ELSEVIER

European Journal of Pharmacology 358 (1998) 213–220

ejp

Angioprotective action of calcium dobesilate against reactive oxygen species-induced capillary permeability in the rat

John Brunet [a], Jean-Claude Farine [b], Ricardo P. Garay [a], Patrick Hannaert [a,*]

[a] *INSERM U400, Faculté de Médecine, 8 rue du Général Sarrail, 94010 Créteil Cédex, France*
[b] *O.M. Laboratories, Meyrin 2, Geneva, Switzerland*

Received 2 July 1998; revised 11 August 1998; accepted 14 August 1998

Abstract

Calcium dobesilate possesses antioxidant properties in vitro, but the in vivo significance and putative angioprotective role of these properties are undefined. Here, calcium dobesilate was tested in a newly developed in vivo model of microvascular permeabilization induced by reactive oxygen species in the rat peritoneal cavity. In this model, microvascular permeabilization is equated to the rate of Evans blue extravasation toward the peritoneal cavity. Basal Evans blue extravasation (rate constant values $k_e = 0.0176 \pm 0.0015$ h^{-1}) was markedly and significantly increased by reactive oxygen species generated in situ, with: (i) phenazine methosulfate/NADH ($\Delta k_e^{(\text{phenazine methosulfate})} = 0.0419 \pm 0.0043$ h^{-1}) and (ii) xanthine/xanthine oxidase ($\Delta k_e^{(XO)} = 0.0383 \pm 0.0010$x h^{-1}). These actions of reactive oxygen species were abolished by locally injected superoxide dismutase (i.p., 300 units/kg). Intraperitoneally given calcium dobesilate (100 mg/kg) inhibited 75–100% of reactive oxygen species-induced Evans blue extravasation. By the intravenous route, calcium dobesilate i.v. (1–50 mg/kg) dose dependently inhibited phenazine methosulfate-induced Evans blue extravasation with an ID_{50} of 2–5 mg/kg (full inhibition was reached at 20–50 mg/kg). After single oral administration, calcium dobesilate (5–500 mg/kg) dose dependently inhibited phenazine methosulfate-dependent Evans blue extravasation with an ID_{50} of 50–100 mg/kg (81% inhibition at 500 mg/kg, $P < 0.003$). After 7 days of oral calcium dobesilate (50 mg/kg once/day) phenazine methosulfate-induced Evans blue peritoneal extravasation was significantly reduced by half. These effects of calcium dobesilate were similar to those observed with a comparative antioxidant molecule, rutin. In conclusion, rat peritoneal microvascular permeability was strongly increased by reactive oxygen species, an effect that was significantly reduced by intraperitoneal, intravenous and oral calcium dobesilate. These results support the hypothesis that the antioxidant properties of calcium dobesilate could play a role in its angioprotective properties in vivo.

Keywords: Calcium dobesilate; Antioxidant; Free radical; Vascular permeability

1. Introduction

Calcium dobesilate (see structure in Fig. 1) exhibits angioprotective properties in diabetic retinopathy (Leite et al., 1990; Vojnikovic, 1991), peripheral microvascular disease (Hachen et al., 1982) and chronic venous insufficiency (Widmer et al., 1990). Among other effects, calcium dobesilate has been shown to enhance nitric oxide synthase-activity in vascular endothelial cells (Ruiz et al., 1997), to reduce capillary permeability and fragility (Beyer et al., 1980; van Bijsterveld and Janssen, 1981), to decrease blood hyperviscosity (Vojnikovic, 1991) in patients with diabetic retinopathy, and to inhibit platelet aggregation (Michal and Gotti, 1988) and microcirculatory thrombus formation (Michal and Giessinger, 1985). Therefore, calcium dobesilate could exert its protective actions through many potential sites (Marmo, 1987). For instance, a reduction of collagen biosynthesis and hydroxylysine incorporation has been observed in retina and glomerula basement membranes of diabetic rats treated with calcium dobesilate (Hasslacher et al., 1981). However, the actual mechanisms of action of calcium dobesilate remain undefined.

Reactive oxygen species have been shown to increase microvascular permeability (Del Maestro et al., 1981a,b) and they seem to play a role in the capillary dysfunctions of diabetic microangiopathy and other microvascular pathologies (for review see Baynes, 1991; Doly and Droy-Lefaix, 1992; Halliwell, 1993). Previous studies have

* Corresponding author. Tel.: +33-1-4981-3633; Fax: +33-1-4981-9426; E-mail: hannaert@im3.inserm.fr

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00604-9

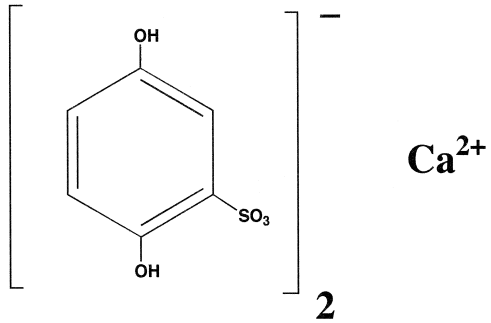

Fig. 1. Chemical structure of calcium dobesilate.

shown that calcium dobesilate possesses antioxidant properties in in vitro assays (Brunet et al., 1994, 1998; Ruiz et al., 1997). Thus, calcium dobesilate was effective in scavenging superoxide anion and hydroxyl radicals in vitro. These antioxidant actions were observed at therapeutically relevant concentrations, suggesting that they can be involved in the vascular protective actions of dobesilate in vivo.

