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Vascular permeabilization by intravenous arachidonate in the rat peritoneal cavity: antagonism by antioxidants

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Abstract

Arachidonic acid was investigated for its vascular permeabilizing potential in the rat peritoneal cavity and for its mechanism of action. The antagonistic potential of antioxidants (vitamin E, vitamin C and troxerutin) was also evaluated. Vascular permeability was equated to the rate of extravasation of Evans blue dye from plasma into the peritoneal cavity. Baseline permeability was linear up to 2 h, with a rate constant (k) of 0.0031 ± 0.0007 h^{-1}. Intravenous arachidonate (from 30 μg/kg to 3 mg/kg) induced an immediate, dose-related and significant increase in permeability (ranging from 80% to 150%), which was comparable to the effect induced by similar doses of serotonin. Aspirin (10 mg/kg) reduced the arachidonate-induced permeability by 75%, but interestingly neither the stable thromboxane A_2 receptor agonist U46619 (prostaglandin H_2 endoperoxide epoxymethane) nor prostacyclin was able to increase peritoneal vascular permeability. In contrast, the permeabilizing action of arachidonic acid was very sensitive to antioxidant agents. Thus, vitamin C and the flavonoid compound troxerutin (100 mg/kg) fully abolished arachidonate-induced permeability, whereas vitamin E had only a partial effect (40–100% inhibition). In conclusion, intravenous administration of arachidonic acid strongly enhanced peritoneal vascular permeability in the rat, apparently via free radical generation. This rat peritoneal model can be used to evaluate the in vivo antinflammatory potential of antioxidant drugs.

Keywords: Antioxidant; Arachidonate; Capillary permeability; Reactive oxygen species

1. Introduction

Increased capillary permeability leading to tissue edema and cell infiltration is a major component of any inflammatory reaction, which relies at least partly upon the generation of reactive oxygen species (Kubes and Gaboury, 1996; Gute et al., 1998). These reactive oxygen species can induce the liberation of arachidonic acid, an important inflammatory permeabilizing agent (Seeger et al., 1987; Rao et al., 1993; Martinez and Moreno, 2001). Arachidonic acid is itself able to generate reactive oxygen species (Caccese et al., 2000; Woo et al., 2000). Thus, in rat pial venular capillaries, arachidonic acid increases cerebral microvascular permeability by free radical formation and subsequent lipid peroxidation (Easton and Fraser, 1998). However, even if the role of reactive oxygen species in the actions of arachidonic acid has been clearly demonstrated, the vascular permeabilization induced by this agent could also result from its metabolites, at least in some vascular territories. Thus: (i) in the lipopolysaccharide-induced dental pulpal inflammation model in the rat, the increase in vascular permeability is not mediated by arachidonic acid itself, but by prostaglandin E_2 and prostacyclin (Okiji et al., 1989); (ii) in isolated rabbit lungs, arachidonic acid increases vascular permeability via peptidoleukotriene formation (Seeger et al., 1987); and (iii) thromboxane A_2 receptor activation increases microvascular permeability in rat lung, kidney and spleen but not in rat brain, liver, mesentery, and cardiac and skeletal muscle (Bertolino et al., 1995).

Shi et al. (1995) found that lipophilic antioxidants were particularly effective in preventing arachidonic acid-induced lipid peroxidation and associated permeability changes in bovine brain microvessel endothelial cell monolayers. These authors proposed this primary culture model as a useful in vitro system to evaluate mechanisms through which mediators of disease or injury states compromise blood–brain

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barrier integrity (Shi et al., 1995). Here, we adapted a previously developed in vivo model (Brunet et al., 1998) to further assess how arachidonic acid induces increases in vascular permeability, i.e., by measuring the rate of extravasation of Evans blue from plasma into the rat peritoneal cavity. Using this technique, we investigated whether arachidonic acid, prostacyclin and/or thromboxane can per se increase capillary permeability. The finding that only arachidonic acid, and not the metabolites tested, was able to increase rat peritoneal permeability prompted us to investigate the antagonistic potential of aspirin (an inhibitor of cyclooxygenase), nordihydroguaiaretic acid (a lipoxygenase inhibitor) and a number of antioxidants (vitamin E, vitamin C and the flavonoid troxerutin).

