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In vitro antioxidant properties of calcium dobesilate

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Summary – Calcium dobesilate, a vascular protective agent, was tested in vitro for its scavenging action against oxygen free radicals. Calcium dobesilate was as potent as rutin to scavenge hydroxyl radicals (IC_{50} = 1.1 vs 0.7 μM, respectively). It was also able to scavenge superoxide radicals, but with 23 times less potency than rutin (IC_{50} = 682 vs 30 μM, respectively). Calcium dobesilate significantly reduced platelet activating factor (PAF)-induced chemiluminescence in human PMN cells and lipid peroxidation by oxygen free radicals in human erythrocyte membranes, although these actions required calcium dobesilate concentrations ≥ 50 μM. Finally, in cultured bovine aortic endothelial cells, magnesium dobesilate reduced the increase in cytosolic free calcium induced by hydrogen peroxide and inhibited phenazine methosulfate-induced cell potassium loss. In conclusion, calcium dobesilate was effective in scavenging hydroxyl radicals in vitro, at therapeutically relevant concentrations. Conversely, higher concentrations of the compound were required to scavenge superoxide radicals or to protect the cells against the deleterious effects of intracellular reactive oxygen species. Further studies in vivo are required to determine if these antioxidant properties of calcium dobesilate can play a role in its vascular protective mechanisms. © 1998 Elsevier, Paris.

calcium dobesilate / antioxidants / free radicals / cell membranes / lipid peroxidation

INTRODUCTION

Calcium dobesilate (see structure in *figure 1*) possesses vascular protective properties in diabetic retinopathy [19, 34], peripheral microvascular disease [14] and chronic venous insufficiency [35]. This compound reduces capillary fragility and permeability, but its mechanism of action is poorly understood. On the other hand, reactive oxygen species seem to play a role in the capillary dysfunctions of diabetic microangiopathy and other microvascular pathologies [for review see 2, 10, 15]. Indeed, supplementation with rutosides or other antioxidants was found: (i) to antagonize the endothelial and tubular deleterious effects of oxidative stress in human insulin-dependent diabetes mellitus [36]; and (ii) to reduce capillary filtration in venous hypertension [29], standing motionless [28] and in the acute hindlimb lymphedema of the rat [5]. Among seven tested flavonoids, Chen et al [7] have found that the natural polyphenolic flavonoid rutin was the strongest scavenger of superoxide radicals. Moreover, rutin was also found efficient in numerous models involving hydroxyl radicals [see 12, 31]. Finally, rutin is commonly used as a reference antiradical and antioxidant compound [see 23].

The above arguments pushed us to investigate whether calcium dobesilate possesses antioxidant properties. Therefore, the compound was tested in vitro for its potential scavenging effect on extra- and intracellular oxygen reactive species. Rutin was used as comparative molecule.

METHODS

Acellular antioxidant assays

Xanthine/xanthine oxidase and PMS/NADH assays

Superoxide radicals generated by reaction between 200 μM xanthine and 0.2 IU/mL xanthine oxidase (or between 1 μM phenazine methosulfate (PMS) and 100 μM hydrogenated nicotinamide-adenine dinucleotide [NADH] were measured

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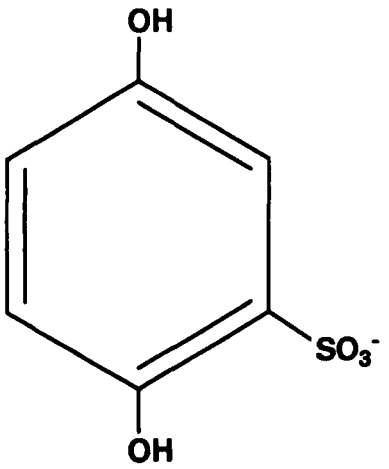

Fig 1. Chemical structure of dobesilate anion. Salts with calcium or magnesium ion were used here. These organic salts are very soluble in water and ethanol, practically insoluble in ether or chloroform.

by reduction of 50 μM nitroblue tetrazolium (NBT), according to a previously published protocol [21]. This reaction was performed at room temperature in a spectrophotometer's cuvette, in presence of 5 mM 3-(N-morpholino)propanesulphonic acid (MOPS)-Tris (hydroxymethyl)-aminomethane (Tris) buffer medium (pH = 7.4 at room temperature).

