ELSEVIER

Biochemical Pharmacology 64 (2002) 277–283

Biochemical Pharmacology

Modulation of L-arginine transport and nitric oxide production by gabexate mesylate

Giuliana Leoncini*, Raffaele Pascale, Maria Grazia Signorello

Department of Experimental Medicine, Biochemistry Section, University of Genoa, Viale Benedetto XV 1, 16132 Genova, Italy

Received 18 March 2002; accepted 15 May 2002

Abstract

Gabexate mesylate, a non-antigenic synthetic inhibitor of trypsin-like serine proteinases, is a drug used efficiently in the treatment of pancreatitis and disseminated intravascular coagulation and as a regional anticoagulant for haemodialysis. Considering the structural similarity between L-arginine and gabexate mesylate, the effect of this drug on L-arginine transport, nitric oxide (NO) formation and constitutive NO synthase activity in human platelets was investigated. Data have shown that gabexate mesylate inhibited competitively L-arginine uptake by increasing the K_m value from 22 ± 2 to 86 ± 6 μM. The K_i value was 158 μM at pH 7.4 and 37°. Furthermore, gabexate mesylate decreased dose and time-dependent nitrite and nitrate formation (NO_x release) and cGMP accumulation in whole cells. In addition, gabexate mesylate inhibited constitutive nitric oxide synthase in a cell-free extract. We concluded that gabexate mesylate could be considered an effective modulator of cellular NO synthesis.

Keywords: L-Arginine uptake; Gabexate mesylate; cGMP; Human platelets; NO formation; NO synthase activity

1. Introduction

NO is a signalling molecule involved in the regulation of several physiological and pathophysiological mechanisms in the cardiovascular and nervous systems [1,2]. In the cardiovascular system NO regulates vascular tone, protects endothelium from vascular injury [3], modulates adhesion of inflammatory cells [4,5], reduces platelet adhesion [6] and plays a basic role in the regulation of platelet activation [7]. In addition, NO controls the arachidonate cascade *in vitro* by inhibiting the lipooxygenase [8,9] and activating the cyclooxygenase pathway [10].

The effective concentration of NO in a given biological tissue is determined not only by its rate of formation but also by its rate of degradation. NO is synthesised from L-arginine by NOS and the availability of intracellular L-arginine is a rate-limiting factor in NO production. In human platelets NO is overall produced by a constitutive nitric oxide synthase (cNOS), a calcium/calmodulin dependent enzyme as other constitutive NOS, but it has different molecular weight [11]. One important scavenging mechanism is the reaction of NO with anion superoxide to generate peroxynitrite. This reaction reducing thrombotic response dependent on reactive oxygen species produces a potent oxidant and potential mediators of tissue injury [12–15]. However, the effects of peroxynitrite are critically dependent on the microenvironment in which this oxidant is produced [16].

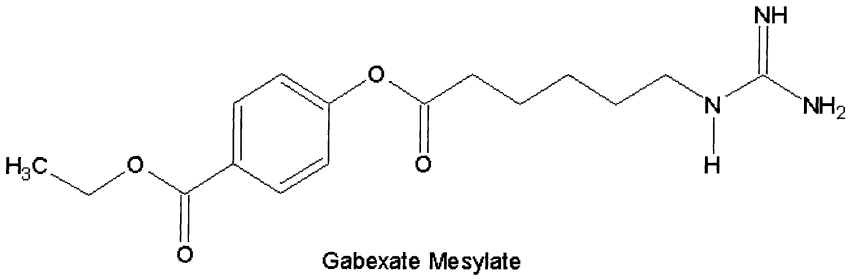
Gabexate mesylate, a non-antigenic synthetic inhibitor of trypsin-like serine proteinases [17], is a drug used in the treatment of pancreatitis and disseminated intravascular coagulation [18]. It reduces endothelial cell activation [19], suppresses liver ischemia-reperfusion injury by lowering the extracellular release of oxygen-free radicals [20], inhibits superoxide anion production in human neutrophils [21], prevents inflammation by inhibiting the TNFα production of activated human monocytes [22] and by blocking selectively human mast cell tryptase [23].

Considering the structural similarity between gabexate mesylate and L-arginine (Fig. 1), it is likely to suppose that this drug may control NO formation by interfering with L-arginine uptake and with the enzymes using L-arginine and/or its derivates as a substrate such as NOS.

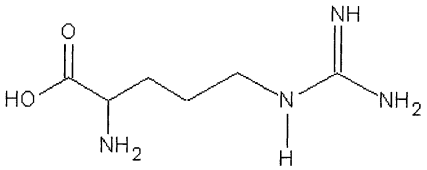
* Corresponding author. Tel.: +39-10-3538154; fax: +39-10-354415.
E-mail address: leoncini@unige.it (G. Leoncini).

