.0°C, thereby representing the first competitive protein inhibitor of NOS activity. Therefore, the clinical use of aprotinin, as a drug, should be under careful control. In addition, aprotinin and aprotinin-like domains are present in a variety of organs, as well as in the Alzheimer's amyloid β-protein precursor. Thus, the present findings open the way to novel mechanisms likely to be involved in the modulation of NOS activity, under physiological and pathological conditions.

Key Words: **rat brain constitutive nitric oxide synthase; rat lung inducible nitric oxide synthase; aprotinin; nitric oxide synthase inhibition.**

Nitric oxide (NO) is a versatile, very important molecule that has broken out onto the scene in many fashions. NO is an unstable nitrogen radical which is generated in different cell types by the concomitant L-arginine/L-citrulline conversion catalyzed by the enzyme NO synthase (NOS). When generated at low levels by constitutive NOS (NOS-I and NOS-III), NO plays important roles in physiological processes, whereas uncontrolled and massive NO production by inducible NOS (NOS-II) is involved in pathological phenomena (for a recent review, see Ref. 1). Thus, the regulation of the NO synthesis is critical in many biological systems.

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Abbreviations: aprotinin, bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor); NO, nitric oxide; NOS, nitric oxide synthase; NOS-I and NOS-III, constitutive NOS; NOS-II, inducible NOS.

Analogs of L-arginine represent the largest and potentially most useful class of NOS inhibitors [1,2]. Recently, it has been observed that some L-arginine-analogs, such as amiloride and gabexate mesylate, clinically used as drugs for serine proteinase-mediated diseases, competitively inhibit NOS activity [3,4]. However, no competitive protein inhibitors of NOS activity are known so far. Here, the effect of aprotinin (Kunitz inhibitor) on NOS activity is reported, aprotinin being one of the most extensively studied globular proteins [5-7]. Present data indicate that aprotinin, clinically used as a trypsin-like serine proteinase inhibitor [5,8,9], inhibits NOS-I and NOS-II, thus representing the first competitive protein inhibitor of NOS activity.

MATERIALS AND METHODS

NOS-I activity was detected in the 20,000 × g supernatant of whole rat brain homogenates. NOS-II activity was determined in the lung homogenate supernatant of rats treated with *E. coli* lipopolysaccharide (10 mg × kg^{-1}). NOS activity was assessed by evaluating the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, in the absence and presence of aprotinin. For NOS-I activity, an aliquot of supernatant was added to a reaction mixture containing 5.0×10^{-2} M Hepes, pH 7.5, 1.0×10^{-3} M NADPH, 1.2×10^{-3} M $CaCl_2$, 1.0 $\mu g \times mL^{-1}$ calmodulin, 1.0×10^{-5} M FAD, 1.0×10^{-5} M FMN, 1.0×10^{-4} M (6R)-5,6,7,8-tetrahydro-1-biopterin and [^{3}H]L-arginine (from 5.0×10^{-6} M to 5.0×10^{-5} M). For NOS-II activity $CaCl_2$ and calmodulin were omitted, and 1.0×10^{-3} M EGTA was added [3,4,10]. The value of the inhibition equilibrium constant (K_i) for aprotinin binding to NOS-I and NOS-II was determined, at pH 7.5 (5.0×10^{-2} M Hepes buffer) and 37.0°C, according to the graphical method of Dixon [11]. NO production was also monitored spectrophotometrically following the NO-mediated conversion of human oxygenated hemoglobin, added to the homogenate supernatant, to methemoglobin [3,4,10]. [^{3}H]L-arginine was obtained from NEN (Boston, MA, USA). Aprotinin and all other products were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade and were used without further purification.