To investigate the above hypothesis, we developed an in vivo model for quantifying microvascular permeability and for determining the deleterious effects of reactive oxygen species. This model is based on the rich microvasculature and convenient experimental accessibility of the peritoneal cavity: intravenously given Evans blue diffuses into the peritoneal cavity where its extravasation kinetics can be monitored. Reactive oxygen reactive species were generated in the peritoneal cavity with phenazine methosulfate or xanthine oxidase, a procedure which strongly increases Evans blue extravasation. Calcium dobesilate was tested in this in vivo model after intraperitoneal, intravenous or oral administration. Rutin was used as reference antioxidant compound.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 280–320 g, were purchased from CERJ (Le Genest St Isle, France). They were allowed to adapt to a humidity- and temperature-controlled room for at least 2 days before the protocol was started. They were fed on a standard diet and tap water ad libitum. Investigations were performed according to the European Community guidelines for ethical animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985).

2.2. Administration of compounds

Compounds were administered either intraperitoneally (immediately before the experiment), intravenously (50 min before the experiment), or orally (one single dose 6 h, or one daily dose for 1 week, before the experiment); in each case, appropriate control groups were given the vehicle.

2.3. Surgical preparation of animals

Rats were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and maintained at an external temperature of 28–32°C. A polyethylene catheter was inserted into the right jugular vein, after which a 10-min stabilization period was allowed.

2.4. Determination of peritoneal microvascular permeability

Basal and reactive oxygen species-dependent vascular permeability was measured by monitoring Evans blue extravasation toward the peritoneal cavity, according to the following procedure. Five to 7 min before the start of the experiment, Evans blue was injected through the jugular catheter (5 μg/g weight in saline). Three to 5 min later, a venous blood sample was collected from the jugular and microcentrifuged (12 000 rpm, 30 s) for spectrocolorimetric measurement of initial plasma concentrations of Evans blue (10–15 absorbance units at 620 nm). Then, 28 ml saline (NaCl 0.9%, pre-equilibrated at 37°C and containing 1 mM NADH) were gently injected into the peritoneal cavity via a needle inserted through the right side of the abdomen (the needle remained in place throughout the whole experiment).

From zero time and every 5 min thereafter (until 45 min), 0.7 ml peritoneal samples were collected via the needle. At time 16 min, reactive oxygen species generation was initiated by injecting phenazine methosulfate (10 μM final concentration) into the peritoneal cavity. Blood samples (0.5 ml) were collected at times −1, 14, 16 and 46 min. Peritoneal and plasma samples were microcentrifuged, and supernatants were diluted 4-fold and 20-fold in saline, respectively. Evans blue content was quantified by reading the optical density at 620 nm. In control experiments with xanthine/xanthine oxidase, the 28 ml saline contained 200 μM xanthine, in which case xanthine oxidase (0.2 units/ml final concentration) was injected at 16 min. In experiments with H_2O_2/Fe^{2+}, the components of a hydroxyl radical generating system, H_2O_2 (100 μM final concentration) and $FeCl_2$ (10 μM final concentration) were injected i.p. together at 16 min (1 ml volume, prepared and mixed when needed). In experiments with sodium hypochlorite, NaOCl (130 μM final concentration) was injected at 16 min (1 ml volume). Preliminary experiments showed that (i) baseline extravasation rate constants were similar with saline + NADH, saline + xanthine or saline alone and (ii) the extravasation slope was not significantly modified by addition of vehicle (saline or dimethylsulfoxide 0.4% v/v final).

Extravasation rate constants (h^{-1}) were calculated as follows. For each 5-min period, the slope of the increase of the peritoneal level of Evans blue with time was divided by the corresponding plasma level (a value which was derived from exponential interpolation between blood sampling points, i.e., −1 and 14 min for baseline, and 16 and 45 min for reactive oxygen species generation period) and the average rate constant for baseline- and reactive oxygen species-periods was calculated: reactive oxygen species-dependent Evans blue extravasation rate constants, $\Delta k_e^{(\text{phenazine methosulfate})}$ in most cases, were calculated by substracting the baseline rate constant from the rate constant calculated in the presence of phenazime methosulfate. Preliminary experiments have shown that: (i) baseline extravasation of Evans blue towards the peritoneal compartment is linear for at least 60 min ($k_e^{(\text{baseline})}$ in the range 0.010–0.030 h^{-1}]); (ii) the peritoneal injection of phenazine methosulfate (in the presence of NADH) or xanthine-oxidase (in the presence of xanthine) induces a reproducible and significant increase of Evans blue extravasation (50–200%).

Scavenger agents, i.e., Mn-SOD (Mn-superoxide dismutase) for the superoxide anion-generating phenazine methosulfate/NADH and xanthine/xanthine oxidase systems, dimethylthiourea for the OH° generating H_2O_2/Fe^{2+} couple, and L-methionine for hypochlorite, were added in the initial 28 ml saline to be injected i.p., a procedure which did not modify baseline Evans blue extravasation.

2.4.1. Cellular composition of peritoneal samples

Intraperitoneal administration of oxygen radical generating systems can induce recruitment of peritoneal granulocytes and macrophages, which can influence peritoneal capillary permeability (see for instance Smith et al., 1991; Siflinger-Birnboim et al., 1993). Therefore, control experiments were performed in order to evaluate the cellular composition of the peritoneal samples as a function of time. A 150-μl aliquot of peritoneal samples was centrifuged (cytospin3, shandon) to project cells onto a glass coverslip. After methanol fixation, the cells were colored according to the Pappenheim method (Giemsa–May–Grünwald reagents: Diff-quick kit, Dade, Maurepas, France). Cells were identified by two experimenters, according to morphological and color criteria. During the baseline period, the total number of cells was $4 \pm 1\ 10^5$ cells/ml ($n = 4$) and the cell composition was $35 \pm 4\%$ macrophages, 41% granulocytes ($16 \pm 3\%$ neutrophils, $23 \pm 3\%$ eosinophils) and $5 \pm 3\%$ lymphoid cells. After phenazine methosulfate injection, the only significant change was an increase in eosinophil content ($37 \pm 7\%$, $P < 0.05$ with respect to baseline value).