The time course of the reduced NBT absorbance (at 560 nm) was recorded at times (min): 0, 1, 2, 3 and 4. Control experiments showed that: (i) NBT absorbance linearly increases with time for at least 4 min (initial rate of the reaction), (ii) NBT reduction was fully blocked by 1 IU/mL superoxide dismutase (SOD), unaffected by 0.1% (v/v) dimethyl sulfoxide (DMSO), and only marginally inhibited (~20%) by 100 mM mannitol (an OH°-radical scavenger). These control experiments confirmed that the assay was appropriate to evaluate potential scavengers of superoxide anion radicals ($O_2^{\circ-}$) [for experimental details see 21].

Xanthine/xanthine oxidase + DMSO/$FeCl_2$ assay

Hydroxyl radicals (and superoxide anions) chemically generated by adding xanthine oxidase (final concentration = 0.01 units/mL) and $FeCl_2$ (0.5 μM) to the above MOPS-Tris medium containing 140 mM DMSO and 200 μM xanthin, were measured by using the fluorescent probe Tempo 9-AC (Sigma, St Louis, MO, USA) at 50 μM. Under such conditions, the fluorescence signal of Tempo 9-AC was shown to depend on OH°-radicals [for details see 26, 27].

Antioxidant assays using cell models

Human Polymorphonuclear Cells

Reactive oxygen species produced by human polymorphonuclear cells (PMNs) upon activation were quantified by their light emission in presence of luminol according to Floch et al [11]. Briefly, human polymorphonuclear cells were isolated from healthy human blood samples (~10 mL harvested on heparinized tubes) by the dextran-method, including erythrocytes lysis and granulocyte re-suspension in Hank's balanced salt solution (HBSS) containing 0.5%, bovine serum albumin (BSA), at a cell concentration of 5 million PMNs/mL. Cell viability, measured with Trypan blue, was > 95%.

Human PMNs, used at final concentration of 1.7 million cells/mL, were first primed with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP: 5 nM; incubation time: 10 min at 37 °C), in order to increase their sensitivity to platelet activating factor [PAF, see 11]. Then, a dose-response curve of chemiluminescence as a function of PAF concentrations was performed on the day-harvested cell sample in order to determine a sub-maximal dose of PAF (C_{16}) for the experiment itself. A Hank's incubation medium containing 0.25% BSA was used to prevent PAF adhesion to the tubes. After preincubation of the cells for 5 min at 37 °C in the presence of the tested-molecule (or solvent), 100 μM luminol and PAF (32 nM) were added to the tubes (final volume = 600 μL) and the luminescence was recorded using a six-channels luminometer (Biolumat LB9505, Berthold, Wildbad, Germany).

Human Erythrocytes

Malondialdehyde (MDA, a marker of lipid peroxydation) was measured in human erythrocytes as previously described [20, 21]. Briefly, red blood cells at a final hematocrit of about 25% were incubated for 2 hours with 1.5 mM PMS and 1 mM diethyldithiocarbamate (DDC), an inhibitor or superoxide dismutase, at 37 °C. Cells were then washed three times with cold 150 mM NaCl. The final cell pellet was lysed with 20 volumes of 5 mM phosphate buffer (pH 7.4). The lysate was submitted to ultracentrifugation (20 min at 30,000 *g*) and the pellet resuspended with 20 volumes of cold 50 mM Na phosphate buffer (pH 7.4). The centrifuged membranes were incubated for 1 hour at 95 °C in the presence of thiobarbituric acid (TBA 0.13% v/v), 0.07 N sulfuric acid, 0.01N NaOH, glacial acetic acid and 0.04% (v/v) Acationox (controls were made in the absence of TBA). After incubation, tubes were rapidly chilled, 5 mL *n*-butanol were added and the tubes were vortexed for 5 min. Absorbance of the butanol phase was measured at 532 nm, and MDA content was calculated from the difference between samples with and without TBA. Parallel calibration curves were done with malondialdehyde diethylacetal, the chromophoric product of the TBA-reaction. Control experiments showed that PMS increased by 3–6 times the amount of membrane MDA (~20 μmol/L of cells).