RESULTS AND DISCUSSION

As shown in Figure 1, aprotinin competitively inhibits NOS-I and NOS-II activity. Aprotinin binding to NOS-I and NOS-II conforms to a simple equilibrium,

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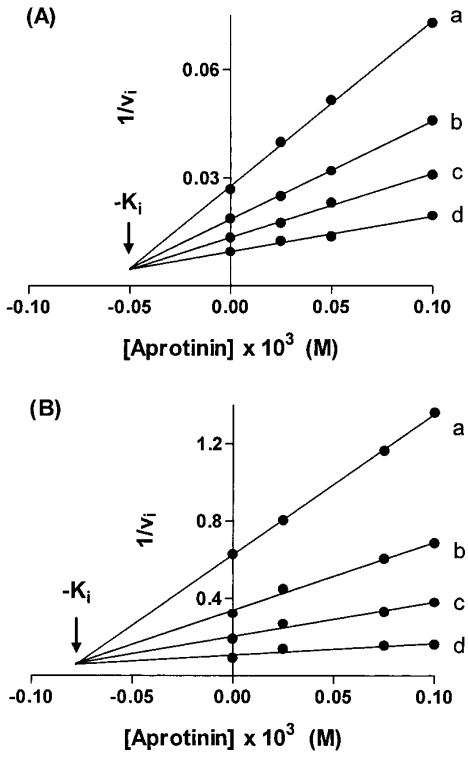

FIG. 1. Dixon plot for NOS-I (panel A) and NOS-II (panel B) competitive inhibition by aprotinin (v_i, nmol $\times$ mg^{-1} $\times$ min^{-1}), at pH 7.5 and 37.0°C. L-arginine concentration was 5.0×10^{-6} M (a), 1.0×10^{-5} M (b), 2.0×10^{-5} M (c), and 5.0×10^{-5} M (d). Values of K_i for aprotinin binding to NOS-I and NOS-II are 5.0×10^{-5} M and 7.8×10^{-5} M, respectively. For further details, see text.

and K_i values are independent of the enzyme, substrate and inhibitor concentrations. As already reported for most NOS inhibitors [2-4,10], NO does not originate from aprotinin. As observed, oxygenated human hemoglobin added to homogenates was not converted to methemoglobin in the presence of aprotinin, instead of L-arginine, as the substrate.

Table 1 shows K_i values for aprotinin binding to NOS-I, NOS-II and trypsin-like serine proteinases. The affinity of aprotinin for NOS-I and NOS-II is higher than that observed for human α-, β- and γ-thrombin, being however lower than that reported for M_r 33,000 and M_r 54,000 species of human urokinase, bovine and human Factor Xa, bovine tryptase, human Glu1-, Lys77-, Val442- and Val561-plasmin, porcine pancreatic β-kallikrein- A and -B, human urinary kallikrein as well as bovine β-trypsin. Aprotinin binds to trypsin-like serine proteinases through the salt bridge occurring between the positively charged Lys15 residue of the inhibitor and the negatively charged Asp189 side chain present invariantly at the enzyme primary specificity subsite [5-7]. Therefore, the different affinity of aprotinin for bovine β-trypsin, bovine and human Factor Xa, human α-, β- and γ-thrombin, human Glu1-, Lys77-, Val442- and Val561-plasmin, the M_r 33,000 and M_r 54,000 species of human urokinase, human urinary kallikrein, as well as porcine pancreatic β-kallikrein-A and -B, reflects different structural factors outside the enzyme catalytic center, involving the secondary specificity subsites. Thus, the affinity decrease of 3 to 10 orders of magnitude with respect to bovine β-trypsin can be ascribed to the influence of serine proteinase loops which limit the inhibitor ac

Accessibility (e.g. external loops in kallikreins, and β and γ loops in human α-thrombin). The removal of such structural constraints restores, at least in part, proteinase affinity [7]. The interaction of aprotinin with the homotetrameric bovine tryptase follows an anti-cooperative behavior. In fact, aprotinin affinity for the fully active serine proteinase is higher than that for the di-inhibited and the tri-inhibited enzyme [12]. Although the NOS-I: and NOS-II:aprotinin binding mode is unknown, it is worthwhile noting that aprotinin displays six fully solvent exposed arginyl residues at positions 1, 17, 20, 39, 42, and 53 [5-7], which might be involved in the enzyme:inhibitor complex formation, as should be expected from NOS specificity properties [13].

