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European Journal of Pharmacology 466 (2003) 207–212
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Vascular permeabilization by intravenous arachidonate in the rat peritoneal cavity: antagonism by ethamsylate

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Received 16 December 2002; received in revised form 18 February 2003; accepted 25 February 2003

Abstract

The hemostatic agent, ethamsylate, inhibits arachidonic acid metabolism by a mechanism independent of cyclooxygenase activity and blocks carrageenan-induced rat paw edema. Here, ethamsylate was investigated for (i) in vivo actions on the free radical-dependent, permeabilizing responses to arachidonic acid and (ii) its antioxidant potential in vitro. Vascular permeability was equated to the extravasation rate of Evans blue from plasma into the rat peritoneal cavity. Antioxidant potential was investigated by classical in vitro tests for superoxide radicals, hydroxyl radicals ($OH^{\bullet}$), and nitric oxide. Intravenous ethamsylate induced a very important and significant reduction of permeability responses to arachidonate, both when given preventively and cumulatively. Thus, (i) ethamsylate significantly reversed arachidonate-induced permeabilization, even at the lowest dose tested (44 ± 5% at 10 mg/kg) and (ii) a maximal reversal (about 70%) was reached between 50 and 200 mg/kg ethamsylate. In contrast, ethamsylate (100 mg/kg) was unable to antagonize the vascular permeabilization induced by serotonin (5-HT). In antioxidant assays, ethamsylate showed scavenging properties against hydroxyl radicals generated by the Fenton reaction (H_2O_2/Fe^{2+}) even at 0.1 μM (−20 ± 3%). $OH^{\bullet}$ scavenging by ethamsylate reached 42 ± 8% at 10 μM and 57 ± 7% at 1 mM and was comparable to that of reference compounds (vitamin E, troxerutin, and mannitol). Conversely, ethamsylate was a poor scavenger of superoxide and nitric oxide radicals. In conclusion, intravenous ethamsylate potently antagonized the peritoneal vascular permeabilization induced by arachidonate, an action likely due to its antioxidant properties, particularly against hydroxyl radical. Such a mechanism can explain previous observations that ethamsylate inhibits carrageenan-induced rat paw edema. Whether it also participates in the hemostatic action of ethamsylate deserves further investigation.

Keywords: Ethamsylate; Antioxidant; Arachidonate; Vascular permeability

1. Introduction

The systemic, nonthrombogenic hemostatic agent, ethamsylate, has been in clinical use for over 30 years, but its mechanism of action is poorly understood. From several studies trying to elucidate its mechanism of action, consistent evidence was provided for its inhibition of arachidonic acid metabolism. Thus, (i) Kovács and Falkay (1981) showed that ethamsylate inhibits prostaglandin biosynthesis in microsomes of pregnant human myometrium in vitro; (ii) Ment et al. (1984) found that the efficacy of ethamsylate to reduce intraventricular hemorrhage in a Beagle puppy model was associated with a reduction of thromboxane A_2 and prostacyclin biosynthesis; and (iii) Rennie et al. (1986) showed that the reduction by ethamsylate of the incidence of intraventricular hemorrhage in low-birth-weight infants with respiratory distress was associated with a decrease in prostacyclin biosynthesis. Curiously, the inhibition of prostaglandin biosynthesis was not due to a reduction in cyclooxygenase activity (Kovács and Falkay, 1981) except at high ethamsylate concentrations (0.5–5 mM).

In 1990, Gard and Trigger showed that ethamsylate was an effective anti-inflammatory agent in the classical model of carrageenan-induced rat paw edema. Interest in the anti-

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doi:10.1016/S0014-2999(03)01545-0

inflammatory properties of ethamsylate was raised by recent reports showing cross-talk between inflammatory and hemostatic mechanisms (for review, see Cicala and Cirino, 1998; Shebuski and Kilgore, 2002). Thus, thrombin functions as an inflammatory proedema mediator in the rat paw model (Cirino et al., 1996) and is also a well-known mediator of arachidonic acid release during hemostasis. Moreover, leukocytes are important constituents of the blood clot and play an active role in hemostasis (Elstad et al., 1995; Li et al., 2000). Finally, tissue factor-initiated coagulation seems to contribute to leukocyte adhesion to the endothelial surface (Kirchhofer et al., 1993).