PMS-induced potassium efflux in human erythrocytes was measured by using a previously published protocol [20].

Endothelial Cell culture

The bovine aortic endothelium cell line was purchased from European Collection of Cell Cultures (ECACC, Sophia-Antipolis, France). Cells were cultured according to standard procedures (90% Dulbecco's Modified Eagle Medium, 10% fetal calf serum), under 5% CO_2/95% air. Sub-passages were done by mild trypsinization (0.25% for 2 min). For measurement of ion movements, cells were seeded in 6-well plates (4.10^4 cells/cm^2). Cytosolic free calcium measurements were performed on suspended cells. In all cases, confluent cells were used.

Measurement of cytosolic free calcium concentrations

Cytosolic free calcium concentrations were measured by using the fluorescent probe Fura2 [13], from the ratio between 340 and 380 nm-excited 505 nm emission (after appropriate blank subtractions). Cells were incubated with the acetoxymethyl ester of the cell-permeant Fura2 (Fura2/AM: 5 μM) for 45 min at room temperature. Then, cells were incubated in absence of Fura2/AM for other 30 min at 37 °C, in order to insure total desterification of the internalized probe. Fluorescence was measured, at 0.5 Hz, using a spectrofluorimeter (Shimadzu RF5000, Roucaire, Vélizy-Villacoublay, France). The cuvette contained 2 million cells in 2 mL of the incubation medium (standard saline). Final calibration (Rmax) was done with 0.1% X100 Triton, followed by addition of 10 mM EGTA (Rmin). Cytosolic free calcium concentration was calculated according to Grynkyewicz et al [13].

Measurement of cell potassium contents

Cells were allowed to equilibrate for 30 min at 37 °C in a standard saline medium containing (mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, MOPS-Tris 10 (pH 7.4 at 37 °C), glucose 5. After this preincubation period, wells were washed with 150 mM NaCl and the efflux medium (containing [mM]: NaCl 135, CsCl 10, MOPS-Tris 10 [pH 7.4 at 37 °C], $CaCl_2$ 1, $MgCl_2$ 1, glucose 5) was added (2 mL per well). Potassium efflux was stopped by quick withdrawal of the external medium, in which potassium concentration was measured by atomic absorption flame photometry (IL457, Spectra, France), followed by a rapid washing with ice-cold 110 mM $MgCl_2$ (3 times 5 mL /well). Cells were lysed by addition of 3 mL 0.02% (v/v H_2O) Acationox® (a cation-free detergent, American Scientific Products, McGraw Park, IL, USA). Lysates were used for measurement of internal Na and K cell contents.

The rate constant of potassium efflux (k_K, in 1/h) was calculated under initial rate conditions by dividing the decrease in internal potassium content by the incubation time and the initial internal potassium content.

Effect of oxygen free radicals on cell potassium contents

Oxygen free radicals were generated by using PMS, in the presence of 5 mM diethyldithiocarbamate (DDC, a SOD inhibitor). Since control experiments showed that exogenous NADH did not enhance PMS effect, most experiments were performed in the absence of added NADH.

Preliminary experiments were carried out to choose significant and reproducible potassium loss in response to oxygen free radicals generated in situ. The optimal conditions were: (i) 1 hour incubation at 37 °C; (ii) 1 mM PMS (likely entering the cell interior and reacting with intracellular NADH); (iii) 5 mM DDC (also present during preincubation), to inhibit intracellular SOD; (iv) 0.1 mM ouabain and bumetanide, in order to block potassium movements through the Na-K pump and the Na-K-Cl cotransport system, respectively.