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 300–350 g were purchased from CERJ (Le Genest, St. Isle, France). Upon arrival, the animals were allowed to adapt to a humidity- and temperature-controlled room for at least 2 days. They were fed on a standard diet and tap water ad libitum. All animals received care in compliance with the principles of the European Community guidelines for ethical animal care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Measurement of peritoneal vascular permeability

Peritoneal vascular permeability was determined according to Brunet et al. (1998) with minor modifications. Rats were anesthesized with sodium pentobarbital (100 mg/kg, i.p.), after a 10- to 15-min premedication with ketamine (50 mg/kg, i.p.), and maintained at an external temperature of 28–32 °C throughout the experiment with a heating blanket. A polyethylene catheter (PE50) was inserted in the jugular vein and used for injections; another catheter was inserted in the carotid for blood sampling. The experimental procedure was initiated after a 10-min stabilization period.

Ten to fifteen minutes before the start of peritoneal sampling, 28 ml saline (NaCl 0.9%, pre-equilibrated at 37 °C) was gently injected into the peritoneal cavity, via a needle inserted through the right abdominal side (this needle remained in place throughout the experiment). Five to ten minutes later, Evans blue (MW 961) was injected through the jugular catheter: 1.0 μl/g body weight of a 50 mg/ml saline solution, yielding 50–80 OD[620 nm] for initial plasma readings. At these concentrations, 5- to 10-fold higher than those in Brunet et al. (1998), Evans blue peritoneal extravasation was linear up to 2 h, a condition required to establish cumulative dose–response curves to arachidonic acid.

One minute after Evans blue injection, a venous blood sample (0.7 ml) was collected via the catheter to measure initial levels of Evans blue in plasmas, and peritoneal samples were collected every 5 min (0.7 ml) or every 10 min (1.4 ml) (see Section 3). Additional blood samples were collected at the middle (60 min, unless otherwise indicated) and at the end of the experiment (120 min, unless otherwise indicated). All samples were microcentrifuged (1 min, 12,000 rpm), and peritoneal and plasma supernatants were diluted twofold and 200-fold, respectively. Evans blue content was quantified by reading the optical density at 620 nm with a spectrocolorimeter (Perkin-Elmer, Fremont, CA, USA).

Evans blue extravasation rate constants (in h^{-1}) were calculated as follows. For each 5 (or 10) min time frame, the slope of Evans blue absorbance as a function of time was calculated and divided by the corresponding plasma level (as derived by exponential interpolation between the appropriate plasma samples).

At the end of the experiment, the macroscopic integrity of the abdominal cavity was visually checked by opening the abdomen.

2.3. Treatment protocols

All drugs were administered intravenously (100 + 100 μl saline so as to empty the needle and the catheter; the injection time was 30 s to 1 min). When given preventively, drugs were injected through the jugular catheter, 10–15 min before the start of the experiment. For cumulative dose–response curves (usually five doses), the test molecule was injected at 21, 41, 61, 81 and 101 min (see Fig. 1).

Sodium arachidonate, serotonin, prostacyclin and the thromboxane A_2 receptor agonist U46619 (prostaglandin H_2 endoperoxide epoxymethane) were investigated for their vascular permeabilizing actions, in cumulative dose–response experiments. Preliminary dose–response experiments with potential arachidonate antagonists allowed us to select doses of test compounds. Thus, aspirin was tested at 3,

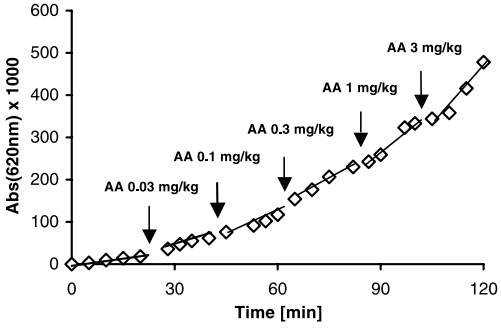

Fig. 1. Vascular permeabilization induced by arachidonic acid in the rat peritoneal cavity. The figure shows a typical experiment, where cumulative doses of intravenous arachidonic acid (AA) increased the extravasation rate of plasma Evans blue in the peritoneal cavity. Evans blue absorbance was measured at 620 nm (see Section 2).

