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Involvement of Nitric Oxide in the Inhibition of Aortic Smooth Muscle Cell Proliferation by Calcium Dobesilate

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Abstract. Vascular smooth muscle cell (SMC) proliferation is a key process in the pathogenesis of atherosclerosis. Numerous factors are involved in the regulation of SMC growth. Nitric oxide (NO) induces the inhibition of SMC proliferation whereas oxidized low-density lipoproteins (LDL) have a mitogenic effect. Calcium dobesilate (Doxium) is an angioprotective agent for treating vascular diseases. It has been shown to increase NO production and to have antioxidant properties but its mechanism of action is not yet fully understood. This study investigated the effect of calcium dobesilate on proliferation of rat aortic SMC in culture. Proliferation was evaluated by cell number and DNA synthesis. Orally administered calcium dobesilate (30, 100, or 200 mg/kg/day for 7 days) induced a dose-dependent decrease of proliferation of SMC in primary culture compared with controls. *In vitro* treatment with calcium dobesilate (0.05–5 mM) inhibited both DNA synthesis and proliferation in a time- and concentration-dependent manner. In both *ex vivo* and *in vitro* models, the inhibition was reversible upon removal of the drug. Calcium dobesilate also stimulated NO production and NO synthase activity. Inhibitors of NO synthesis attenuated the inhibitory effect of calcium dobesilate (300 μM) on DNA synthesis. In addition, calcium dobesilate (2.5–40 μM) induced a dose-dependent protection of cooper-induced LDL oxidation. These results showed that calcium dobesilate inhibits SMC proliferation, partly by a NO-dependent mechanism, and suggest that it could be effective in the treatment of pathological disorders associated with vascular SMC proliferation.

Introduction

Abnormal vascular smooth muscle cell (SMC) proliferation in the tunica intima is an important component of arterial disorders such as atherosclerosis, restenosis after angioplasty, and in accelerated arteriopathy after cardiac transplantation [1]. Factors affecting SMC proliferation are believed to be important in controlling the development of these arterial disorders. The proliferation is believed to result partly from the increase of mitogens and partly from the decrease of inhibitory mediators. Nitric oxide (NO) is a multifunctional factor that contributes to the control of vascular functions such as platelet adhesion, vascular reactivity, and endothelial permeability [2]. NO also inhibits SMC growth *in vitro* and experimental neointimal hyperplasia *in vivo,* suggesting a role for NO as a regulator of SMC proliferation [3]. An inducible form of nitric oxide synthase (NOS) produces NO in vascular SMC. This enzyme is expressed following arterial balloon injury or exposure to inflammatory cytokines. The biological responses are likely to involve the activation of soluble guanylate cyclase and the production of cGMP in the target cells [2].

In restenosis, SMC proliferation is a prominent component of neointimal hyperplasia following balloon angioplasty. Thus, the ability of factors to directly inhibit SMC proliferation could be a mechanism to decrease this disease. In contrast, SMC proliferation is detected only at very low levels in primary human atherosclerotic plaques [1]. Among the multiple risk factors of atherosclerosis, low-density lipoproteins (LDL) levels are thought to play a primary atherogenic role after undergoing oxidative modifications [4]. It is well established that LDL promotes the growth of arterial SMC [5]. Furthermore, oxidized LDL appears to have a powerful mitogenic effect or to induce cell death by apoptosis, depending on the degree of change by oxidation [6]. Thus, the supply of antioxidants appears to have an inhibitory effect on the development of atherosclerotic plaques.

Calcium dobesilate (calcium dihydroxy-2,5 benzenesulfonate) is a vascular protective agent currently used for the treatment of diabetic retinopathy and chronic venous insufficiency. The drug has been shown to inhibit platelet aggregation [7] and thrombus formation in the microcirculation [8]. However, its mechanism of action is poorly understood. Recent *in vitro* studies have shown that calcium dobesilate increases the constitutive NOS-activity in macro- and mi-

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crovascular endothelial cells [9] and enhances endothelium-dependent relaxation in rabbit isolated aorta [10]. Furthermore, calcium dobesilate has been shown to express antioxidant properties in human erythrocytes and polymorphonuclear cells [11,12] and to protect human polymorphonuclear cells from apoptosis [12].