Ethamsylate shares a common sulfonic moiety with calcium dobesilate, a compound showing potent antioxidant properties in vitro (Brunet et al., 1998a) and in vivo (Brunet et al., 1998b; Szabo et al., 2001). Moreover, reactive oxygen species can induce the liberation of arachidonic acid (Martinez and Moreno, 2001), and Easton and Fraser (1998) have shown that arachidonic acid increases cerebral microvascular permeability by the formation of free radicals and subsequent lipid peroxidation. Finally, intravenous administration of arachidonate strongly enhances peritoneal vascular permeability in the rat, a permeabilizing action blocked by pretreatment with antioxidant compounds (see accompanying manuscript, Alvarez-Guerra et al., 2002). Taken together, we suspected that ethamsylate possesses antioxidant properties which explain, at least in part, its inhibition of arachidonic acid metabolism (Kovács and Falkay, 1981; Ment et al., 1984; Rennie et al., 1986) and its anti-inflammatory action in the model of carrageenan-induced rat paw edema (Gard and Trigger, 1990). Therefore, we used a recently characterized rat peritoneal model (see accompanying manuscript, Alvarez-Guerra et al., 2002) to investigate whether ethamsylate antagonizes the vascular permeabilization induced by arachidonic acid. The finding that ethamsylate is effective in this in vivo model prompted us to investigate its antioxidant potential in vitro.

2. Materials and methods

2.1. In vivo protocols

Rats, methods for surgical preparation, measurement of peritoneal vascular permeability, and administration of drugs were as described in an accompanying manuscript (Alvarez-Guerra et al., 2002). Ethamsylate was intravenously given either in cumulative or noncumulative doses.

2.2. Antioxidant assays

2.2.1. Superoxide radical assay

Superoxide anion radicals were generated by the xanthine/xanthine oxidase reaction according to a previously published method (Brunet et al., 1998a).

2.2.2. Hydroxyl radical ($OH^{\bullet}$) assay

Hydroxyl radicals were generated by a Fenton-type reaction and measured with the "deoxyribose oxidative degradation" method (Halliwell, 1995 ; Hermes-Lima et al., 1999). Briefly, $OH^{\bullet}$ radical was chemically generated by a mixture of hydrogen peroxide (H_2O_2, 2 mM) and ferrous ion (11.5 μM), performed in 10 mM HEPES buffer (pH 7.4 at room temperature) in the presence of 10 mM deoxyribose (reactional volume = 1 ml). In this reaction, $OH^{\bullet}$ reacts with deoxyribose, giving products that further react with thiobarbituric acid, producing a colorimetrically quantifiable chromogene (at 532 nm). $OH^{\bullet}$-scavenging compounds compete with deoxyribose, thus reducing thiobarbituric acid-dependent chromogene. Zero times were systematically run with controls to correct for possible absorbance by the scavenging compound in the mixture, and possible direct reaction(s) of compounds with thiobarbituric acid (see Halliwell, 1995).

Fenton-type (H_2O_2/Fe^{2+}) reactions are notoriously rapid (usually completed in seconds) and the experimental signal levels off thereafter (Lopes et al., 1999). Therefore, experiments were performed with both a "short" (30 s) and a "long" period (5 min) at 37 °C, and "zero times" were performed in the absence of H_2O_2 and Fe^{2+} ($FeSO_4$) at 4 °C. After short or long incubations, the tubes were transferred to 4 °C (ice-cold water) and the reaction was stopped by rapidly adding 1 ml of 4% phosphoric acid (vol/vol), followed by 1 ml of 1% thiobarbituric acid (wt/vol, in 50 mM NaOH). After 20 min of boiling, the optical density was measured at 532 nm.

2.2.3. Nitric oxide radical assay

Nitric oxide was generated using the $NO^{\bullet}$ donor molecule, *S*-nitroso-*N*-acetylpenicillamine (SNAP; Ignarro et al., 1988). SNAP spontaneously releases $NO^{\bullet}$ radical under physiological conditions, according to a bimolecular reaction (in contrast to other $NO^{\bullet}$ donors, which simultaneously release superoxide anion, SNAP releases only $NO^{\bullet}$, thus avoiding artifacts due to superoxide anion per se and/or peroxynitrite).