10 and 20 mg/kg, nordihydroguaiaretic acid was tested at 10 and 100 mg/kg, and the antioxidants vitamin E, vitamin C and troxerutin were tested at the single dose of 100 mg/kg.

2.4. Compounds

Sodium arachidonate from porcine liver (99% purity) was purchased from Sigma (St. Louis, MO, USA). U46619 was from Biomol (Plymouth Meeting, PA, USA). Troxerutin was kindly provided by Laboratoires NEGMA (Magny-les-Hameaux, France). All other compounds were from Sigma or from InterChim (Montluçon, France). Aspirin was neutralized with NaOH.

2.5. Statistical analysis

Results are expressed as means ± 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. Peritoneal vascular permeabilization by arachidonic acid

Evans blue was intravenously injected and the rate constant for its extravasation toward the peritoneal cavity (k) was determined as described in the Section 2. Arachidonic acid was tested in this model, either preventively or in cumulative intravenous doses.

Fig. 1 shows a typical experiment, where cumulative doses of arachidonic acid (i.v. bolus, from 30 μg/kg to 3 mg/kg) induced a pronounced, dose-dependent acceleration of Evans blue extravasation. In six different experiments, a significant increase in vascular permeability was already observed at the dose of 30 μg/kg of arachidonic acid (5.3 ± 0.8 vs. $2.6 \pm 0.2 \times 10^3$ h^{-1} in controls; $P < 0.05$, Student's t-test), whereas a maximum value of $7.2 \pm 0.7 \times 10^3$ h^{-1} was reached with 3 mg/kg of arachidonic acid.

For comparison, we tested the reference permeabilizing agent serotonin (5-HT). In four different experiments, 5-HT induced a maximal permeability increase of $9.5 \pm 0.7 \times 10^3$ h^{-1} at the dose of 100 μg/kg (against $3.1 \pm 0.6 \times 10^3$ h^{-1} in controls; intravenous 5-HT doses higher than 100 μg/kg produced cardiorespiratory distress). Therefore, the 177% maximal increase in permeability with arachidonic acid compared well with the 206% permeability increase with 5-HT.

Non-cumulative, preventive administration of arachidonic acid (15 min) in different rats ($n = 3$ per dose) induced modestly higher permeabilizing effects (5–30%) as compared with arachidonic acid given in cumulative doses. Thus, a single intravenous bolus of 0.1 mg/kg arachidonic acid increased k to $6.3 \pm 0.9 \times 10^{-3}$ h^{-1} (baseline value = 3.1 ± 0.7 h^{-1}), whereas the dose of 1 mg/kg yielded $9.3 \pm 1.8 \times 10^{-3}$ h^{-1} (compare with Fig. 1). Therefore, the agonist potential of compounds (prostacyclin and U46619) was evaluated using cumulative dose–response curves (to reduce the number of rats, to increase the number of agonist doses and to ensure a more reproducible test of agonist drugs). Conversely, experiments to evaluate the antagonist potential of compounds (aspirin, nordihydroguaiaretic acid and anti-oxidants) were done with compounds given preventively (15 min) in non-cumulative doses.