The aim of this study was to investigate the effect of calcium dobesilate on rat aortic SMC proliferation in culture after *in vivo* oral administration and after *in vitro* treatment, and to determine whether the production of NO is involved in this response. In addition, we evaluated the antioxidant effect of calcium dobesilate on copper-mediated LDL oxidation.

Materials and Methods

Animals

Adult male Wistar rats weighing 350–370 g, were used in all experiments. Laboratory chow and water were supplied *ad libitum*. Animals for *ex vivo* experiments were divided into four groups and treated with an oral administration of 30, 100, or 200 mg/kg per day of calcium dobesilate or 0.9% NaCl, respectively, for 1 week. Twenty-four hours or 20 days later, the rats were killed by cervical dislocation and thoracic aortae were harvested for SMC preparation.

Cell Culture

Aortic SMC were isolated by enzymatic dissociation and cultured as described by Parés-Herbuté et al. [13]. Cells were seeded at a density of $2 \times 10^4/cm^2$ and cultured in equal parts of minimum essential medium (MEM) containing Earle's salts and Ham's F-10 medium supplemented with 2 mmol/L glutamine, 0.05 mmol/L vitamin C, 0.05 mmol/L L-proline, 100 U/ml penicillin G, and 10% (vol/vol) fetal calf serum (FCS). The culture plates were incubated in a humidified incubator at 37°C in 5% CO_2 and 95% room air. The cells were positively identified as smooth muscle by indirect immunofluorescent staining for α-actin, using a mouse monoclonal antibody to α-actin. Experiments were performed with cells in primary culture and in passage 2 seeded into either 4-well plates for the measurement of growth and nitrite production or in 75-cm^2 flask for the citrulline assay.

Ex Vivo Experiments

Aortic SMC from calcium dobesilate-treated rats were cultured in medium supplemented with 10% FCS. Medium was changed every 48 hours. Cell proliferation was evaluated at the indicated times by cell counting after trypsinization using a Coulter counter.

In Vitro Experiments

Cells in the second passage were grown in 10% FCS medium. Twenty-four hours after plating, the medium was changed to one containing 10% FCS, with or without calcium dobesilate (50–400 μg/ml = 120–960 μM). After different time intervals, cells were trypsinized, resuspended, and counted.

DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine. Cells were grown in 10% FCS medium. Twenty-four hours later the medium was changed to one containing 0.5% FCS to stop cell growth, and cells were incubated for 72 hours. At this time the medium was replaced by 10% FCS in the presence or absence of known concentrations of calcium dobesilate (50–5000 μM) and the incubation was continued for a further 24 hours. [^{3}H]Thymidine (0.5 μCi/ml, 25 Ci/mmol) incorporation was measured during the last 4 hours of the incubation period. [^{3}H]Thymidine incorporation into newly synthesized DNA was stopped by removal of the labeled medium and precipitation of acid-insoluble material with 10% trichloroacetic acid. Cells were digested with 0.3 mol/L NaOH and the radioactivity of the solution was measured in a liquid scintillation counter.

To determine the role of NO in the effect of calcium dobesilate on DNA synthesis, quiescent SMC were incubated with or without calcium dobesilate (300 μM) in the presence or absence of L-NMMA (3 mM) or aminoguanidine (0.5 mM)—two inhibitors of NO synthesis—, indomethacin (1 μg/ml)—a cyclooxygenase inhibitor—or methylene blue (10 μM)—a soluble guanylate cyclase inhibitor. Methylene blue was added to the wells 4 hours before the end of the incubation period.

In some experiments, when cells reached confluence, the culture medium was replaced with serum-free medium containing 0.1% (wt/vol) fatty acid-free bovine serum-free albumin, with or without calcium dobesilate. After 24 hours, cells were prepared for citrulline assay. In other experiments, the culture medium was collected for nitrite assay 48 hours after the addition of calcium dobesilate.