2.5. Compounds

Calcium dobesilate (calcium dihydroxy-2-5 benzenesulfonate) was provided by O.M. Laboratories (Meyrin 2, Geneva, Switzerland). All other chemicals were from Merck or Sigma (distributed through Coger, Paris, France).

2.6. Statistical analysis

Results are given as means ± S.E.M. (n indicates the number of experiments). Statistical differences between mean values were determined by using non-paired Student's t-test. Statistical significance was accepted for $P < 0.05$.

3. Results

3.1. Evans blue extravasation induced by reactive oxygen species in the rat peritoneal cavity

Evans blue was intravenously injected into rats and its extravasation into the peritoneal cavity was monitored by the increase in absorbance (620 nm) of peritoneal fluid samples. Control experiments showed that: (i) under basal conditions (without reactive oxygen species- generating system), peritoneal absorbance increased slowly and linearly for at least 60 min, (ii) the Evans blue extravasation rate constant (k_e) was $0.0176 \pm 0.0015\ h^{-1}$ ($n = 74$).

Fig. 2 shows the effect of the reactive oxygen species generating system (phenazine methosulfate + NADH) on peritoneal Evans blue extravasation. It can be seen that reactive oxygen species generation by intraperitoneally injected phenazine methosulfate strongly increased Evans blue extravasation: phenazine methosulfate-dependent in-

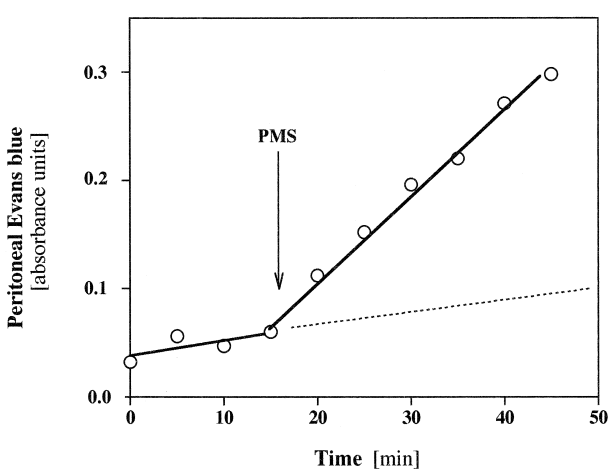

Fig. 2. Extravasation of intravenously given Evans blue toward the peritoneal cavity as a function of time. Control experiments showed that baseline extravasation was linear for more than 60 min (symbolized by dotted line); baseline rate constant was $k_e = 0.0176 \pm 0.0015\ h^{-1}$ ($n = 74$). Reactive oxygen species generated in situ by intraperitoneal injection of phenazine methosulfate (phenazine methosulfate, PMS, in the presence of NADH) or xanthine oxidase (XO, in the presence of xanthine) markedly and significantly increased the rate constant for Evans blue extravasation: $\Delta k_e^{(PMS)} = 0.0419 \pm 0.0043\ h^{-1}$ ($P < 0.0001$; $n = 65$); $\Delta k_e^{(XO)} = 0.0383 \pm 0.0010x\ h^{-1}$ ($P < 0.001$; $n = 9$).

Table 1
Reactive oxygen species-induced Evans blue peritoneal extravasation

Condition	Increase in rate constant (Δk_e in h^{-1}) for each free-radical-generating system	
	ROS-generating system	ROS + Scavenger
H_2O_2/Fe^{2+} (1 mM/10 μM)	0.0346 ± 0.0041 ($n = 10$)	0.0005 ± 0.0047 ($n = 5$)[a]
NaClO (130 μM)	0.0236 ± 0.0025 ($n = 6$)	-0.0028 ± 0.0028 ($n = 3$)[b]
PMS/NADH (1 mM/10 μM)	0.0419 ± 0.0025 ($n = 65$)	0.0024 ± 0.0036 ($n = 4$)[c]
X/XO (200 μM/0.2 u/ml)	0.0383 ± 0.0010 ($n = 9$)	0.0048 ± 0.0026 ($n = 3$)[c]

Values are given as means ± S.E.M. with the number of experiments in parentheses. Statistical significance was tested using Student's *t*-test. ROS, reactive oxygen species; X/XO, xanthine/xanthine oxidase.
[a] Scavenger = dimethylthiourea (10 mM), $P < 0.0001$.
[b] Scavenger = L-methionine (2 mM), $P < 0.001$.
[c] Scavenger = Mn-SOD (3.5 u/ml), $P < 0.001$.

crease of k_e ($\Delta k_e^{(PMS)}$) was 0.0419 ± 0.0043 h^{-1} ($n = 65$). The effect of phenazine methosulfate was fully blocked ($n = 4$, $P < 0.01$) by superoxide dismutase (Mn-SOD) 3.5 U/ml saline, i.e., 300 units/kg, i.p., with no effect on baseline Evans blue extravasation. Similar results were obtained with xanthine/xanthine oxidase, another system primarily generating superoxide anion (see Table 1).