3.2. Inhibitors of arachidonic acid metabolism

3.2.1. Aspirin

Preliminary experiments showed that (i) aspirin partially antagonized the permeability responses to arachidonic acid, and (ii) aspirin per se did not induce changes in basal peritoneal capillary permeability (preventively given aspirin 10 mg/kg, baseline k_{Eb} was 2.9 ± 1.3 vs. $3.1 \pm 0.7 \times 10^{-3}$ h^{-1}, $n = 8$ per group of rats). To increase the experimental precision, a 120-min period was used to measure vascular permeability after a single dose of arachidonic acid (1 mg/kg), with aspirin given preventively (15 min before) in different groups of rats. Fig. 2 (top) compares the vascular permeabilization produced by arachidonic acid given alone (single intravenous bolus of 1 mg/kg) and given 15 min after three doses of intravenous aspirin (3, 10 and 20 mg/kg; $n = 6$). It can be seen that aspirin dose dependently and significantly antagonized by 70–80% the permeabilizing action of arachidonate.

3.2.2. Nordihydroguaiaretic acid

The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) was tested for its ability to prevent the peritoneal vascular permeabilization induced by arachidonic acid (from 30 μg/kg to 3 mg/kg, $n = 3$). Fig. 2 (bottom) shows the effect of preventive administration of intravenous NDGA (100 mg/kg bolus i.v., 15 min before successive intravenous doses of arachidonic acid). It can be seen that NDGA fully abolished the peritoneal vascular permeabilization produced by arachidonic acid.

In another set of experiments, 10 mg/kg NDGA given preventively inhibited by 70% the permeabilizing effect of arachidonic acid (1 mg/kg, $n = 3$, $P < 0.05$), supporting the idea that NDGA 100 mg/kg achieved its maximal effect. Finally, it is interesting to mention that pretreatment of rats with both aspirin (10 mg/kg) and NDGA (10 mg/kg) completely inhibited the arachidonic acid response ($n = 3$, $P < 0.05$).

3.3. Arachidonic acid metabolites

To evaluate the potential contribution of thromboxane A_2 receptors, we investigated the permeabilizing potential of U46619, a stable thromboxane A_2 receptor agonist. Intra-

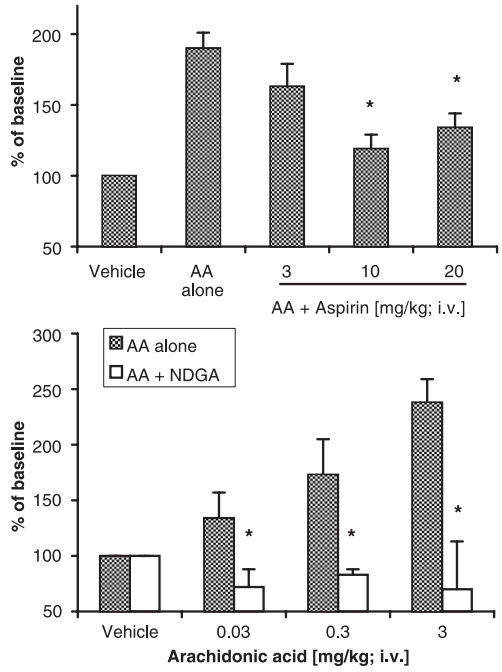

Fig. 2. *Top*: Partial prevention by aspirin of vascular permeabilization induced by arachidonic acid (AA) in the rat peritoneal cavity. Intravenous aspirin was preventively given 15 min before an intravenous bolus of 1 mg/kg arachidonic acid ($n=6$). Permeability values were normalized as percentage of baseline, to reduce interindividual variation. Aspirin dose dependently and significantly antagonized by 70–80% the permeabilizing action of arachidonate ($P=0.0035$, one factor ANOVA analysis followed by Scheffe's *F*-test comparing with arachidonic acid alone; * indicates $P<0.05$). *Bottom*: Blockade by nordihydroguaiaretic acid (NDGA) of the permeabilizing action of arachidonic acid (AA, from 30 μg/kg to 3 mg/kg). Intravenous NDGA 100 mg/kg was preventively given 15 min before different intravenous doses of arachidonic acid ($n=3$). *$P<0.05$ vs. NDGA + the same arachidonic acid dose (two-tailed Student's *t*-test). NDGA fully abolished the peritoneal vascular permeabilization induced by arachidonic acid.