The reaction was carried out for 60 min at 37 °C, in a 5 mM morpholinopropanesulfonic acid (MOPS)–Tris buffer (pH 7.4) containing 0.5 mM SNAP. Hemoglobin (50 μM) was used as reference $NO^{\bullet}$ scavenger. The reaction was stopped by transferring the tubes to ice-cold water (4 °C). The tubes were carefully capped and stored for 3–4 days at −20 °C before nitrite/nitrate determinations. Each experiment was done in triplicate.

Nitric oxide contents were equated to the nitrite/nitrate contents, measured according to the Griess reaction (Privat et al., 1997; Guevara et al., 1998). Nitrite standards were prepared in the range 0–200 μM (KNO_2) to establish a calibration curve, which yielded a determination coefficient of 0.99 (run in duplicates). The average slope of the calibration curve (0.00594 OD U/μM) was used to convert sample OD readings into [$NO_2 + NO_3$] concentrations (μM).

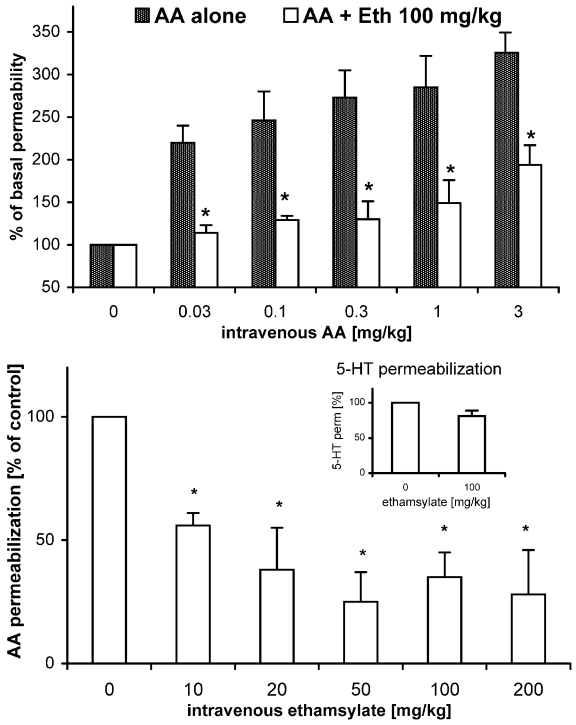

Fig. 1. Top: Vascular permeabilization induced by cumulative doses of arachidonic acid in the rat peritoneal cavity and antagonism by intravenous ethamsylate (100 mg/kg; $n=3$). AA indicates arachidonic acid. Each sequential dose of arachidonic acid was given every 20 min. Vascular permeabilization is expressed as the percent increase in basal permeability (baseline values of permeability rate constants ranged from 2.5 to 7.5×10^{-3} h^{-1}). *$P<0.05$ vs. baseline (two-tailed Student's t test). Bottom: Percent reduction in arachidonate-induced permeabilization induced by cumulative doses of intravenous ethamsylate ($n=3$). AA indicates arachidonic acid. Vascular permeabilization was induced by arachidonate 3 mg/kg, i.v. (a dose increasing the permeability rate constant up to 32.5×10^{-3} h^{-1}). Each sequential dose of ethamsylate was given every 10 min. Ethamsylate significantly reversed arachidonate-induced permeabilization, even at the lowest dose tested (10 mg/kg; $P=0.0022$, one-factor ANOVA followed by Scheffe's F test comparing with arachidonic acid alone; *$P<0.05$). Inset: Lack of antagonism by ethamsylate (100 mg/kg) against vascular permeabilization induced by serotonin (0.1 mg/kg 5-HT; $n=4$).

2.3. Compounds

Ethamsylate (diethylammonium salt of dihydroxy-2,5 benzenesulphonate) was obtained from OM PHARMA (Meyrin, Geneva, Switzerland). Sodium arachidonate from porcine liver (99% purity) was purchased from Sigma (St. Louis, MO, USA). All other compounds were from Sigma or InterChim (Montluçon, France).