In order to evaluate the reactive oxygen species specificity of the permeability effects, two other reactive oxygen species-generating systems were tested: (i) $H_2O_2 + Fe^{2+}$, a hydroxyl radical-generating system and (ii) hypochlorite, another reactive oxygen species (see Halliwell, 1993, 1995). Table 1 summarizes the results in terms of the increase in peritoneal Evans blue extravasation and inhibition of this increase by the appropriate scavengers (data from phenazine methosulfate/NADH experiments are included for comparison). It can be seen that all reactive oxygen species-generating systems induced comparable peritoneal Evans blue extravasation (although hypochlorite was least effective), and that the appropriate scavenger agents were able to inhibit these effects.

3.2. Effects of calcium dobesilate by the intraperitoneal route

Calcium dobesilate (100 mg/kg) was tested by the intraperitoneal route for its protective effects against the peritoneal Evans blue extravasation induced by phenazine methosulfate/NADH or xanthine/xanthine oxidase. Table 2 shows that: (i) calcium dobesilate 100 mg/kg reduced by 73% phenazine methosulfate-dependent Evans blue extravasation ($\Delta k_e^{(PMS)} = 0.0682 \pm 0.0129$ h^{-1}, $n = 13$, and 0.0185 ± 0.0099 h^{-1}, $n = 11$, for control and dobesilate groups respectively, $P < 0.01$), and (ii) the same dose of rutin (100 mg/kg i.p.) reduced phenazine methosulfate-dependent Evans blue extravasation to the same extent (75% inhibition, see Table 2). Similar results were obtained for xanthine/xanthine oxidase-induced Evans blue peritoneal extravasation: calcium dobesilate (100 mg/kg i.p.) abolished xanthine/xanthine oxidase-dependent Evans blue extravasation ($\Delta k_e^{(XO)} = 0.0610 \pm 0.0090$ h^{-1}, $n = 9$, and -0.0237 ± 0.0219 h^{-1} $n = 3$, for control and dobesilate groups respectively, $P < 0.01$). It is important to mention that: (i) neither calcium dobesilate nor rutin was able to significantly modify basal values of Evans blue extravasation and (ii) calcium ions, in similar amounts as provided by calcium dobesilate 100 mg/kg, were without a significant effect on phenazine methosulfate-dependent Evans blue extravasation (data not shown).

3.3. Effect of calcium dobesilate by the intravenous route

Rats were intravenously given a single bolus of calcium dobesilate, 50 min before Evans blue extravasation was determined. Fig. 3 shows the effect of increasing doses of calcium dobesilate on phenazine methosulfate-dependent peritoneal Evans blue extravasation. It can be seen that (i) calcium dobesilate dose dependently inhibited the effect of phenazine methosulfate, with an $ID_{50\%}$ of 2–5 mg/kg ($n = 6$), and (ii) full inhibition was attained between 20 and 50 mg/kg. Under the same conditions, 50 mg/kg rutin i.v. inhibited only 46% of the effect of phenazine methosulfate ($n = 5$, $P < 0.05$), whereas 20 mg/kg was marginally active (28% inhibition, n.s.).

To investigate the possible contribution of calcium to the effect of calcium dobesilate, we tested high doses of calcium chloride, i.e., 20 and 50 mg/kg, corresponding to

Table 2
Effects of intraperitoneally given calcium dobesilate and rutin on the rate constant for PMS-dependent peritoneal Evans blue extravasation

Condition	Δk_e(PMS) (h^{-1})	Statistical significance
Vehicle	0.0682 ± 0.0129 (13)	–
Calcium dobesilate 100 mg/kg	0.0185 ± 0.0099 (11)	$P < 0.01$
Rutin 100 mg/kg	0.0170 ± 0.0182 (5)	$P < 0.05$

Values are given as mean ± S.E.M. (the number of experiments is indicated in brackets). PMS, phenazine methosulfate. Δk_e(PMS), the rate constant for the PMS-dependent increase of peritoneal Evans blue extravasation. Statistical significance was tested by using non-paired Student's *t*-test.

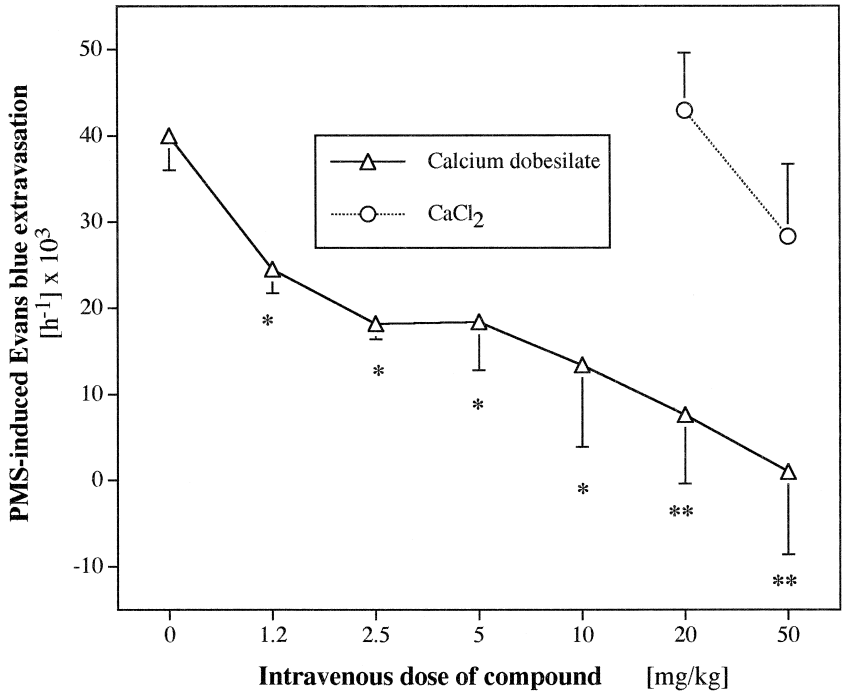

Fig. 3. Intravenously given calcium dobesilate dose dependently reduced the rate constant for phenazine methosulfate-induced peritoneal Evans blue extravasation (triangles). The ID_{50} for this effect was 2–5 mg/kg; $CaCl_2$ had no significant effect (circles). Under the same conditions, the ID_{50} for rutin was 50 mg/kg (see text).