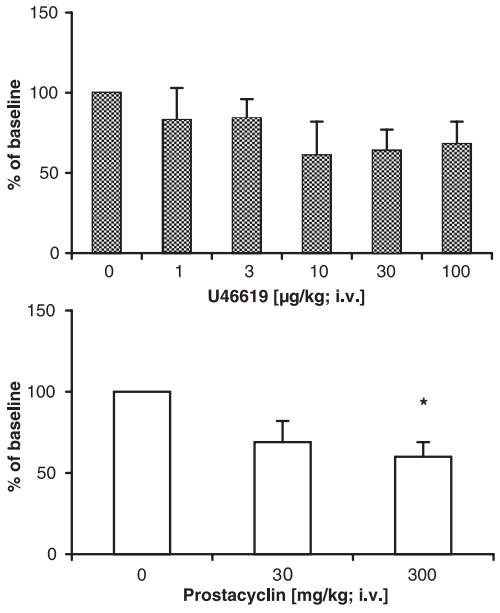

Fig. 3. *Top*: Lack of permeabilizing potential of U46619, a stable thromboxane A_2 receptor agonist. Intravenous U46619 was acutely given in cumulative bolus doses, from 1 to 100 μg/kg ($n=4$). High intravenous doses of U46619 induced a modest, non-significant decrease in vascular permeability (one factor ANOVA analysis vs. baseline). *Bottom*: Lack of permeabilizing potential of prostacyclin. Intravenous prostacyclin induced a slight decrease in peritoneal vascular permeability ($n=3$), which reached statistical significance at 300 μg/kg prostacyclin ($P=0.023$, one-factor ANOVA analysis followed by Scheffe's *F*-test comparing with vehicle; * indicates $P<0.05$).

venous U46619 was acutely given in cumulative bolus doses, from 1 to 100 μg/kg ($n=4$). Fig. 3 (top) shows that U46619 was unable to increase peritoneal capillary permeability over the range of concentrations tested. In contrast, high intravenous doses of U46619 induced a modest, non-significant decrease in vascular permeability (one-factor ANOVA analysis, Fig. 3, top).

Fig. 3 (bottom) shows that acute intravenous administration of prostacyclin (30 and 300 μg/kg, $n=3$) induced also a slight decrease in peritoneal vascular permeability, a decrease which reached statistical significance at 300 μg/kg prostacyclin (−40%).

3.4. Antioxidants

Three classical antioxidants (vitamin E, vitamin C and the flavonoid troxerutin) were tested for their ability to antagonize the vascular permeabilization induced by arachidonic acid (30 μg/kg to 3 mg/kg). All compounds were given preventively (100 mg/kg bolus i.v., $n=3$–4). Fig. 4 shows that all three antioxidants strongly reduced (or even abolished) the permeabilizing response to arachidonic acid. At each arachidonate dose, all compounds induced statistically significant actions ($P<0.05$, for the sake of clarity, statistical significance is not represented). The order of

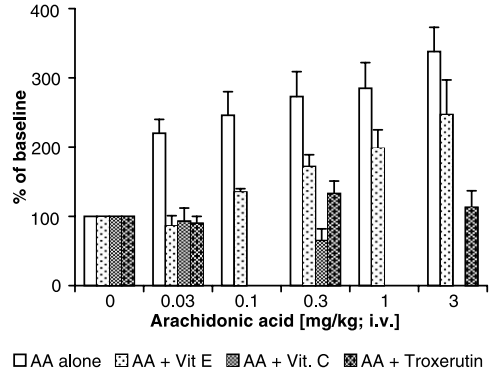

Fig. 4. Prevention by antioxidants (vitamin E, vitamin C and the flavonoid troxerutin) of vascular permeabilization induced by arachidonic acid (AA) in the rat peritoneal cavity. Compounds were given preventively (100 mg/kg i.v. bolus, $n=3$–4). At each arachidonic acid dose, all compounds induced statistically significant antagonistic actions ($P<0.05$; for the sake of simplicity, statistical significance was not represented). Vitamin C and troxerutin were the most effective, abolishing the permeabilizing responses at all arachidonic acid doses, whereas vitamin E was 40–80% effective after arachidonic acid ≥0.1 mg/kg.

potency was the following: vitamin C>troxerutin>vitamin E. Neither vitamin C or vitamin E nor troxerutin modified baseline k values (0.0045 ± 0.0006, 0.0023 ± 0.0004 and 0.0027 ± 0.0004 h^{-1}, respectively).