Abbreviations: BH_4, 5,6,7,8-tetrahydrobiopterin; DTT, dithiothreitol; FMN, flavin mononucleotide; NO, nitric oxide; NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; plts, platelets; PMSF, phenylmethylsulfonyl fluoride

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01108-5



Gabexate Mesylate



Arginine

Fig. 1. Chemical structures of gabexate mesylate and arginine.

In the present study, we investigated the effect of gabexate mesylate on L-arginine uptake and on the total NO formation and cGMP accumulation in human platelets. In addition, the drug effect on cNOS activity of a cell-free system was evaluated.

2. Materials and methods

2.1. Blood collection and preparative procedures

Human blood from normal healthy volunteers, who declared to have not taken drugs known to interfere with platelet function, was collected in 130 mM aqueous trisodium citrate anticoagulant solution (9:1). Washed platelets were prepared as previously described [24]. Briefly platelet-rich plasma, obtained by centrifugation of the whole blood at 100 *g* for 25 min, was centrifuged at 1000 *g* for 15 min. Pellet was washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose). Platelets obtained by the centrifugation (1000 *g* for 15 min) were resuspended in pH 7.4 HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Glucose, 10 mM HEPES). All chemicals were from Sigma Chemicals Co.

2.2. L-Arginine uptake assay

L-Arginine uptake was measured as previously described [25]. Washed platelets (2.0×10^8 plts), prewarmed at 37°, were incubated in the presence of 1.0 μCi/mL L-[2,3,4-^{3}H] arginine (NEN-Perkin-Elmer), L-arginine, gabexate mesylate (Lepetit), thrombin and collagen (Mascia Brunelli), when required. After the incubation for 1 min at 37°, if not otherwise indicated, aliquots of 1.0 mL were withdrawn, immediately filtered through a Titertek™ (Flow Laboratories) filter and washed twice with large volumes of cold PBS, containing 10 mM L-arginine. The radioactivity corresponding to the incorporated L-[2,3,4-^{3}H]arginine was directly measured by liquid scintillation counting of the filter in a Packard model TRI-CARB 1600 TR Liquid Scintillation Analyzer. The kinetic parameters of L-arginine transport were calculated by the Lineweaver–Burk plot. The values of K_i (dissociation equilibrium inhibition constant) were obtained by measuring the inhibitory effect of gabexate mesylate on L-arginine uptake and processing data with the Dixon plot.

2.3. Measurement of NO_x production

To evaluate the effect of gabexate mesylate on NO production in whole platelets the level of nitrite and nitrate was measured. Washed platelets, resuspended at 1.0×10^9 plts in pH 7.4 HEPES buffer containing 2 mM $CaCl_2$ and prewarmed at 37°, were incubated with L-arginine or PBS in the presence or absence of gabexate mesylate as indicated. Incubation was stopped by sonicating samples in ice. Suitable aliquots of supernatant, added to equal volumes of pH 9.7 assay buffer (15 g/L Glycine–NaOH) containing cadmium beds, were incubated overnight at room temperature under horizontal shaking. Cadmium beds were activated immediately before each experiments by subsequent washings with 0.2 N H_2SO_4, bidistillated water and assay buffer. Nitrite and nitrate accumulation, determined by the Griess reagent (1% sulphanilamide in 2.5% H_3PO_4, 0.1% naphtylenediamine dihydrochloride), was measured at 540 nm using a sodium nitrite calibration curve.

2.4. Measurement of cGMP levels

cGMP intracellular level was measured as previously described [26]. Washed platelets resuspended at

1.0×10^9 plts in pH 7.4 HEPES buffer containing 2 mM $CaCl_2$, were incubated at 37° in the presence of L-arginine or PBS and gabexate mesylate when required. The reaction was stopped by the addition of cold 2 M perchloric acid. Supernatants recovered after a brief centrifugation were neutralised with 2 M NaOH and analysed for the cGMP content by radioimmunoassay (RIA) kit (Amersham-Pharmacia Biotech).

2.5. cNOS assay in a cell-free system

cNOS activity was measured according to the fluorimetric method of Chen *et al*. [27] with some modifications. Washed platelets (2.0×10^9 plts) were sonicated twice for 15 s in ice, in the presence of 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin and 100 μM dithiothreitol (DTT) and centrifuged for 600 *g* for 20 min. Suitable aliquots of the supernatant, treated with 100 μM DTT for 30 min at room temperature, were mixed with 10 μM FAD, 10 μM flavin mononucleotide (FMN), 0.1 μM 5,6,7,8-tetrahydrobiopterin (BH_4) and 1 mM $CaCl_2$. Incubation was started by the addition of L-arginine and gabexate mesylate, when required. After 5 min at 37° the mixtures, added to equal volumes of pH 9.7 assay buffer (15 g/L Glycine–NaOH), were incubated overnight at room temperature under horizontal shaking in the presence of activated cadmium beds. At the end of incubation, samples were diluted with cold bidistilled water and mixed with 2,3-diaminonaphthalene (50 μg/mL in 0.62 M HCl) to form the fluorescent product 2,3-naphthotriazole. After 15 min at 37° the reaction was stopped with 2.8 N NaOH addition. Relative fluorescence intensities in different samples were assayed with a Perkin-Elmer model LS50B fluorescence spectrometer (365 nm excitation and 405 nm emission). Protein concentration was measured according to the Lowry method [28].