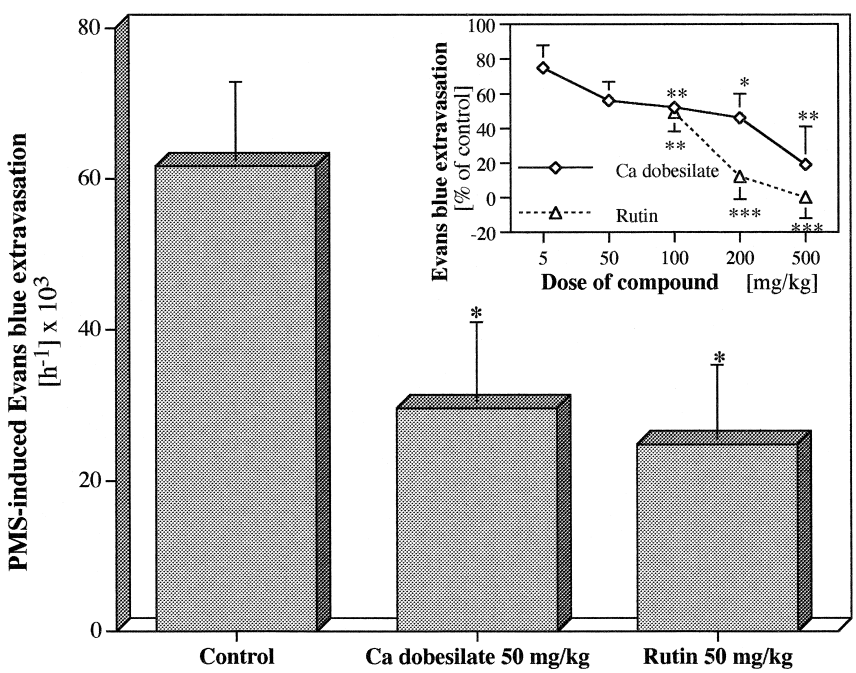

Fig. 4. Orally given calcium dobesilate reduced the rate constant for phenazine methosulfate-dependent peritoneal Evans blue extravasation. A group of rats treated for 7 days with calcium dobesilate 50 mg/kg p.o. once a day ($n = 9$) was compared to a control group receiving vehicle (5% arabic gum, $n = 10$) and to a group of rats receiving rutin 50 mg/kg p.o. once a day ($n = 9$). Both calcium dobesilate and rutin significantly reduced Evans blue extravasation. Baseline Evans blue extravasation was similar in all three groups. $*$ indicates $P < 0.05$ (non-paired Student's t-test). Inset: Acute effect of orally given compounds. phenazine methosulfate-dependent peritoneal Evans blue extravasation was measured 6 h after a single oral administration of compounds or vehicle. $*$ $P < 0.05$; $**$ $P < 0.01$; $***$ $P < 0.001$ (non-paired Student's t-test).

about 50 and 125 mg/kg calcium dobesilate, respectively. Fig. 3 shows that these amounts of calcium had no significant effect on phenazine methosulfate-dependent Evans blue extravasation. Finally, we observed that Mn-SOD (i.v., 90 units/kg) inhibited by 95% phenazine methosulfate-induced Evans blue extravasation ($n = 5$, $P < 0.01$).

3.4. Effect of calcium dobesilate by the oral route

A single-dose protocol was designed in order to search for an optimal per os dose. Thus, different groups of animals were treated with increasing single doses of calcium dobesilate p.o. (from 5 to 500 mg/kg) and the protective efficacy against peritoneal phenazine methosulfate-induced oxidative challenge was tested 6 h later. Rutin was used for comparison. The inset of Fig. 4 shows that calcium dobesilate P.o. was able to dose dependently and significantly inhibit phenazine methosulfate/NADH-dependent Evans blue extravasation into the peritoneal cavity. At the highest dose tested (500 mg/kg), the inhibition was nearly complete ($81 \pm 22\%$, $n = 5$, $P < 0.003$). The ID_{50} of this inhibitory effect was about 100 mg/kg (44% inhibition was observed at 50 mg/kg, $n = 5$, $P < 0.02$). Rutin (100–500 mg/kg) was tested according to the same protocol: 100 mg/kg rutin exhibited a 50% inhibitory effect ($P < 0.01$) and total inhibition was observed at 500 mg/kg ($P < 0.001$).

In the final set of experiments, drugs were given for 1 week. Thus, rats were orally given 50 mg/kg calcium dobesilate or rutin (vs. a control group receiving the vehicle) once a day for 7 days. On the 7th day, 3 h after the last oral dose, rats were submitted to peritoneal oxidative challenge and Evans blue extravasation was monitored. Fig. 4 (main panel) shows that calcium dobesilate and rutin p.o. were both able to significantly inhibit phenazine methosulfate/NADH-induced Evans blue extravasation into the peritoneal cavity. Calcium dobesilate reduced Evans blue extravasation by $55 \pm 17\%$ ($n = 9$, $P < 0.028$ vs. control), whereas rutin inhibited it by $63 \pm 16\%$ ($n = 9$, $P < 0.011$ vs. control).