Aprotinin represents the first competitive protein inhibitor of NOS-I and NOS-II activity, suggesting that L-arginine-containing peptides and proteins may mod-

TABLE 1

Values of K_i for Aprotinin Binding to NOS-I, NOS-II, and Trypsin-like Serine Proteinases

Enzyme	K_i (M)
NOS-I[a]	5.0×10^{-5}
NOS-II[a]	7.8×10^{-5}
Bovine Factor Xa[b]	4.8×10^{-6}
Bovine tryptase (fully active enzyme)[c]	8.3×10^{-9}
Bovine tryptase (aprotinin di-inhibited enzyme)[c]	3.7×10^{-7}
Bovine tryptase (aprotinin tri-inhibited enzyme)[c]	4.5×10^{-5}
Bovine β-trypsin[d]	6.0×10^{-14}
Human α-thrombin[d]	8.0×10^{-4}
Human β-thrombin[d]	4.0×10^{-4}
Human γ-thrombin[d]	1.1×10^{-4}
Human urokinase M_r 33,000 species[d]	2.0×10^{-5}
Human urokinase M_r 54,000 species[d]	2.0×10^{-5}
Human Factor Xa[b]	4.8×10^{-6}
Human Glu1-plasmin[e]	1.0×10^{-9}
Human Lys77-plasmin[e]	8.3×10^{-10}
Human Val442-plasmin[e]	7.6×10^{-10}
Human Val561-plasmin[e]	6.3×10^{-9}
Human urinary kallikrein[d]	9.1×10^{-11}
Porcine pancreatic β-kallikrein-A[d]	7.7×10^{-10}
Porcine pancreatic β-kallikrein-B[d]	9.1×10^{-10}

[a] Values of K_i have been obtained at pH 7.5 and 37.0°C. Present study.

[b] Values of K_i have been obtained at pH 8.0 and 21.0°C [17].

[c] Values of K_i have been obtained at pH 8.0 and 30.0°C [12].

[d] Values of K_i have been obtained between pH 7.5 and 8.0, and between 21.0°C and 25.0°C [7].

[e] Values of K_i have been obtained at pH 8.0 and 21.0°C [18].

ulate NO synthesis catalyzed by NOS. In this respect, intracellular aprotinin and aprotinin-like domains are present in a variety of organs, such as brain, parotid glands, lung, pancreas and spleen, as well as in tissues, especially in those ones rich in mast cells [5]. These serine proteinase inhibitors are present also in plasma and other biological fluids [5]. Moreover, an aprotinin-like inhibitor domain has been identified in the Alzheimer's amyloid β-protein precursor [14]. In many cases, aprotinin and aprotinin-like domains represent one of the most abundant proteins [5], and may be co-localized with NOS [1]. The present findings open the way to novel mechanisms which may be involved in the modulation of NOS activity, under physiological and pathological conditions. Aprotinin specificity for NOS could be also relevant in consideration of its clinical use in the treatment of acute pancreatitis, shock, hyperfibrinolytic hemorrhage, and inflammatory processes, as a trypsin-like serine proteinase inhibitor [5,8,9]. Thus, the pharmacological effect of aprotinin may be due not only to the inhibition of trypsin-like serine proteinases [5,8,9], but also to NOS-II inactivation. On the other hand, aprotinin has already been reported to inhibit cytokine-induced NOS-II mRNA expression. This effect has been attributed to the inhibition of NF-κB dissociation from I-κB, thereby not allowing NF-κB activation [15,16]. On the basis of these considerations, aprotinin has been suggested to permeate cell membranes [15]. As a whole, since inhibition of NOS-I and NOS-II activity may occur also *in vivo,* as happens with trypsin-like serine proteinases as well, aprotinin should be administered under careful control.

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