2.6. Tests to assay platelet viability

To check up platelet viability upon gabexate mesylate treatment the efficiency of the glycolitic pathway was measured by the production of L-lactate according to Hohorst [29]. To verify the membrane damage the activity of lactic dehydrogenase released from platelets was quantified by the method of Vassault [30].

2.7. Data analysis

Data reported in this paper are the mean ± SD of at least three independent determinations each performed in duplicate. Reported drawings are also representative of triplicate experiments. Statistical analysis was performed using *one-way* ANOVA followed by Fisher's post-hoc test and Student's *t*-test as appropriate. A P value <0.05 was considered to be statistically significant.

3. Results

3.1. The effect of gabexate mesylate on L-arginine uptake

The NO formation is strictly dependent on the availability of L-arginine to NOS. Thus, the L-arginine uptake can be considered a very important control step of L-arginine/NO pathway. Compounds able to regulate L-arginine uptake can modulate intracellular NO production. Therefore, we have tested the effect of gabexate mesylate on L-arginine transport in human platelets. As shown in Fig. 2, gabexate mesylate inhibited L-arginine transport of resting platelets, being the effect time and dose-dependent. Platelet stimulation with agonists (thrombin or collagen) did not change the L-arginine uptake behaviour and the response to gabexate (data not shown). To clarify the characteristics of the gabexate inhibition we determined the kinetic parameters of the drug inhibitory effect on L-arginine uptake. It was shown that gabexate behaved like a competitive inhibitor of L-arginine transport. The K_m value was increased from 22 ± 2 to 86 ± 6 μM in the presence of gabexate mesylate, while the V_{max} value was unaffected (126 ± 4 pmol/2.0×10^8 plts/min for control and 134 ± 5 pmol/2.0×10^8 plts/min with gabexate mesylate) (Fig. 3). Data obtained from other experiments processed with the Dixon plot allowed us to define the K_i value that was 158 μM (Fig. 4).

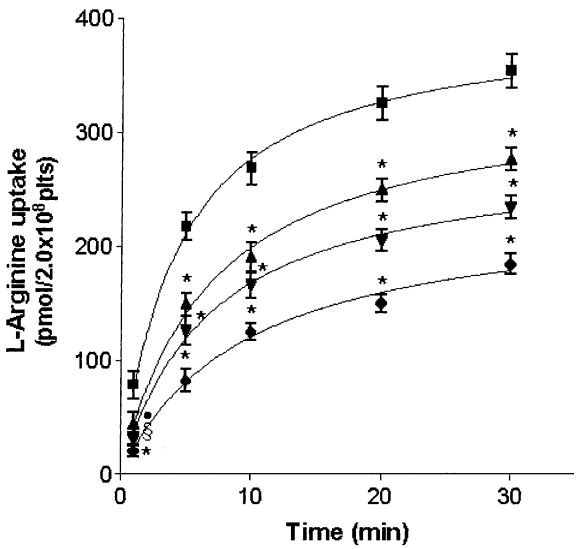


Fig. 2. Time course of L-arginine uptake in the presence or absence of gabexate mesylate. Washed platelets (2.0×10^8 plts), prewarmed at 37°, were incubated with 40 μM L-arginine in the presence of PBS (■), 300 μM (▲), 500 μM (▼), 1000 μM (◆) gabexate mesylate. At the indicated times aliquots of 1.0 mL were withdrawn and rapidly filtered as described in Section 2. Data are the mean ± SD of three experiments (each of them in duplicate). (*) $P < 0.0005$; (§) $P < 0.0025$; (●) $P < 0.01$ vs. none by unpaired Student's *t*-test. ANOVA showed significant variation of L-arginine uptake upon platelet treatment with various concentrations of gabexate mesylate ($P < 0.0001$ in all cases).