Nitrite Determination

The production of nitrite was determined by diazotization and absorbance reading at 540 nm. Aliquots of cell supernatant were mixed with an equal volume of Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamine-dihydrochloride in 5% phosphoric acid) and incubated at room temperature for 10 minutes. Concentrations were determined relative to a standard curve using sodium nitrite. The background nitrite values of each media were determined and subtracted from experimental values.

Citrulline Assay

Cytosolic NOS activity was estimated from the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described [14]. Briefly, cytosolic preparations were made from 10,000 g supernatants of homogenates of SMC. The cytosols (30 μl) were incubated with L[^{3}H]arginine (1 μCi) and cofactors for 30 minutes at 37°C. To separate L-[^{3}H]citrulline from L-[^{3}H]arginine, the reaction mixture was further incubated with AG 50W-X8 (Na^+ form, Bio-Rad S.A.) cation exchange resin and centrifuged at 10,000 g. L-[^{3}H]-citrulline in the supernatant was quantified by liquid scintillation counting.

Lipoproteins and LDL Oxidation

LDL was isolated from EDTA-plasma of normolipemic patients by sequential ultracentrifugation [15]. LDL oxidation was evaluated by following the formation of conjugated dienes after addition of copper according to Esterbauer et al. [16]. The susceptibility of LDL to *in vitro* oxidation was determined by the lag phase. It was defined as the intercept of the baseline and the slope of the absorbance curve in the propagation phase and was expressed in minutes.

Data Analysis

Data are expressed as mean ± SEM. Differences between two means were determined using the Student's unpaired t test; $p < 0.05$ was considered statistically significant.

Results

Proliferation of Aortic SMC from Calcium Dobesilate-treated Rats

In vivo administration of oral calcium dobesilate (30, 100, or 200 mg/kg/day) induced a dose-dependent decrease of growth rate and cell density at the quiescent phase of aortic SMC in primary culture, when cultures were prepared 24 hours after the last oral dose of drug (Fig. 1). After 10 days

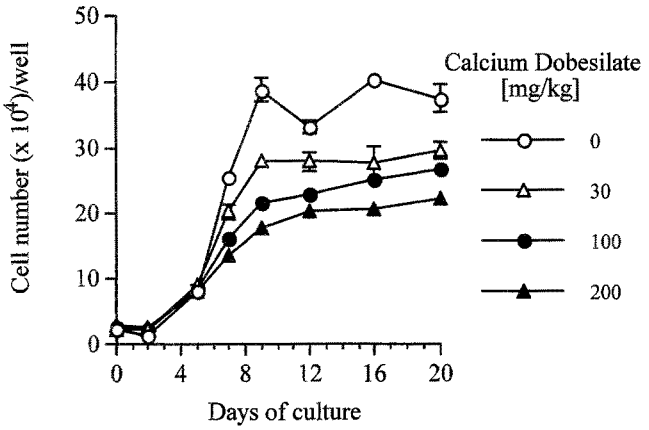

Fig. 1. Growth curves of cultured aortic SMC from rats orally treated with 0, 30, 100, or 200 mg/kg/day of calcium dobesilate. Primary cultures were prepared 24 hours after the last oral dose. SMC were seeded into 4-well plates (4×10^4 cells/well) in medium containing 10% FCS. Each point represents mean ± SEM of two separate experiments containing four replicates.

in culture, the number of SMC from animals treated with 200 mg/kg/day of calcium dobesilate was decreased by 49.0% ($p < 0.01$) when compared with the number of SMC from untreated control rats. When cultures were prepared 20 days after the last oral dose, no significant difference in cell proliferation was observed between SMC from treated rats and those from control rats. After 12 days in culture, the number of SMC from 0, 30, 100, and 200 mg/kg calcium dobesilate-treated rats was $13.6 \pm 0.7 \times 10^4$, $13.9 \pm 0.4 \times 10^4$, $14.6 \pm 0.4 \times 10^4$, and $14.3 \pm 0.9 \times 10^4$ cells/well, respectively (n = 8).