4. Discussion

In order to assess if the in vitro antioxidant properties of calcium dobesilate can translate into a vascular protective action in vivo, we developed a new rat model for measuring capillary permeability and thus allowing a precise and reproducible measurement of capillary permeabilization induced by reactive oxygen species. This quantitative model is based on the extravasation of Evans blue toward the peritoneal cavity, which is monitored as a function of time. Reactive oxygen species generated inside the peritoneal cavity by the exogenous couples phenazine methosulfate/NADH or xanthine/xanthine oxidase, strongly increased (2–3-fold) peritoneal Evans blue extravasation. This result confirms previous observations by Del Maestro et al. (1981a,b) that xanthine oxidase-generated reactive oxygen species increase vascular permeability to macromolecules in the hamster cheek pouch model, and further supports previous reports pointing at an active role of reactive oxygen species in alterations of the endothelial barrier in pathophysiological situations such as inflammation, edema and vascular diseases (Nelson et al., 1992; Tate et al., 1982; Suttorp et al., 1996).

Although the cellular and molecular mechanisms involved in reactive oxygen species-induced vascular permeabilization are still under debate, it is likely that reactive oxygen species act by altering structural and functional elements of the endothelial barrier, such as endothelial cells (e.g., by increasing cytosolic calcium, or by peroxidative damage of the cell membrane) and/or extracellular matrix macromolecules. Furthermore, reactive oxygen species are intermediates in the production of vasoactive arachidonic acid metabolites (e.g., prostaglandines) able to promote the extravasation of plasma macromolecules and edema formation (Nagakura et al., 1990).

Two arguments suggest that the superoxide anion ($O_2^{\circ -}$) is responsible for the above increase in peritoneal capillary permeability. First, phenazine methosulfate/NADH and xanthine/xanthine oxidase systems primarily produce superoxide anions. Second, the effects of phenazine methosulfate/NADH and xanthine/xanthine oxidase were abolished by superoxide dismutase i.p. (Mn-SOD, enzymatic scavenger of $O_2^{\circ -}$). However, it is well known that the $O_2^{\circ -}$ species is involved in several intricate reactions that ultimately result in the generation of other reactive oxygen species, such as hydrogen peroxide (H_2O_2) or the highly reactive hydroxyl radical (OH°; Halliwell, 1995). Indeed, we observed that H_2O_2/Fe^{2+}, an OH°-generating system, was as efficient as phenazine methosulfate/NADH and xanthine/xanthine oxidase in increasing Evans blue extravasation (see Table 1). Furthermore, Del Maestro et al. (1981a,b) concluded from their elegant studies that xanthine oxidase-induced vascular permeability was mediated by the secondarily generated hydroxyl radical (from $O_2^{\circ -}$ and H_2O_2). Therefore, we cannot exclude that the phenazine methosulfate/NADH and/or xanthine/xanthine oxidase-dependent increase of capillary permeability reported here was, at least partly, caused by $O_2^{\circ -}$-derived reactive oxygen species acting at the target site(s). In this regard, the observation that Mn-SOD was as efficient when given i.v. or i.p. ($\approx$ 2–3 units/ml, assuming an i.v. distribution volume of 15 ml/100 g body weight), suggests that primarily and/or secondarily generated reactive oxygen species diffuse toward specific targets located within the vascular wall (endothelial cells, junctions and/or extracellular matrix components). Finally, primarily generated $O_2^{\circ -}$ might have also reacted with the endothelial-derived relaxing factor nitric oxide (NO°) to irreversibly produce the powerful and toxic oxidant peroxynitrite

($ONOO^-$; Beckman and Koppenol, 1996). It has been suggested that continuously released NO° may be an important modulator of endothelial permeability (Oliver, 1992). For instance, removal of endogenous NO° increases whereas application of exogenous NO° reduces endothelial permeability (Suttorp et al., 1996). Taken together, the above arguments illustrate the difficulties in identifying the molecular mechanisms involved in reactive oxygen species- (and NO°)-dependent changes in microvascular permeability. Since our purpose was to evaluate the potential in vivo angioprotective effects of calcium dobesilate, no further attempt was made to investigate these complex biochemical and cellular mechanisms.

Calcium dobesilate was compared with the flavonoid rutin. Rutin is a well-established antioxidant that is active in both in vitro (Maridonneau-Parini et al., 1986; Chen et al., 1990; Grinberg et al., 1994; Schmitt et al., 1995) and ex-vivo models (Halliwell, 1993; Afanas'ev et al., 1995). Moreover, flavonoids, particularly rutosides, also have vascular protective properties (Casley-Smith et al., 1993; Rehn et al., 1993; Yaqoob et al., 1993; Renton et al., 1994) and the mechanism of action of these compounds seems to reside in their ability to scavenge reactive oxygen species. Indeed, dietary supplementation with rutosides (or other antioxidants) is found: (i) to antagonize the endothelial and tubular deleterious effects of oxidative stress on endothelial and tubular function in human insulin-dependent diabetes mellitus (Yaqoob et al., 1993) and (ii) to reduce capillary filtration in venous hypertension (Renton et al., 1994), in the standing motionless model (Rehn et al., 1993), and in the acute hindlimb lymphedema of the rat (Casley-Smith et al., 1993).