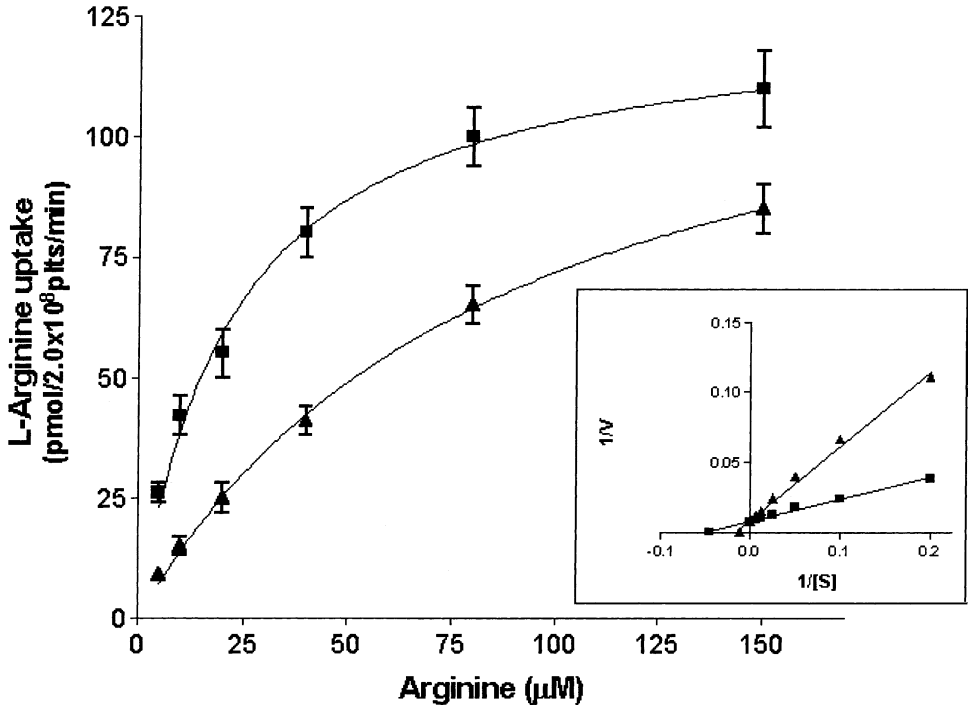


Fig. 3. The gabexate mesylate effect on the kinetic parameters of L-arginine uptake. Washed platelets (2.0×10^8 plts), prewarmed at 37°, were incubated for 1 min at 37° in the presence of various L-arginine concentrations (5–150 μM) and 500 μM gabexate mesylate when required. The uptake was measured as described in Section 2. The corresponding Lineweaver–Burk plot is reported in the inset. Data are the mean ± SD of four experiments carried out in duplicate.

3.2. The gabexate mesylate effect on NO production

To evaluate whether the gabexate inhibition of L-arginine uptake may limit cellular synthesis of NO, some experiments have been performed incubating platelets with various concentrations of the drug. At the end of incubation nitrite and nitrate accumulation was measured. Data have shown that gabexate mesylate dose-dependently inhibited NO production in whole platelets. As shown in Fig. 5, 100 μM gabexate decreased NO formation of more than 40% and the maximal inhibition (88%) was reached in the presence of 1000 μM gabexate after 15 min incubation. A

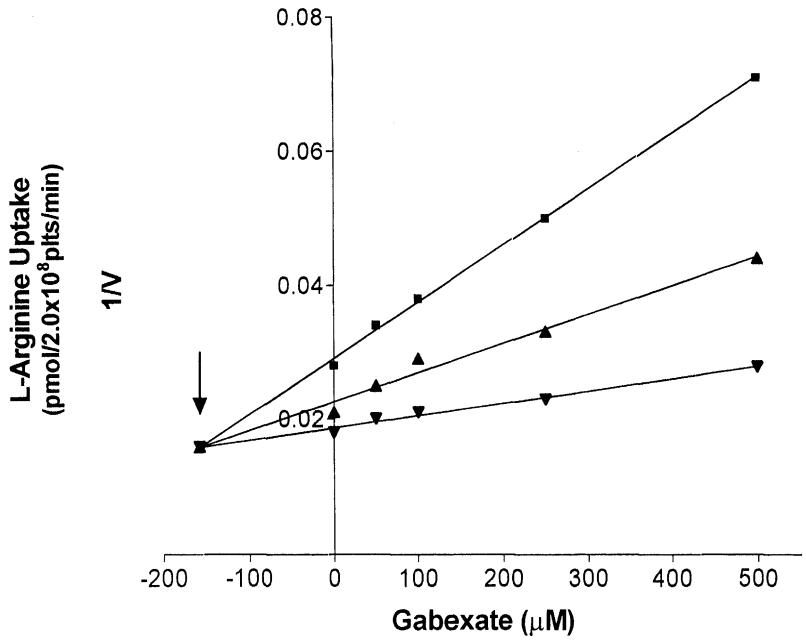


Fig. 4. Dixon plot for L-arginine uptake inhibition by gabexate mesylate. Washed platelets (2.0×10^8 plts), prewarmed at 37°, were incubated for 1 min in the presence of 10 μM (■), 20 μM (▲) or 40 μM (▼) L-arginine and gabexate mesylate at the indicated concentrations. L-Arginine uptake was measured as described in Section 2. Arrow indicates the K_i value.