Effect of Calcium Dobesilate on Aortic SMC Proliferation

Calcium dobesilate (50–400 μg/ml = 120–960 μM) induced a time- and concentration-dependent inhibition of SMC proliferation in culture (Fig. 2a). The inhibitory effect ($p < 0.01$) was observed from 100 μg/ml calcium dobesilate and after 3 days of culture. At 400 μg/ml concentration, calcium dobesilate almost abolished cell growth (94%, $p < 0.01$) completely. Calcium dobesilate at 50 μg/ml induced 28% inhibition ($p < 0.05$) of SMC proliferation only on day 3 of culture.

Culture with 200 μg/ml calcium dobesilate for 7 days inhibited SMC proliferation by 53% ($p < 0.01$). Subsequent culture in drug-free medium (Fig. 2b) resulted in an accelerated growth rate approaching that of the untreated SMC. This result indicates that the antiproliferative effect of calcium dobesilate is reversible and is not due to a toxic effect.

Calcium dobesilate (50–5000 μM) also decreased [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 3), with an IC50 value of 400 μM (169 μg/ml).

Effect of Calcium Dobesilate on NO Production by Aortic SMC

The effect of calcium dobesilate on the activity of inducible NOS was studied by the nitrite formation and the generation of labeled L-citrulline from labeled L-arginine. Treatment of confluent cultures of SMC with calcium dobesilate (10–10,000 μM) for 48 hours (Fig. 4a) caused the dose-dependent accumulation of nitrite into the incubation medium. Nitrite production was significantly increased after exposure to 100 μM calcium dobesilate ($p < 0.05$). The cytosolic fractions from homogenates of SMC that had been treated with calcium dobesilate (1 mM) for 24 hours stimulated the conversion of L-arginine to L-citrulline by 53% ($p < 0.01$), (Fig. 4b).

Effect of NO Pathway Inhibitors on DNA Synthesis of Calcium Dobesilate-Treated SMC

Incubation with 3 mM L-NMMA or 0.5 mM aminoguanidine, two inhibitors of NO synthesis, significantly attenuated ($p < 0.01$) the inhibition of [^{3}H]thymidine incorporation induced by calcium dobesilate (300 μM) (Table 1). The soluble guanylate cyclase inhibitor methylene blue (10 μM) also attenuated ($p < 0.01$) the inhibited [^{3}H]thymidine incorporation in calcium dobesilate-treated cells. In contrast, in the presence of indomethacin (1 μg/ml), a cyclooxygenase inhibitor, the inhibitory effect of calcium dobesilate remained (Table 1).

Effect of Calcium Dobesilate on LDL Oxidation

Calcium dobesilate (2.5–40 μM) induced a dose-dependent increase of the lag phase of copper-induced LDL oxidation. In the presence of 10 μM calcium dobesilate, the lag phase was increased by 30 minutes (150 ± 3.9 vs 120 ± 19.8 minutes, $p < 0.05$). The concentrations of calcium dobesilate required to increase the lag phase were lower than those needed with the vitamin E analogue trolox (data not shown).

Discussion

In this study we have shown that calcium dobesilate inhibits rat aortic SMC proliferation in culture. This inhibition is partially mediated by stimulating NOS activity and NO production.

Aortic SMC isolated from atherosclerotic lesions or from diabetic rats retain an enhanced proliferative activity in primary culture [13]. Thus, primary culture of aortic SMC is used as a model for testing drugs *in vivo* for possible antiatherogenic activity. Orally administered calcium dobesilate decreased proliferation of aortic SMC from treated rats in primary culture compared with those from untreated animals. Calcium dobesilate did not affect the viability of the aortic SMC and its effect was reversible, indicating that the inhibition of cell proliferation was not due to cell toxicity. Significantly, this effect was observed at the dose of 30 mg/kg/day. The clinical dose was assumed to be 500 mg × 4/day (2 g/day; about 33 mg/kg/day) [17]. Therefore, the effective dose of calcium dobesilate in the rat to inhibit SMC proliferation in primary culture is similar to the clinical dose.