4. Discussion

Intravenous administration of arachidonic acid strongly enhanced peritoneal vascular permeability in the rat, an effect blocked by pretreatment with antioxidant compounds (troxerutin and vitamins C and E). Neither thromboxane A_2 nor prostacyclin was responsible for the permeabilizing action of arachidonic acid. These results are in line with the observation of Easton and Fraser (1998), that arachidonic acid increases cerebral microvascular permeability in rat pial venular capillaries by the formation of free radicals and subsequent lipid peroxidation.

In previous studies (see for instance Unterberg et al., 1987; Easton and Fraser, 1998), micromolar concentrations of circulating arachidonate were needed to increase vascular permeability (30–300 μM, depending on the molecular weight of the tracer used). This can be explained by the fact that serum albumin possesses multiple (≥ 20) high-affinity sites for arachidonic acid, with K_m in the micromolar range, a feature explaining most of the anti-permeabilizing effects of albumin in vivo (Beck et al., 1998). Here, Evans blue (961 Da) was used as permeability marker, a molecule bigger than fluorescein (332 Da, the marker used by Unterberg et al., 1987) or Lucifer yellow (476 Da; used by Easton and Fraser, 1998), but smaller than the commonly used albumin or dextran derivatives. Although it is difficult to be precise about the actual amounts of arachidonic acid reaching receptor targets under our experimental conditions, we can expect that intravenous bolus doses of 0.03–3 mg/kg would yield plasma arachidonate concentrations in the micromolar range (3–300 μM assuming 10 ml plasma volume, for a 320-g rat).

Vascular permeabilization induced by arachidonic acid was mostly inhibited by aspirin (70%), suggesting the participation of cyclooxygenase. However, neither U46619 nor prostacyclin was able to reproduce the permeabilizing action of arachidonic acid. Moreover, data from the literature further suggested that the vascular permeabilization induced by arachidonic acid is not due to a cyclooxygenase metabolite. Thus, Bertolino et al. (1995) showed that U-46619 increased the vascular leakage of albumin in rat lung, but not in rat mesentery. Moreover, prostacyclin and other vasodilator prostaglandins, such as prostaglandin E_2, activate adenylate cyclase, an effect known to reduce capillary permeability and to dampen the permeabilizing effects of inflammatory agonists such as bradykinin or platelet-aggregating factor (Farmer et al., 2001; Irie et al., 2001).

It is well established that cyclooxygenase activity contributes to free radical generation and lipid peroxidation (see for example Chemtob et al., 1993; Roshchupkin and Murina, 1998). Thus, the reduction of arachidonate-dependent vascular permeability induced by aspirin can be simply explained by previous results (Shi et al., 1995; Easton and Fraser, 1998) showing that arachidonic acid increases microvascular permeability by the formation of free radicals and subsequent lipid peroxidation. Therefore, at least part of the free radical formation induced by arachidonic acid in our rat model (about 70%) could be due to cyclooxygenase activity. In this respect, it is important to note that arachidonic acid can induce free radical generation independently of cyclooxygenase. Thus, it has been shown that arachidonic acid can directly "activate" NADPH oxidase ("priming"), one of the key enzymes responsible for leukocyte superoxide anion production (Dana et al., 1998; Daniels et al., 1998). In addition, endothelial adhesion of activated leukocytes can produce reactive oxygen species, which are thought to participate in the capillary permeability increase.