Measurement of free iron concentrations

To investigate potential Fe^{2+} chelation by calcium dobesilate, changes in free Fe^{2+} concentrations were monitored by using the fluoresceinated derivative of the naturally occurring microbial siderophore desferrioxamine B (FL-DFO, Molecular Probes, Eugene, OR, USA). Non-fluorescent desferrioxamine was used as reference iron chelator. The reaction was carried out at room temperature in a solution of (mM): MOPS-Tris 5 (pH 7.4 at 20 °C) and FL-DFO 0.001. $FeCl_2$ was added at a final concentration of 100 nM. Fluorescence was measured at 515 nm (excitation wavelength: 493 nm).

Compounds

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

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM) (n indicates the number of experiments). Statistical differences between two mean values were determined by unpaired Student's t-test. Statistical significance was accepted for $P < 0.05$.

RESULTS

Scavenging actions of calcium dobesilate on superoxide and hydroxyl radicals

Superoxide

Superoxide anion radicals were chemically generated (0.4 μM/min) by the couple xanthine/xanthine oxidase (X/XO) and calcium dobesilate was tested for its scavenging action in dose response curves. *Figure 2* shows that calcium dobesilate reduced superoxide levels in a concentration-dependent manner. This superoxide scavenging action was observed in the millimo-

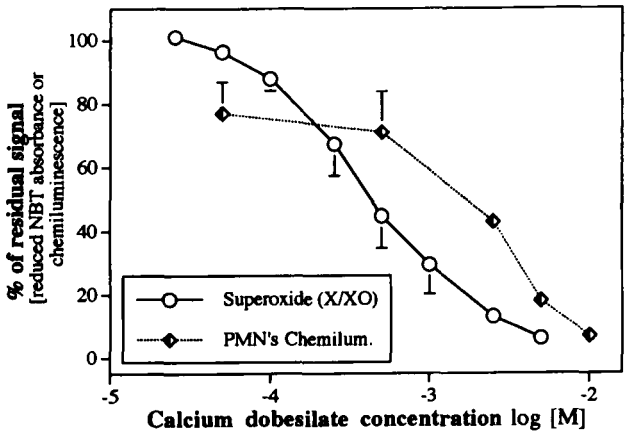

Fig 2. Reduction by calcium dobesilate of superoxide radicals chemically generated (0.4 μM/min) by the couple xanthine/xanthine oxidase and of the chemiluminescence of platelet activating factor (PAF)-activated human polymorphonuclear cells. The initial rate of superoxide anion generation was equated to the rate of increase in reduced nitroblue tetrazolium (NBT). PAF concentration was 32 nM. Values are given as mean ± standard error of mean (SEM) (n = 6 and 4 for the superoxide xanthine/xanthine oxidase [X/XO] and human polymorphonuclear cells [PMN] chemiluminescence experiments respectively).

lar range of compound concentrations (IC_{50} = 0.69 ± 0.20 mM, n = 6). Indeed, *table I* shows that calcium dobesilate was 23 times less potent than the reference molecule: rutin (IC_{50} = 29.8 ± 9.0 μM, n = 6) as scavenger of superoxide radicals.

It is important to mention that: (i) calcium chloride was without significant action on superoxide levels, even at concentrations of 10 mM; and (ii) calcium dobesilate showed similar scavenging potency against superoxide anions chemically generated by the couple PMS/NADH (IC_{50} = 0.5–1 mM).

Hydroxyl radical

Hydroxyl radicals were chemically generated by adding DMSO and $FeCl_2$ to the couple xanthine/xanthine oxidase. *Figure 3* (upper panel) shows that calcium dobesilate reduced the OH°-induced fluorescence signal in a concentration-dependent manner. This scavenging action was observed at concentrations three orders of magnitude lower as those active on superoxide (*figure 2*). *Table I* shows that calcium dobesilate was almost as potent as rutin in this assay (IC_{50} = 1.1 vs 0.74 μM, for calcium dobesilate and rutin respectively). It is important to mention that neither calcium dobesilate (0.05–50 μM), nor rutin (1 μM) quenched the fluorescent probe signal (TEMPO-9AC).