In our rat peritoneal model, rutin was found to exert a protective action when administered intraperitoneally, intravenously and orally. Calcium dobesilate, tested under the same conditions, also proved remarkably efficient in protecting the peritoneal vasculature from the permeabilizing effects of reactive oxygen species (without exhibiting a significant effect on baseline Evans blue permeability). Thus, intraperitoneal calcium dobesilate (100 mg/kg) inhibited reactive oxygen species-dependent peritoneal Evans blue extravasation by more than 70%, an effect independent of calcium ions. Because reactive oxygen species were generated inside the peritoneal cavity, it is not surprising that i.p. calcium dobesilate was active at 100 mg/kg. Indeed, the final concentration of calcium dobesilate in the peritoneal cavity was estimated to be 2–3 mM, a range in which it scavenges superoxide anion in vitro (Brunet et al., 1994; cf. also Ruiz et al., 1997). In terms of activity, rutin was comparable to calcium dobesilate, leading to a 75% inhibition at 100 mg/kg (i.e., $\approx$ 2 mM final concentration). Conversely, when administered intravenously, calcium dobesilate was about 10 times more efficient than rutin (ID_{50} 1–5 mg/kg vs. $\approx$ 50 mg/kg). This can be explained by the observation that only 20–25% of plasma dobesilate is bound to plasma proteins (Benakis et al., 1974), and thus is more likely to reach reactive oxygen species site(s) of action than circulating rutin (preferentially bound to plasma proteins).

In the single-dose oral administration protocol, the protective efficacy of calcium dobesilate against phenazine methosulfate-induced oxidative challenge was tested after 6 h and compared to that of rutin. Under these conditions, the ID_{50} for calcium dobesilate was about 100 mg/kg, whereas it was close to 50 mg/kg in the repeated-dose protocol (similar to rutin). Thus, chronically given calcium dobesilate was only 2-fold more potent than it was when administered as a single dose.

With regard to the antioxidant mechanism of calcium dobesilate, it is important to recall that a previous in vitro study showed that calcium dobesilate potently scavenges superoxide anion (Brunet et al., 1994; Ruiz et al., 1997) and hydroxyl radicals (Brunet et al., 1998). Moreover, this last study suggested that calcium dobesilate acts predominantly in the extracellular compartment, as expected from its hydrophilic properties (Brunet et al., 1998). Therefore, in the present in vivo model of reactive oxygen species-induced capillary permeability, calcium dobesilate can be hypothesized to act by preferentially scavenging extracellular hydroxyl radicals and/or superoxide anions. This hypothesis requires further investigation, including an accurate analysis of reactive oxygen species-driven cellular and molecular events. Overall, since experimental and clinical studies have clearly shown that calcium dobesilate possesses vascular protective features, particularly in microvessels (Hasslacher et al., 1981; Hachen et al., 1982; Marmo, 1987; Leite et al., 1990; Widmer et al., 1990; Vojnikovic, 1991) and in pathologies where reactive oxygen species are involved (Baynes, 1991; Doly and Droy-Lefaix, 1992; Brenner et al., 1993; Halliwell, 1993), our results suggest that scavenging of pathophysiological reactive oxygen species in vivo is one molecular mechanism involved in the angioprotective effects of calcium dobesilate.

In conclusion, rat peritoneal microvascular permeability was strongly increased by reactive oxygen species, and this effect was significantly reduced by intraperitoneal, intravenous and oral calcium dobesilate. These results support the hypothesis that the antioxidant properties of calcium dobesilate could play a role in its angioprotective properties in vivo.

References

Afanas'ev, I.B., Ostrachovitch, E.A., Abramova, L.G., Kirkina, N.E., 1995. Different antioxidant activities of bioflavonoid rutin in normal and iron-overloading rats. Biochem. Pharmacol. 50 (1995), 627–635.

Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. Diabetes 40, 405–412.

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am. J. Physiol. 271, C1424–C1437.

Benakis, A., Glasson, B., Bouvier, C., Ritschard, J., Krahenbuhl, B., Jung, A., Hachen, H., 1974. Métabolisme and pharmacocinétique du dobésilate de calcium chez l'homme. Thérapie 29, 211–219.

Beyer, J., Meissner, H.J., Cordes, U., 1980. Calcium dobesilate reduces microvascular permeability to plasma proteins in diabetes mellitus. Med. Wochenschr. 105, 1604–1608.

Bijsterveld, J., Janssen, P.T., 1981. The effect of calcium dobesilate on albumin leakage of the conjunctival vessels. Curr. Eye Res. 1, 425–430.

Brenner, H., Burkart, V., Rothe, H., Kolb, H., 1993. Oxygen radicals production is increased in macrophages from diabetes-prone BB rats. Autoimmunity 15, 93.

Brunet, J., Garay, R., Hannaert, P., 1994. Calcium dobesilate has oxygen free radical scavenger properties. Can. J. Physiol. Pharmacol. 72 (S1), 596.

Brunet, J., Farine, J.C., Garay, R., Hannaert, P., 1998. In vitro antioxidant properties of calcium dobesilate. Fundam. Clin. Pharmacol. 12, 205–212.

Casley-Smith, J.R., Curri, S., Foldi, M., 1993. Topical treatment of acute hindlimb lymphedema of the rat using a troxerutin-phosphatidylcholine complex in liposomal-like microdispersion. Lymphology 26, 25–27.

Chen, Y.T., Zheng, R.L., Jia, Z.J., Ju, Y., 1990. Flavonoids as superoxide scavengers and antioxidants. Free Radic. Biol. Med. 9, 19–21.

Del Maestro, R.F., Bjork, J., Arfors, K.E., 1981a. Increase in microvascular permeability induced by enzymatically generated free radicals: I. In vivo study. Microvasc. Res. 22, 239–254.