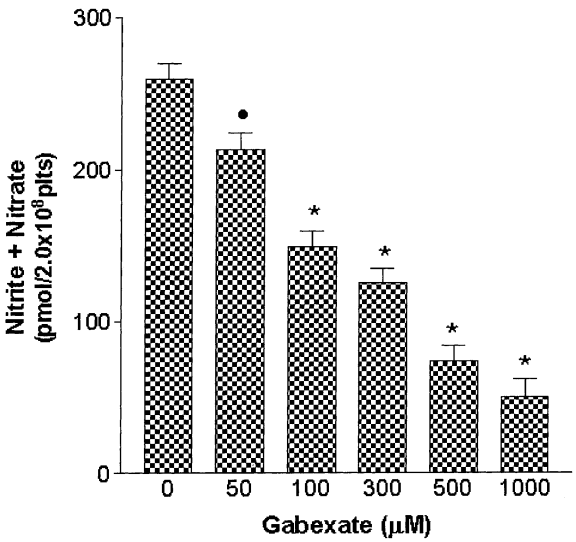


Fig. 5. Effect of gabexate mesylate on NO formation in platelets. Washed platelets resuspended in pH 7.4 HEPES buffer containing 2 mM $CaCl_2$ (1.0×10^9 plts) and prewarmed at 37°, were incubated for 15 min with 40 μM L-arginine and gabexate mesylate as indicated. Incubation was stopped by sonicating samples in ice and nitrite and nitrate levels of supernatants measured as reported in Section 2. Each bar represents the mean ± SD of three experiments carried out in duplicate. (*) $P < 0.0005$; (●) $P < 0.0025$ vs. none by unpaired Student's *t*-test. ANOVA showed significant variation of NO formation upon platelet treatment with different concentrations of gabexate mesylate ($P < 0.0001$ in all cases).

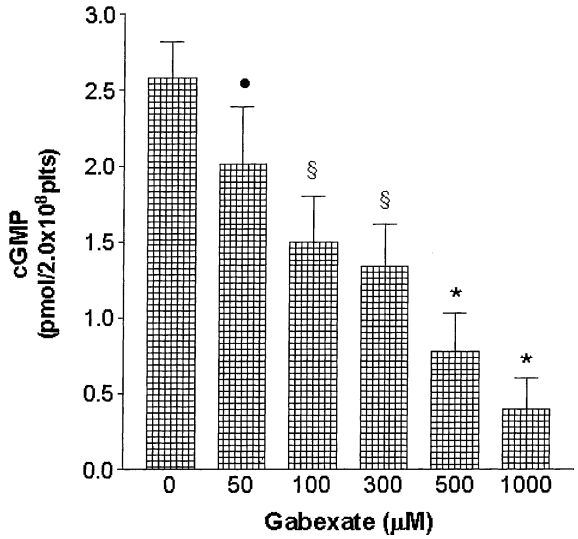


Fig. 6. Effect of gabexate mesylate on cGMP levels in platelets. Washed platelets, resuspended at 1.0×10^9 plts in pH 7.4 HEPES buffer containing 2 mM $CaCl_2$, were incubated with 40 μM L-arginine and gabexate mesylate as indicated. After 30 min at 37° incubation was stopped by adding 2 M perchloric acid. Supernatants neutralized with 2 N NaOH were analysed for the cGMP content by radioimmunoassay (RIA) kit. (*) $P < 0.0005$; (§) $P < 0.0025$; (●) $P < 0.01$ vs. none by unpaired Student's *t*-test. ANOVA showed significant variation of NO formation upon platelet treatment with different concentrations of gabexate mesylate ($P < 0.0001$ in all cases).

prolonged exposure of platelets to gabexate did not improve the effect. Moreover, it should be stressed that gabexate mesylate (100–1000 μM) incubated with platelets in the absence of L-arginine did not affect basal NO levels (data not shown).

3.3. The gabexate mesylate effect on cGMP levels

As additional evidence for the inhibition of NO production, the gabexate mesylate effect on cGMP levels was evaluated in platelets incubated in the presence of L-arginine. Gabexate mesylate inhibited in a dose-dependent manner cGMP formation (Fig. 6). The results obtained are in agreement with data reported in Fig. 5. The effects of gabexate mesylate on NO_x formation and on cGMP accumulation were closely correlated ($y = 0.009837x - 0.779868$; $r^2 = 0.99$; $P < 0.0001$). On the other hand, gabexate mesylate in the absence of L-arginine did not change cGMP basal level.