Figure 3 (lower panel) shows the effect of calcium dobesilate on the Fe2+-sensitive fluorescence signal of fluoresceinated desferrioxamine B (FL-DFO, see Methods). It can be seen that calcium dobesilate up to concentrations as high as 100 μM was unable to significantly decrease Fe2+-dependent FL-DFO fluorescence signal. Conversely, Fe2+-dependent FL-DFO fluorescence signal was strongly reduced by non-fluorescent desferrioxamine.

Antioxidant functions of calcium dobesilate in cell models

Human polymorphonuclear cells

Calcium dobesilate was tested for its inhibitory effects on luminol-dependent chemiluminescence of PAF-activated human polymorphonuclear cells, as described in Methods. *Figure 2* shows that calcium dobesilate inhibited luminol-dependent chemiluminescence in a similar manner as it acts as a superoxide scavenger. Significant inhibition ($P < 0.05$) was noticed for calcium dobesilate concentrations ≥ 500 μM. Chemiluminescence inhibition of PAF-activated human PMNs reached 90% for 10.7 mM calcium dobesilate.

Human erythrocytes

Figure 4 shows the effect of increasing concentrations of calcium dobesilate (0.5–10 mM) on PMS-dependent membrane MDA formation in human erythrocyte membrane. It can be seen that high concentrations of calcium dobesilate (5 and 10 mM) decreased MDA contents by 55% ($P < 0.05$) and 85% ($P < 0.01$) respectively. It is important to mention that calcium dobesilate per se (0.5–5 mM) did not significantly modified basal membrane MDA contents (data not shown).

Figure 4 also shows that calcium dobesilate was ineffective to reduce PMS-induced potassium efflux in human red blood cells.

Cultured endothelial cells

Basal cytosolic free calcium contents in bovine aortic endothelial cells were 170 ± 30 nM (n = 12). Calcium dobesilate induced a slight and dose-dependent reduction of cytosolic free calcium concentration (–20% at 5 mM). Conversely, magnesium dobesilate (0.05–5 mM) was unable to significantly change basal values of cytosolic free calcium, even after 15 min preincubation. Moreover, magnesium dobesilate (0.005–0.5 mM) was unable to significantly modify basal potassium contents (after 30 or 60 min of incubation). Therefore, subsequent experiments in these cells were carried out by using magnesium dobesilate.

PMS possesses intrinsic fluorescence, thus hampering the measurements of cytosolic calcium contents with the Fura2 fluorescent probe. Thus, hydrogen peroxide was used to increase cytosolic free calcium con-

Table I. Free radical scavenger actions of calcium dobesilate vs rutin.

	Scavenging IC_{50} [μM]	
Compound	Superoxide radicals Xanthine/Xanthine oxidase assay	Hydroxyl radicals Xanthine/Xanthine oxidase + DMSO/$FeCl_2$ assay
Calcium dobesilate	682 ± 172 (6)	1.1 ± 0.1 (4)
Rutin	29.8 ± 9.0 (6)	0.74 ± 0.14 (3)

Values are given as mean ± SEM. The number of experiments is indicated in brackets.