2.4. Statistical analysis

Results are expressed as mean ± S.E.M. of n experimental determinations. Statistical analysis of the data was performed by using an analysis of variance (ANOVA) program and two-tailed Student's t tests.

3. Results

3.1. Effects of ethamsylate on vascular permeability responses to arachidonate

Ethamsylate was tested for its ability to antagonize vascular permeabilization induced by arachidonic acid. The results obtained were compared with those obtained when ethamsylate was tested against the vascular permeabilization induced by serotonin (5-HT). In all experiments, baseline values of permeability rate constants ranged from 2.5 to 7.5×10^{-3} h^{-1}. Moreover, control experiments showed that after arachidonic acid *alone*, Evans blue extravasation remained linear for at least 120 min.

In a first set of experiments ($n=3$), ethamsylate was given preventively (100 mg/kg, i.v. bolus) and, 15 min later, intravenous arachidonate was tested in cumulative dose–response curves (30 μg/kg–3 mg/kg) for its effects on Evans blue extravasation from plasma to the peritoneal cavity. Fig. 1 (top) compares the percent increase in basal permeability when arachidonate was given alone and when it was given after the ethamsylate bolus. It can be seen that preventive ethamsylate induced a very important and significant reduction of permeability responses at all doses of arachidonate tested. Based on these experiments, an arachidonate dose of 3 mg/kg, i.v. was selected for further experiments.

In a second set of experiments ($n=3$), increasing doses of ethamsylate (10–20–50–100–200 mg/kg) were tested against the vascular permeabilization induced by arachidonate 3 mg/kg, i.v. This dose of arachidonate increased the permeability rate constant up to 32.5×10^{-3} h^{-1} and, 10 min, later ethamsylate was intravenously given at cumulative doses. Fig. 1 (bottom) shows the percent reduction in arachidonate-induced permeabilization as a function of the intravenous dose of ethamsylate. It can be seen that (i) ethamsylate significantly reversed arachidonate-induced permeabilization, even at the lowest dose tested (at 10 mg/

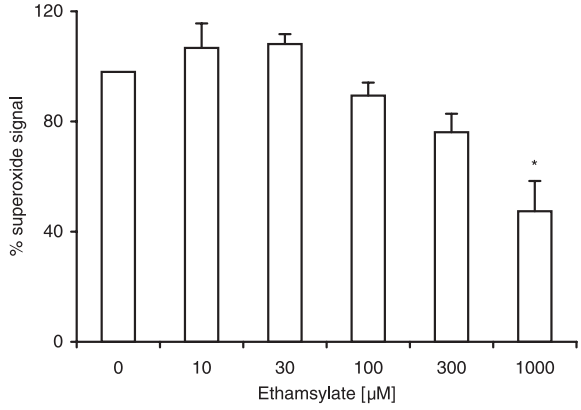

Fig. 2. Effect of ethamsylate on superoxide radicals ($O_2^{\bullet-}$) generated by xanthine/xanthine oxidase ($n=3$). High concentrations of ethamsylate significantly reduced the superoxide signal ($P=0.0001$, one-factor ANOVA followed by Scheffe's F test comparing with control; *$P<0.05$).

kg, it reversed 44 ± 5% of arachidonate permeabilization), and (ii) a maximal reversal (of about 70%) was reached between 50 and 200 mg/kg ethamsylate.

In a third series of experiments, ethamsylate (100 mg/kg, i.v. bolus) was tested against the vascular permeabilization induced by 5-HT 0.1 mg/kg ($n=4$; this dose of 5-HT induced a significant threefold increase in vascular permeability). In contrast to the results obtained with arachidonate, ethamsylate was without significant effect on the serotonin-induced permeabilization (inset to Fig. 1, bottom).

3.2. Ethamsylate and free radicals

3.2.1. Superoxide radical

Ethamsylate (10 μM–1 mM; $n=3$) was tested for its scavenging properties against superoxide radicals ($O_2^{\bullet -}$) generated by xanthine/xanthine oxidase. Fig. 2 shows that ethamsylate was a very modest superoxide radical scavenger; that is, high ethamsylate concentrations (1 mM) were required to reduce $O_2^{\bullet -}$ levels by half. For comparison, superoxide dismutase (SOD; 1 IU/ml) scavenged $O_2^{\bullet -}$ by more than 90% ($P<0.01$ vs. control).