3.4. Effect of gabexate mesylate on cNOS activity in a cell-free system

In addition, we wanted to measure the inhibiting effect of gabexate mesylate on the cNOS activity of a platelet cell-free system. Data reported in Fig. 7 show a typical saturation curve (A) and the related Lineweaver–Burk plot (B) for cNOS in the presence or absence of gabexate mesylate. The K_m value increased from 0.82 ± 0.07 to 6.0 ± 0.4 μM in the presence of gabexate mesylate, while the V_{max} value was unchanged (672 ± 12 pmol/mg protein for control and 664 ± 16 pmol/mg protein with gabexate mesylate). Data of other experiments processed with the Dixon plot defined a K_i value of 48 ± 5 μM (data not shown).

3.5. Tests to assay platelet viability

Platelets treated with varying concentrations of gabexate (100–1000 μM) were not different from control platelets in the lactate produced or in the lactic dehydrogenase released during 15 or 30 min of incubation at 37°. Lactate produced was 24 ± 2.0 nmol/10^9 plts/min and 23 ± 1.5 nmol/10^9 plts/min in controls and in platelets treated with gabexate mesylate, respectively. Moreover, the leakage of lactate dehydrogenase that occurred during the incubation and centrifugation of the platelets was 8 and 10% of the total activity after 15 or 30 min at 37°, respectively, both in the presence and in the absence of the drug (100–1000 μM).

4. Discussion

NO is a free radical that can participate in several types of redox reactions, some that mediate its biological effects and others that limit its activity. Inactivation of NO occurs largely through oxidative reactions mediated by reactive oxygen intermediates such as superoxide, hydrogen peroxide and lipid peroxyl radicals generated from lipid

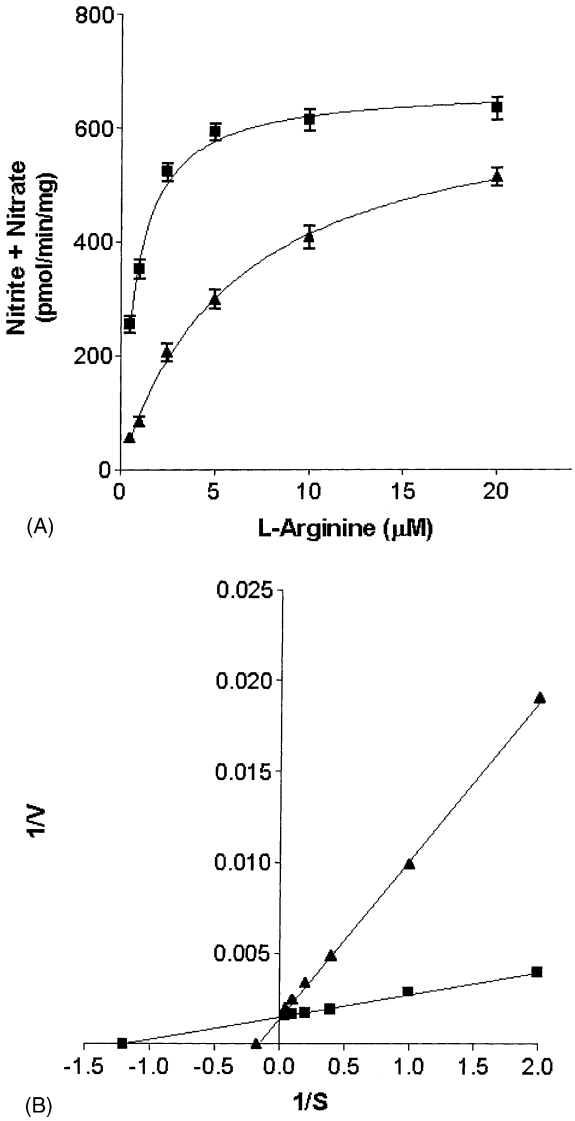


Fig. 7. The effect of gabexate mesylate on the kinetic parameters of cNOS in a cell-free system. Suitable aliquots of platelet sonicate were incubated in the presence of L-arginine (0.5–20 μM), PBS (●) or 300 μM gabexate mesylate (▼) for 5 min at 37°. cNOS activity was determined as described in detail in Section 2. Each point represents the mean ± SD of three experiments carried out in duplicate: (A) saturation curve; (B) Lineweaver–Burk plot.

peroxides [31]. Resting and activated platelets are themselves a rich source of superoxide and hydrogen peroxide [32]. These species have been found increased in platelets of various diseases [33,34]. On the other hand, several diseases such as cerebral infarction, diabetes mellitus and neurodegenerative disorders are associated with NO overproduction [35].

The formation of peroxynitrite by the reaction of either endothelial or platelet-derived NO with either endothelial or platelet-derived superoxide has been postulated to not only mediate cytotoxicity, but also cause tissue injury in physiological and pathological states [36]. It has been supposed that in addition to reactive oxygen species, reactive nitrogen oxide species (RNOS) generated from NOS may contribute to post-ischemic myocardial injury [37]. Recent studies have demonstrated that NOS inhibition significantly attenuates post-ischemic myocardial injury [38].