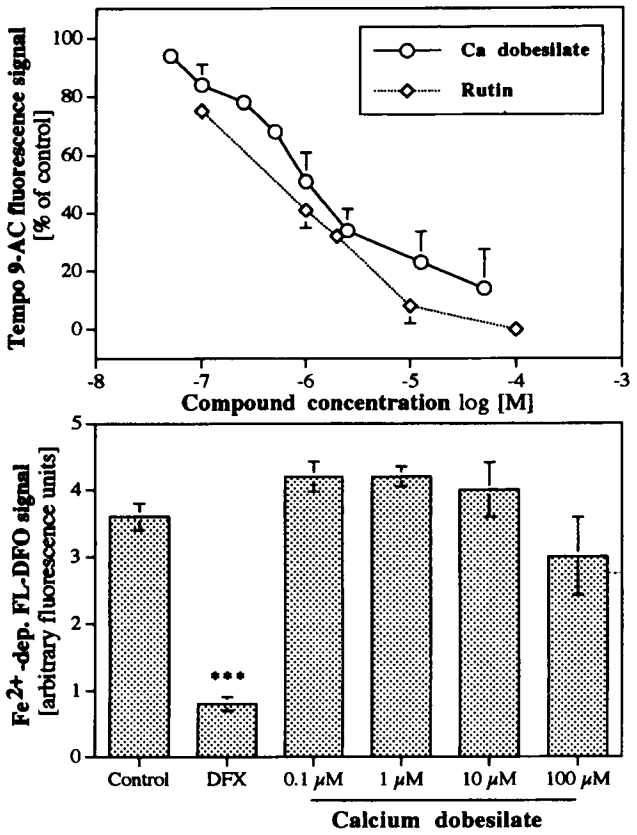

Fig 3. Upper panel: Scavenging action by calcium dobesilate on hydroxyl radicals chemically generated by the couple xanthine/xanthine oxidase in presence of dimethyl sulfoxide (DMSO) and $FeCl_2$. The initial rate of hydroxyl radical generation was equated to the rate of increase in Tempo 9-AC fluorescence [26, 27; see also Methods and Discussion). Rutin was used as reference molecule. Values are given as mean ± SEM (n = 4 and 3 for calcium dobesilate and rutin, respectively). It can be seen that calcium dobesilate was almost as potent as rutin to scavenge hydroxyl radicals. Lower panel: Lack of effect of calcium dobesilate on the Fe2+-sensitive fluorescence signal of fluoresceinated desferrioxamine B (FL-DFO). Fe2+-dependent FL-DFO fluorescence signal was strongly reduced by non-fluorescent desferrioxamine (DFX).

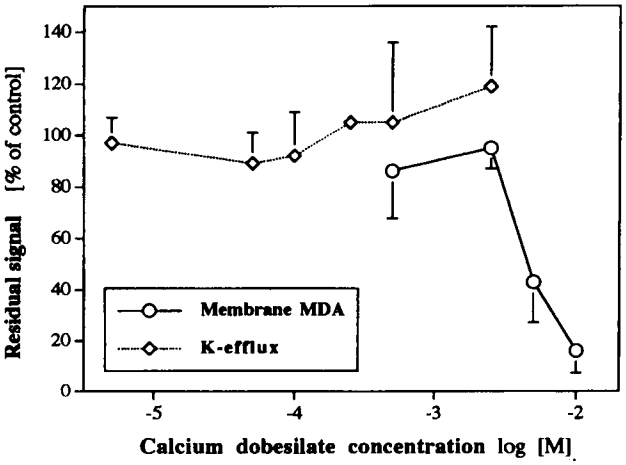

Fig 4. Effect of calcium dobesilate on the lipid peroxydation and increase in potassium permeability induced by phenazine methosulfate (PMS)-dependent oxygen free radicals in human erythrocytes. Peroxidation of unsaturated membrane phospholipids by oxygen free radicals was evaluated by measurement of PMS-dependent membrane malondiadehyde (MDA) formation, one product of the reaction. Values are given as mean ± SEM (n = 3–4 for each condition).

tents in cultured endothelial cells. In the absence of added iron, high concentrations of hydrogen peroxide (0.2% w/v) were required to induce a reproducible and significant increase in cytosolic free calcium (324 ± 21 nM, n = 9). *Figure 5* shows that preincubation with magnesium dobesilate (0.55 mM) for 15 min strongly inhibited the cytosolic calcium increase (~50%).

Incubation of cultured endothelial cells for 1 h with PMS (which reacts with internal NADH to give superoxide anions) plus DDC (an inhibitor of superoxide dismutase) induced a cell potassium loss of 20% (n = 14, $P < 0.01$). *Figure 5* shows that addition of 0.55 mM magnesium dobesilate significantly reduced this cell potassium loss (by ~50%, $P < 0.05$).