3.2.2. Hydroxyl radical

Ethamsylate (30 nM–1 mM; $n=5$) was tested for its scavenging properties against hydroxyl radicals generated by the Fenton reaction (H_2O_2/Fe^{2+}). Fig. 3 (top) shows that ethamsylate was a potent $OH^{\bullet}$ scavenger, significantly reducing the $OH^{\bullet}$ signal even at 0.1 μM (−20 ± 3%). The $OH^{\bullet}$-scavenging action reached 42 ± 8% at 10 μM and 57 ± 7% at 1 mM ethamsylate.

For comparison, Fig. 3 (bottom) shows the $OH^{\bullet}$-scavenging properties of reference antioxidants. It can be seen that vitamin E (30 μM), troxerutin (10 μM), and mannitol (50 mM) significantly inhibited the $OH^{\bullet}$ signal by 81 ± 5% ($n=7$), 55 ± 4% ($n=5$), and 67 ± 9% ($n=7$), respectively.

3.2.3. Nitric oxide radical

Ethamsylate (10 nM–1 mM) was tested for its scavenging properties against $NO^{\bullet}$ radicals generated by SNAP ($n=3$). Ethamsylate was a very modest $NO^{\bullet}$ scavenger; that is, ethamsylate concentrations ≥100 μM were required to significantly reduce $NO^{\bullet}$ levels. For comparison, hemoglobin (50 μM) inhibited $NO^{\bullet}$ levels by 92 ± 11% ($n=6$; $P<0.0001$).

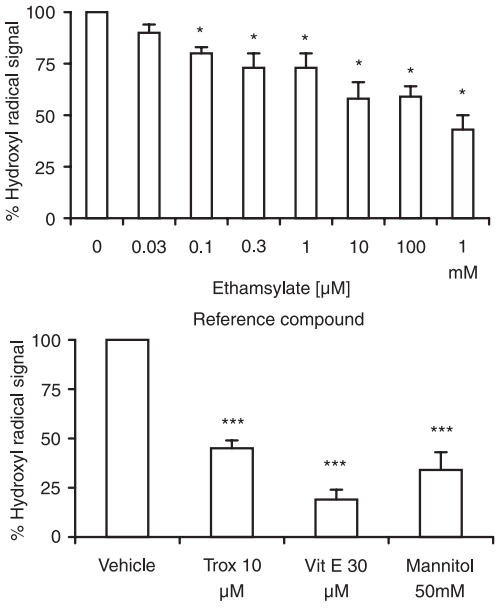

Fig. 3. Top: Effect of ethamsylate on hydroxyl radicals ($OH^{\bullet}$) generated by the Fenton reaction (H_2O_2/Fe^{2+}). Ethamsylate significantly reduced the $OH^{\bullet}$ signal, even at 0.1 μM ($P=0.0001$, one-factor ANOVA followed by Scheffe's F test comparing with control; $^*P<0.05$; $n=5$). Bottom: $OH^{\bullet}$-scavenging properties of reference antioxidants. Vitamin E (30 μM), troxerutin (10 μM), and mannitol (50 mM) significantly inhibited the $OH^{\bullet}$ signal by 81 ± 5% ($n=7$), 55 ± 4% ($n=5$), and 67 ± 9% ($n=7$), respectively. $^*P<0.05$ vs. control (two-tailed Student's t test).

4. Discussion

Intravenous ethamsylate potently antagonized peritoneal vascular permeabilization induced by arachidonate. The antagonism by ethamsylate was seen both when the drug was given preventively and cumulatively. No such antagonistic action was observed against peritoneal vascular permeabilization induced by serotonin. Finally, a battery of in vitro antioxidant tests revealed that ethamsylate preferentially scavenges hydroxyl radicals.