The selection of new compounds able to regulate NO formation in the cells could represent a very important topic. Drugs structurally similar to L-arginine could have particular significance. For such reason we have studied gabexate mesylate and its probable effects on L-arginine/NO pathway in human platelets. These cells are a simple model to clarify relevant metabolic and functional mechanisms. On the other hand, NO possesses anti-aggregating properties but whether it combines with anion superoxide largely produced by platelets could produce citotoxicity effects. Thus, the modulation of L-arginine/NO pathway could have a particular significance.

Our study demonstrates that gabexate mesylate inhibited L-arginine uptake with a K_i value of 158 μM. Since data are not available in literature on the gabexate mesylate bioavailability upon oral administration or ointment treatment of the drug, it is difficult to establish whether the gabexate mesylate effect could have relevance "*in vivo*". Nevertheless, the inhibition of L-arginine uptake could have as a consequence a diminished intracellular NO formation "*in vitro*". There was clear evidence for the inhibitory effect of gabexate on NO formation as determined directly by the detection of nitrite and nitrate formation and indirectly by the assessment of cGMP accumulation in intact cells. NO formation in human platelets seems to be regulated by the L-arginine transport that supplies cNOS with substrate. Up-to date recycling and degradation mechanisms of L-arginine have not been demonstrated in human platelets. Therefore, the aminoacid uptake represents the most remarkable regulatory step of NO production in human platelets and gabexate an effective modulator of L-arginine/NO pathway. Finally, it was shown that gabexate mesylate competitively inhibited the cNOS activity of a platelet cell-free extract, as demonstrated in rat C6 glioma cells by Colasanti *et al.* [39]. These authors have shown that gabexate mesylate inhibited competitively constitutive and inducible NOS and dose-dependently nitrite and nitrate production, but they did not test the drug effect on L-arginine transport.

In conclusion, gabexate mesylate can be considered an antioxidant agent exerting protective effect against tissue injury by decreasing NO formation and oxygen-free radical release. Nevertheless, the inhibition of L-arginine transport could result in a L-arginine depletion of the cells. For such reason gabexate mesylate as other L-arginine analogues should be administered under careful control.

Acknowledgments

This work was supported by the Ministero della Ricerca Scientifica (MURST-PRIN, 2000) Rome, Italy.

References

[1] Moncada S, Higgs A. The L-arginine–nitric oxide pathway. N Engl J Med 1993;329:2002–12.

[2] Schmidt HH, Walter U. NO at work. Cell 1994;78:919–25.

[3] Loscalzo J. Nitric oxide insufficiency, platelet activation and arterial thrombosis. Circ Res 2001;88:756–62.

[4] Suematsu M, Tamatani T, Delano FA, Miyasaka M, Forrest M, Suzuki H, Schmid-Schönbein GW. Microvascular oxidative stress preceding leukocyte activation elicited by *in vivo* nitric oxide suppression. Am J Physiol 1994;266:H2410–5.

[5] Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci USA 1991;88:4651–5.

[6] Groves PH, Banning AP, Penny WJ, Newby AC, Cheadle HA, Lewis MJ. The effects of exogenous nitric oxide on smooth muscle cell proliferation following porcine carotid angioplasty. Cardiovasc Res 1995;30:87–96.

[7] Salvemini D, De Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. Proc Natl Acad Sci USA 1989;86:6328–32.

[8] Maccarrone M, Corasaniti MT, Guerrieri P, Nisticò G, Finazzi Agrò A. Nitric oxide-donor compounds inhibit lipoxygenase activity. Biochem Biophys Res Commun 1996;219:128–33.

[9] Nakatsuka M, Osawa Y. Selective inhibition of the 12-lipoxygenase pathway of arachidonic acid metabolism by L-arginine or sodium nitroprusside in intact human platelets. Biochem Biophys Res Commun 1994;200:1630–4.

[10] Maccarrone M, Putti S, Finazzi Agrò A. Nitric oxide donors activate the cyclo-oxygenase and peroxidase activities of prostaglandin H synthase. FEBS Lett 1997;410:470–6.

[11] Muruganandam A, Mutus B. Isolation of nitric oxide synthase from human platelets. Biochim Biophys Acta 1994;1200:1–6.

[12] Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 1995;268:L699–722.

[13] Beckman JS, Wink DA, Crow JP. Nitric oxide and peroxynitrite. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. Chichester: Wiley, 1996, p. 61–70.

[14] McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, Wahl SM. Suppression of arthritis by an inhibitor of nitric oxide synthase. J Exp Med 1993;178:749–54.

[15] Hooper DC, Bagasra O, Marini JC, Zborek A, Ohnishi ST, Kean R, Champion JM, Sarker AB, Bobroski L, Farber JL, Akaike T, Maeda H, Koprowski H. Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: implications for the treatment of multiple sclerosis. Proc Natl Acad Sci USA 1997;94:2528–33.