Calcium dobesilate inhibited the proliferation of cultured rat aortic SMC. The inhibition was reversible and not ac-

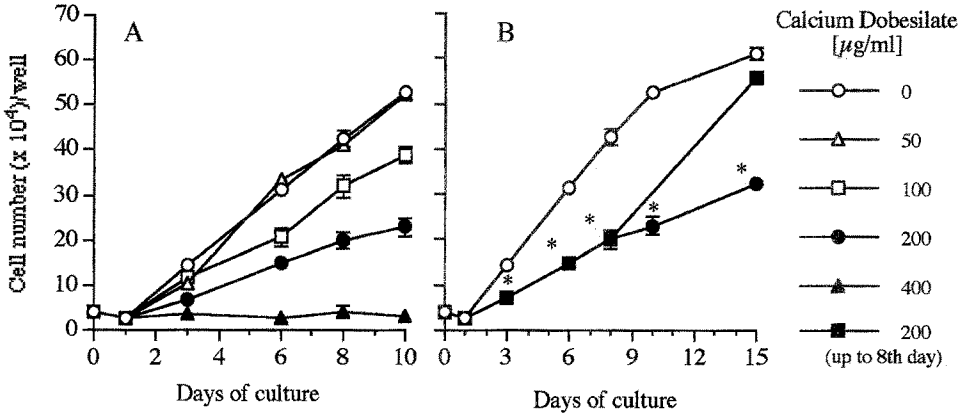

Fig. 2. (A) Effect of calcium dobesilate (50–400 μg/ml) on the growth curve of rat aortic SMC in culture. The drug was added 24 hours after seeding. **(B)** Reversibility of the effect of 200 μg/ml calcium dobesilate on SMC proliferation. Drug was added 24 hours after seeding. After 8 days in culture, some treated cells were washed and maintained in the absence of calcium dobesilate. Each point represents mean ± SEM of two separate experiments containing four replicates.

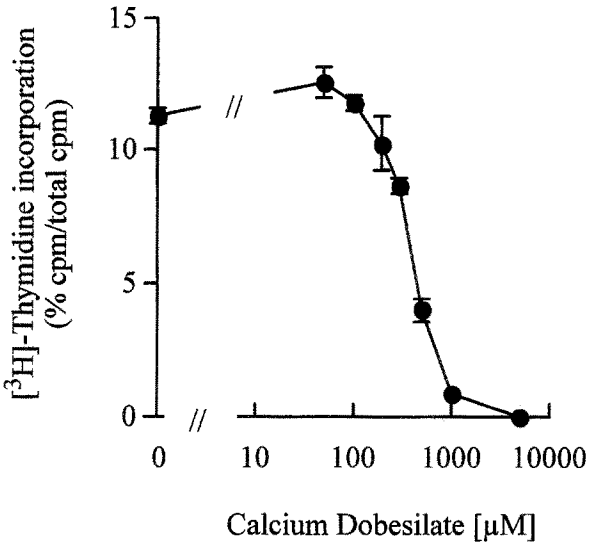

Fig. 3. Effect of calcium dobesilate on [3H]thymidine incorporation into DNA of rat aortic SMC in culture. Twenty-four hours after seeding, the cells were arrested by 72 hours incubation in medium containing 0.5% FCS. SMC were then incubated with increasing concentrations of calcium dobesilate for an additional 24 hours and labeled during the last 4 hours of the incubation period. [^{3}H]Thymidine incorporation was determined as described in Methods. Each point represents mean ± SEM of two separate experiments containing four replicates.