DISCUSSION

Experimental and clinical studies revealed that calcium dobesilate possesses vascular protective properties, particularly for microvessels [14, 17, 19, 22, 34, 35]. A reduction in collagen biosynthesis and hydroxylysine incorporation in retina and glomerula base-

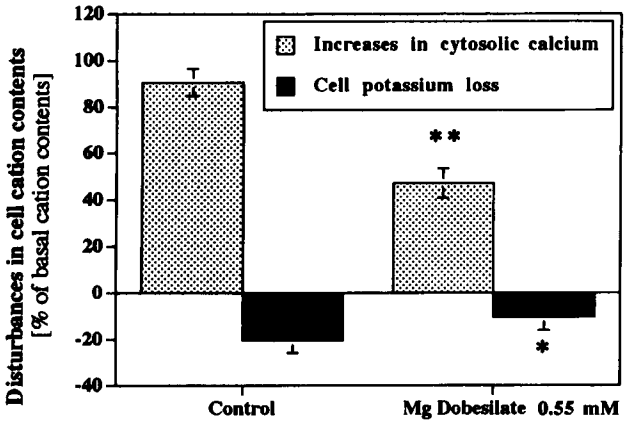

Fig 5. Inhibition by magnesium dobesilate of the cytosolic free calcium increase and cell potassium loss induced by oxygen free radicals in cultured endothelial cells. PMS reacts with internal NADH to produce superoxide anions (superoxide dismutase was inhibited by adding diethyldithiocarbamate [DDC]). Values are given as mean ± SEM (n = 3–4). * and ** indicate $P < 0.05$ and < 0.01 respectively (non-paired Student's t-test).

ment membranes of diabetic rats [17] is probably one of the apparently multiple underlying mechanisms of calcium dobesilate action.

Flavonoids, particularly rutosides, also exert vascular protective properties [5, 6, 28, 29, 36] and the mechanism of action of these compounds seems to reside in their ability to scavenge reactive oxygen species. Thus, rutin has been shown to act as antioxidant in various in vitro [7, 12, 21, 32] and ex-vivo models [1, 15].

Dobesilate is a diphenolic anion *(figure 1)* and hydroxylated and or aromatic compounds are known to scavenge oxygen reactive species. Moreover, the above considerations suggested that calcium dobesilate could act as vascular protective agent, at last in part, through antioxidant functions. Therefore, the compound was tested for antioxidant properties by using simple in vitro tests and taking rutin as reference compound. The obtained results clearly revealed that calcium dobesilate possesses free radical scavenging properties, but its profile is complex and depends on the radical species and the cell model in consideration.

Calcium dobesilate was effective to reduce superoxide anion radicals ($O_2^{\circ-}$, generated by the reaction couples xanthine/xanthine-oxidase or PMS/NADH), but the scavenging action was observed in the millimolar range of concentrations. Indeed, calcium dobesilate was 23 times less potent as ($O_2^{\circ-}$)-scavenger than the reference molecule: rutin *(table I)*. This is not surprising because, except flavonoids, few antioxidant compounds appear active against superoxide radicals [16, 21]. On the other hand, although two different tests were used, we cannot exclude that millimolar concentrations of calcium dobesilate compete with the natural target for the oxidizing species.

Calcium dobesilate acted with slightly less antioxidant potency against reactive oxygen species in PAF-activated human polymorphonuclear cells. This rather similar potency can be explained by the fact that oxygen free radicals are generated outside the cell (particularly $O_2^{\circ-}$). In this model, it is possible to quantitize oxygen free radical production by their luminescence under controlled experimental conditions [11].

It is well documented that oxygen free radical generation by PMN cells (and macrophages) is involved in most inflammatory processes. Moreover, the recent observation of an exacerbated oxygen free radical production by macrophages from diabetes-prone BB rats provides an additional conceptual link between oxidative stress and diabetes [4]. Therefore, the observation that calcium dobesilate exhibited an inhibitory action on PAF-dependent PMN cell chemiluminescence can explain its previously reported anti-inflammatory actions and the inhibition of macrophage migration [25].