[16] Moro MA, Darley-Usmar VM, Goodwin DA, Read NG, Zamora-Pino R, Feelisch M, Radomski MW, Moncada S. Paradoxical fate and biological action of peroxynitrite on human platelets. Proc Natl Acad Sci USA 1994;91:6702–6.

[17] Cortesi R, Ascenzi P, Colasanti M, Persichini T, Venturini G, Bolognesi M, Pesce A, Nastruzzi C, Menegatti E. Cross-enzyme inhibition by gabexate mesylate: formulation and reactivity study. J Pharm Sci 1998;87:1335–40.

[18] Martindale J. In: James E, Reynolds F, editors. The extra pharmacopoeia, 31st ed. London: The Royal Pharmaceutical Society, 1996.

[19] Aosasa S, Ono S, Seki S, Takayama E, Tadakuma T, Hiraide H, Mochizuki H. Inhibitory effect of protease inhibitor on endothelial cell activation. J Surg Res 1998;80:182–7.

[20] Jung SE, Yun IJ, Youn YK, Lee JE, Ha J, Noh DY, Kim SJ, Oh SK, Choe KI. Effect of protease inhibitor on ischemia-reperfusion injury to rat liver. World J Surg 1999;23:1027–31.

[21] Ohashi I, Nishijima J, Murata A, Toda H, Kato T. Inhibitory effect of a synthetic protease inhibitor (gabexate mesylate) on the respiratory burst oxidase in human neutrophils. J Biochem (Tokyo) 1995;118:1001–6.

[22] Aosasa S, Ono S, Mochizuki H, Tsujimoto H, Ueno C, Matsumoto A. Mechanism of the inhibitory effect of protease inhibitor on tumor necrosis factor alpha production of monocytes. Shock 2001;15:101–5.

[23] Erba F, Fiorucci L, Pascarella S, Menegatti E, Ascenzi P, Ascoli F. Selective inhibition of human mast cell tryptase by gabexate mesylate, an antiproteinase drug. Biochem Pharmacol 2001;61:271–6.

[24] Leoncini G, Maresca M, Buzzi E, Piana A, Armani U. Platelets of patients affected with essential thrombocythemia are abnormal in plasma membrane and adenine nucleotide content. Eur J Haematol 1990;44:116–20.

[25] Giovine M, Signorello MG, Pozzolini M, Leoncini G. Regulation of L-arginine uptake by Ca^{2+} in human platelets. FEBS Lett 1999;461:43–6.

[26] Leoncini G, Signorello MG, Roma G, Di Braccio M. Effect of 2-(1-piperazinyl)-4H-pyrido[1,2-a]pyrimidin-4-one (AP155) on human platelets *in vitro*. Biochem Pharmacol 1997;53:1667–72.

[27] Chen L, Salafranca MN, Mehta JL. Cyclooxygenase inhibition decreases nitric oxide synthase activity in human platelets. Am J Physiol 1997;273:H1854–9.

[28] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

[29] Hohorst H.J., L(+)lactate. In: Bergmeyer HU, editor. Methods of enzymatic analysis, 2nd ed. Weinheim: Verlag Chemie, 1965, p. 266–70.

[30] Vassault A., Lactate dehydrogenase. In: Bergmeyer HU, editor. Methods of enzymatic analysis, vol. III, 3rd ed. Weinheim: Verlag Chemie, 1983, p. 277-82.

[31] Kelm M. Nitric oxide metabolism and breakdown. Biochim Biophys Acta 1999;1411:273–89.

[32] Leoncini G, Maresca M, Colao C. Oxidative metabolism of human platelets. Biochem Int 1991;25:647–55.

[33] Leoncini G, Maresca M, Colao C, Piana A, Armani U. Increased hydrogen peroxide formation in platelets of patients affected with essential thrombocythaemia (ET). Blood Coagul Fibrinolysis 1992;3:271–7.

[34] Leoncini G, Signorello MG, Piana A, Carrubba M, Armani U. Hydrogen peroxide formation in platelets of patients with non-insulin-dependent diabetes mellitus. Platelets 1998;9:213–7.

[35] Christopherson KS, Bredt DS. Nitric oxide in excitable tissues: physiological roles and disease. J Clin Invest 1997;100:2424–9.

[36] Moncada S, Higgs EA. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J 1995;9:1319–30.

[37] Darley-Usmar V, Halliwell B. Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions and the vascular system. Pharm Res 1996;13:649–62.

[38] Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. J Biol Chem 1996;271:29223–30.

[39] Colasanti M, Persichini T, Venturini G, Menegatti E, Lauro GM, Ascenzi P. Effect of gabexate mesylate (FOY), a drug for serine proteinase-mediated diseases, on the nitric oxide pathway. Biochem Biophys Res Commun 1998;246:453–6.