In addition to cyclooxygenase, two other enzymatic pathways metabolize arachidonic acid, the lipoxygenase and the cytochrome P450 pathways. Aspirin may enhance arachidonic acid metabolism by these competitive pathways (Seeger et al., 1987; Samuelsson, 1991; Rao et al., 1993; Ivey et al., 1998), particularly via a displacement toward leukotrienes that exert permeabilizing effects. Such an effect could explain the incomplete inhibition by aspirin in our model. However, the permeabilizing effects of leukotrienes in response to inflammatory agents usually develop late and are leukocyte dependent (Stenson et al., 1986; Rao et al., 1993; Lloret and Moreno, 1995; Wang et al., 1999). Moreover, it must be noted that two of these studies (Rao et al., 1993; Lloret and Moreno, 1995) concluded that prostaglandins had a role in the early response (mouse skin models).

The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) inhibited by 70% and 100% the permeabilizing response to arachidonic acid at 10 and 100 mg/kg, respectively. Although NDGA inhibits cyclooxygenase at high concentrations (Hope et al., 1983; Salari et al., 1984), such an effect is unlikely to explain our results since Chacur et al. (2001) found NDGA to have selective lipoxygenase inhibitory actions in rats at 30 mg/kg, i.v. (see however, De Matos et al., 1997; De Lima et al., 1992). Interestingly, NDGA is not only a lipooxygenase inhibitor, but also a very efficient free radical scavenger (Burba and Becking, 1969; Robison et al., 1990). This latter property can explain the inhibitory action found in our in vivo permeabilization model.

Finally, other candidates are isoprostanes (in vivo non-enzymatic products of free radical catalyzed peroxidation of arachidonic acid, see Morrow et al., 1994), which can directly modify the physical properties of membranes (membrane fluidity and/or cellular Ca^{2+} uptake). However, isoprostanes seem unlikely candidates in our model, since they do not increase capillary permeability, at least in pulmonary vessels in response to ischemia–reperfusion (Becker et al., 1998), and because direct membrane effects of arachidonic acid usually require concentrations as high as

50–100 μM, and in our case most circulating arachidonate would be linked to albumin (see above).

In agreement with the above arguments, vitamin C and troxerutin completely abolished arachidonate-induced vascular permeability and vitamin E inhibited it by 40–100% (depending on the dose of arachidonic acid used). These results further revealed the role of reactive oxygen species, rather than arachidonate metabolites.

Arachidonic acid is a mediator of capillary permeabilization in response to several agents such as bradykinin, thrombin, platelet-aggregating factor, venoms and also ischemia–reperfusion (Balsa et al., 1997; Cirino, 1998; Lugrin et al., 1996; Wang et al., 1999; De Araujo et al., 2000). Moreover, vascular permeabilization is an essential component of the inflammatory reaction, leading to tissue edema and cell infiltration. Actually, most inflammatory mediators increase vascular permeability, and pathophysiological events able to trigger the inflammatory cascade, e.g. sepsis, reperfusion injury or coagulation, are associated with an increased capillary permeability. The determinant role of arachidonic acid metabolites in inflammation is further highlighted by the observation that their biosynthesis is inhibited by most antiinflammatory agents.

As a consequence of the above properties, arachidonic acid per se is very often used as a pro-inflammatory mediator in experiments, either endogenously released (e.g. in response to cellular Ca^{2+} increase) or exogenously given (Woo et al., 2000; Xing et al., 1997). Therefore, the present rat peritoneal model can be used to evaluate the in vivo anti-inflammatory potential of antioxidant drugs. The technique is simple, precise and very reproducible. It can be also used to test other permeabilizing agonists (5-HT for instance) or antagonists.

In conclusion, intravenous administration of arachidonate strongly enhanced peritoneal vascular permeability in the rat, apparently via free radical generation. The permeabilizing action of arachidonate was blocked by pretreatment with antioxidant compounds (troxerutin and vitamins C and E). This rat peritoneal model can be used to evaluate the in vivo antinflammatory potential of antioxidant drugs.

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