Calcium dobesilate was tested against the OH°-dependent increase in fluorescence signal of Tempo 9-AC. This assay is based on the following features: (i) hydroxyl radicals are generated via the iron-catalyzed Haber-Weiss reaction from superoxide anion (xanthine/xanthine oxidase) and hydrogen peroxide (derived from spontaneous dismutation of superoxide anion); and (ii) DMSO selectively traps hydroxyl radicals producing methyl radicals which are able to react with the nitroxide moiety of Tempo-9AC thus increasing its fluorescence signal [26, 27].

Calcium dobesilate was active in the Tempo 9-AC assay in the micromolar range of concentrations (IC_{50} = 1.1 μM) and was almost as potent as rutin *(table I)*. This action of calcium dobesilate was not due to iron chelation since calcium dobesilate concentrations as high as 100 μM were unable to significantly reduce the Fe2+-sensitive fluorescence signal of fluoresceinated desferrioxamine B (*figure 3*, lower panel). Therefore, the action of calcium dobesilate can be explained by direct hydroxyl radical scavenging. However, we cannot exclude scavenging of methyl radicals.

To investigate if calcium dobesilate could act as antioxidant in intracellular compartments, the compound was tested against the following disturbances of intracellular oxygen reactive species: (i) membrane lipid peroxidation; (ii) potassium loss; and (iii) cytosolic calcium increase. In some experiments, magnesium dobesilate was used to control the potential

effects of excess calcium ions (for instance, an increase in cytosolic free calcium concentration which can activate calcium-dependent potassium channels and induce cell potassium loss). Calcium and/or magnesium dobesilate were both efficient to protect the cells against oxygen reactive species in the models studied.

In human erythrocytes, calcium dobesilate reduced PMS-dependent lipid peroxydation, although the effect was observed at high concentrations *(figure 4)*. This low potency of calcium dobesilate is intriguing because hydroxyl radicals catalyze membrane lipid peroxydation. One possible explanation is that calcium dobesilate has difficult access to the cell interior, as expected from its hydrophilic properties (see structure in *figure 1*).

In cultured endothelial cells, high concentrations of hydrogen peroxide (~50 mM) were required to induce a marked and reproducible increase in cytosolic free calcium concentration (seemingly through intracellular OH° radical generation). This cytosolic calcium increase was strongly inhibited by high concentrations of magnesium dobesilate. Moreover, magnesium dobesilate was also able to inhibit the PMS-induced cell potassium loss.

It is important to mention that in the above cell toxicity tests calcium dobesilate can act by a more complex mechanism, ie, that its protective effect is not exclusively linked to an antioxidant effect, but also to an action on some downstream step.

Taken together our results strongly suggest that calcium dobesilate has antioxidant properties via a direct scavenging effect, particularly against external hydroxyl radicals. This can explain, at least in part, the observation in animal models that calcium dobesilate reduces the myocardium infarcted area after coronary artery occlusion [18, 30] and decreases the levels of biochemical markers of early acute myocardial infarction in humans [33].

Further studies are required to investigate whether the antioxidant effects of calcium dobesilate in vitro translate into significant beneficial actions in vivo. In this respect, it is important to recall that in vivo actions of rutin in rats require oral doses as high as 1g/kg/d [24]. Conversely, after a single 500 mg oral dose in humans, the blood concentration of calcium dobesilate peaks at ~35 μM at the 6th hour, with a plateau phase between the 3rd and 10th hour [3]. In addition, the drug is only 20–25% bound to plasma proteins. Thus, the present in vitro antioxidant properties of calcium dobesilate can be of therapeutical relevance. In particular, one is tempted to tentatively predict that calcium dobesilate should exhibit in vivo protective effects against the deleterious effects of oxygen free radicals at the capillary level, including proteins leak and microvascular edema formation [8, 9].

CONCLUSION

Calcium dobesilate was effective in scavenging hydroxyl radicals in vitro. This effect was observed at therapeutically relevant dobesilate concentrations. Conversely, higher concentrations of the compound were required to scavenge superoxide radicals or to protect the cells against the deleterious effects of intracellular reactive oxygen species. Further studies are required to decide if these antioxidant properties of calcium dobesilate can participate in its vascular protective mechanisms in vivo.

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