Ethamsylate proved to be a very good scavenger of hydroxyl radical, but not of superoxide and nitric oxide radicals. Thus, ethamsylate in concentrations as low as 0.1–10 μM inhibited 20–42% of the Fenton reaction-generated $OH^{\bullet}$ signal. Surprisingly, complete inhibition of the $OH^{\bullet}$ signal was not attained at the highest ethamsylate concentration assayed (1 mM). This may be due to the high H_2O_2/Fe^{2+} concentrations used (2 mM/10 μM), which produced high amounts of $OH^{\bullet}$ radical, thus limiting the apparent ethamsylate-scavenging action. This view is supported by the incomplete inhibition induced by the "classical $OH^{\bullet}$ scavenger," mannitol (67% inhibition, despite the use of 50 mM), and by other reference antioxidant compounds (30 μM vitamin E and 10 μM troxerutin yielded 81 and 55% inhibition, respectively). Further investigation could clarify this point.

In an accompanying manuscript (Alvarez-Guerra et al., 2002), we reported that the peritoneal vascular permeabilization induced by arachidonate was blocked by pretreatment with antioxidant compounds (vitamins C and E, and troxerutin, a soluble form of the potent antioxidant flavonoid, rutin). Easton and Fraser (1998) found that arachidonic acid increases cerebral microvascular permeability in rat pial venular capillaries by the formation of free radicals and subsequent lipid peroxidation. Therefore, the antioxidant activity of ethamsylate per se can explain its antagonistic

properties against arachidonate-induced vascular permeabilization.

Gard and Trigger (1990) showed that ethamsylate inhibits carrageenan-induced rat paw edema by a mechanism different from that of indomethacin. Boughton-Smith et al. (1993) showed that the generation of oxygen free radicals by arachidonic acid has a role in carrageenan-induced paw edema in the rat, and that carrageenan paw edema is sensitive to antioxidants (Boughton-Smith et al., 1993). Therefore, the results of Gard and Trigger (1990) can be explained by an antioxidant action of ethamsylate against arachidonate-induced permeability.

In humans receiving a single oral dose of 500 mg of ethamsylate, the plasma ethamsylate concentration peaks at 60 μmol after 4 h, with 95% of the compound being bound to plasma proteins (Vidal, 1997). This gives a plasma concentration of 3 μmol (i.e., an ethamsylate concentration able to scavenge 30–40% of the hydroxyl radical signal in the experiments represented in Fig. 3, top). Therefore, the hydroxyl radical-scavenging action of ethamsylate can explain its anti-inflammatory actions in vivo (Gard and Trigger, 1990) and perhaps the reduction of prostaglandin biosynthesis independent of cyclooxygenase (Kovács and Falkay, 1981).

Ment et al. (1984) found that the efficacy of ethamsylate to reduce intraventricular hemorrhage in a Beagle puppy model was associated with a reduction in thromboxane A_2 and prostacyclin biosynthesis, and Rennie et al. (1986) showed that the reduction by ethamsylate of the incidence of intraventricular hemorrhage in low-birth-weight infants with respiratory distress was associated with a decrease in prostacyclin biosynthesis. However, it appears too early to speculate about whether the results of the present investigation explain the hemostatic mechanism of ethamsylate (for recent reviews of cross-talk between inflammatory and hemostatic mechanisms, see Cicala and Cirino, 1998; Shebuski and Kilgore, 2002). Caccese et al. (2000) showed that collagen-induced platelet aggregation is associated with hydroxyl and superoxide radical formation, which is dependent on arachidonic acid release and metabolism. Moreover, free radicals are also involved in other stages of hemostasis. For instance, the endothelial-permeabilizing action of leukocytes relies, for a great part, on oxygen reactive species such as superoxide anion, hydroxyl radical, and hydrogen peroxide. Therefore, whether the antioxidant/anti-inflammatory properties of ethamsylate participate in its hemostatic action deserves further investigation.

In conclusion, intravenous ethamsylate potently antagonized the peritoneal vascular permeabilization induced by arachidonate. Moreover, ethamsylate exhibited antioxidant properties in several in vitro assays, particularly against hydroxyl radicals. The antioxidant activity of ethamsylate can explain its antagonistic properties against arachidonate-induced vascular permeabilization and the previous observation (Boughton-Smith et al., 1993) that ethamsylate inhibits carrageenan-induced rat paw edema. Whether the antioxidant/anti-inflammatory properties of ethamsylate participate in its hemostatic action deserves further investigation.

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