Del Maestro, R.F., Bjork, J., Arfors, K.E., 1981b. Increase in microvascular permeability induced by enzymatically generated free radicals: II. Role of superoxide anion radical, hydrogen peroxide and hydroxyl radical. Microvasc. Res. 22, 255–270.

Doly, M., Droy-Lefaix, M.T., 1992. Lipid peroxidation in the pathology of the retina. Free radicals in the brain aging. In: Packer, L., Prilipko, L., Christen, Y. (Eds.), Neurological and Mental Disorders. Springer-Verlag, Berlin, pp. 123–139.

Grinberg, L.N., Rachmilewitz, E.A., Newmark, H., 1994. Protective effects of rutin against hemoglobin oxidation. Biochem. Pharmacol. 48, 643–649.

Hachen, H., Hachen, F.I.C.A., Lorenz, P., 1982. Etude clinique and pléthysmographique en double-aveugle du Dobésilate de Calcium chez des patientes atteintes de troubles microcirculatoires périphériques. Angiologie 33, 480–487.

Halliwell, B., 1993. The role of oxygen radicals in human disease with particular reference to the vascular system. Haemostasis 23, 118–126.

Halliwell, B., 1995. Antioxidant characterization. Methodology and mechanism. Biochem. Pharmacol. 49, 1341–1348.

Hasslacher, C., Kopischke, H., Bürklin, E., 1981. Hemmung der gesteigerten Synthese kapillärer Basalmembran diabetischer Ratten in vivo. Vasa 10, 148–150.

Leite, E., Mota, M., Faria de Abreu, J., Cunha-Vaz, J., 1990. Effect of calcium dobesilate on the blood-retinal barrier in early diabetic retinopathy. Int. Ophthalmol. 14, 81–88.

Maridonneau-Parini, I., Braquet, P., Garay, R.P., 1986. Heterogeneous effects of flavonoids on K loss and lipid peroxydation induced by oxygen-free radicals in human red cells. Pharm. Res. Commun. 18, 61–72.

Marmo, E., 1987. Calcium dobesilate in medical treatment: experimental and clinical aspects. Eur. Rev. Med. Pharmacol. Sci. 9, 1–60.

Michal, M., Giessinger, N., 1985. Effect of calcium dobesilate and its interaction with aspirin on thrombus formation in vivo. Thromb. Res. 40, 215–226.

Michal, M., Gotti, C., 1988. Effect of calcium dobesilate on platelet function. Thromb. Res. 51, 593–605.

Nagakura, T., Ebisawa, M., Reason, D.C., Iikura, Y., 1990. Chemical mediators in hypersensitivity reactions. Acta Paediatr. Jpn 32, 209–215.

Nelson, C., Wei, E., Povlishock, J., Kontos, H., Moskowitz, M., 1992. Oxygen radicals in cerebral ischemia. Am. J. Physiol. 263, H1356–H1362.

Oliver, J.A., 1992. Endothelium-derived relaxing factor contributes to the regulation of endothelial permeability. J. Cell. Physiol. 151, 506–511.

Rehn, D., Nocker, W., Diebschlag, W., Golden, G., 1993. Time course of the anti-oedematous effect of different dose regimens of *O*-(beta-hydroxyethyl) rutosides in healthy volunteers. Arzneimittelforschung 43, 335–338.

Renton, S., Leon, M., Belcaro, G., Nicolaides, A.N., 1994. The effect of hydroxyethylrutosides on capillary filtration in moderate venous hypertension: a double blind study. Int. Angiol. 13, 259–262.

Ruiz, E., Lorente, R., Tejerina, T., 1997. Effects of calcium dobesilate on the synthesis of endothelium-dependent relaxing factors in rabbit isolated aorta. Br. J. Pharmacol. 121, 711–716.

Schmitt, A., Salvayre, R., Delchambre, J., Nègre-Salvayre, A., 1995. Prevention by alpha-tocopherol and rutin of glutathione and ATP-depletion induced by oxidized LDL in cultured endothelial cells. Br. J. Pharmacol. 116, 1985–1990.

Siflinger-Birnboim, A., Bode, D.C., Malik, A.B., 1993. Adenosine 3', 5'-cyclic monophosphate attenuates neutrophil-mediated increase in endothelial permeability. Am. J. Physiol. 264, H370–H375.

Smith, J.K., Carden, D.L., Korthuis, R.J., 1991. Activated neutrophils increase microvascular permeability in skeletal muscle: role of xanthine oxidase. J. Appl. Physiol. 70, 2003–2009.

Suttorp, N., Hippenstiel, S., Fuhrmann, M., Krüll, M., Podzuweit, T., 1996. Role of nitric oxide and phosphodiesterase isoenzyme II for reduction of endothelial hyperpermeability. Am. J. Physiol. 270, C778–C785.

Tate, R., Vanbenthuyse, K., Shasby, M., McMurtry, I., Repine, J., 1982. Oxygen-radical-mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. Am. Rev. Respir. Dis. 126, 802–806.

Vojnikovic, B., 1991. Doxium® (calcium dobesilate) reduces blood hyperviscosity and intra-ocular pressure in patients with diabetic retinopathy and glaucoma. Ophthalmol. Res. 23, 12–20.

Widmer, L., Biland, L., Barras, J.P., 1990. Doxium® 500 dans le traitement de l'insuffisance veineuse chronique: étude multicentre en double aveugle contre placebo. Int. Angiol. 9, 105–110.

Yaqoob, M., Ptak, A., McClelland, P., Stevenson, A., Mason, H., White, M., Bell, G., 1993. Relationship between markers of endothelial dysfunction, oxidative injury and tubular damage in patients with insulin-dependent diabetes mellitus. Clin. Sci. 85, 557–562.