companied by a significant lactate dehydrogenase release (data not shown), indicating that the antiproliferative effect of calcium dobesilate was not due to a toxic effect. The inhibitory effect was observed at relatively high concentrations (0.1–1 mM). Similar calcium dobesilate concentrations were also required to increase the constitutive NOS activity in endothelial cells [9]. Clinical studies have shown that after a single dose of 500 mg of dobesilate, serum concentrations in man were in the range 25–137 μM [18]. However, the beneficial effects of dobesilate are apparent after a treatment for weeks, suggesting that accumulative doses are required for significant results. So, calcium dobesilate effects were observed in a potential therapeutic dose range. The calcium dobesilate-induced inhibition of SMC proliferation was associated with increases in nitrite accumulation and L-citrulline formation. The effect of calcium dobesilate on DNA synthesis was partially inhibited with two NO synthesis inhibitors, L-NMMA and aminoguani-

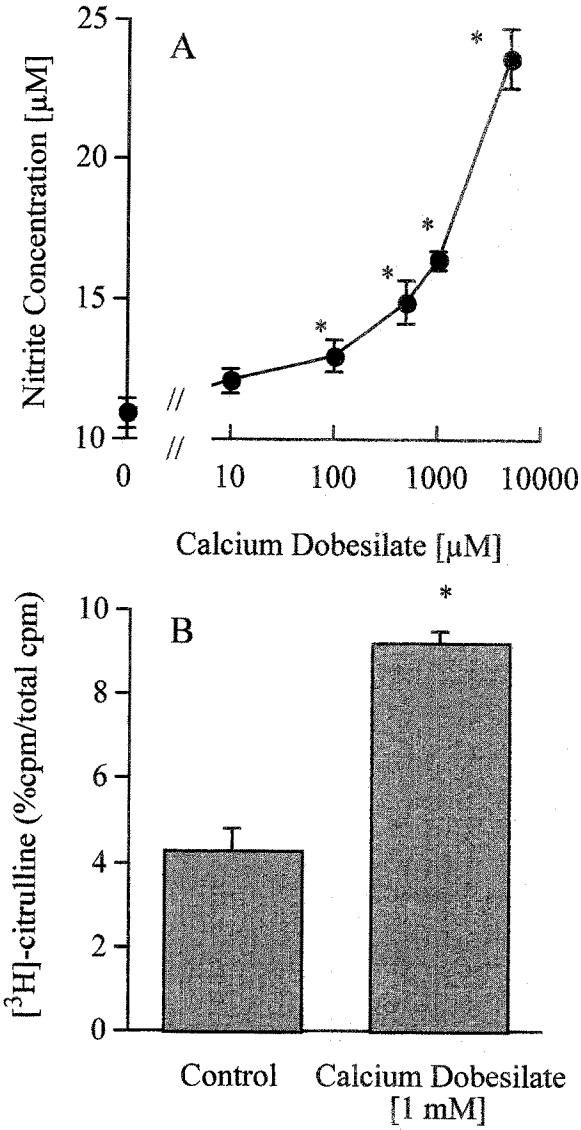

Fig. 4. **(A)** Effect of calcium dobesilate (10–10,000 μM) on the release of nitrite. Nitrite concentration in the medium was determined after a 48-hour incubation. **(B)** Formation of L-citrulline from L-arginine by the cytosolic fractions from homogenates of untreated confluent SMC (control) and of confluent SMC exposed to calcium dobesilate (1 mM) for 24 hours. Values are mean ± SEM of two separate experiments containing four replicates. *Significant ($p < 0.01$) stimulatory effect of calcium dobesilate.

Table 1. Effect of L-NMMA, aminoguanidine, methylene blue, or indomethacin on calcium dobesilate-induced inhibition of [^{3}H]thymidine incorporation of rat aortic SMC in culture

	Calcium dobesilate (300 μM)				
	Control	L-NMMA (3 mM)	Aminoguanidine (0.5 mM)	Methylene blue (10 μM)	Indomethacin (1 μg/ml)
[^{3}H]Thymidine incorporation (% inhibition)	54.1 ± 1.89	39.9 ± 1.67*	24.3 ± 5.27*	36.4 ± 1.11*	49.4 ± 1.33

L-NMMA, N^G-monomethyl-L-arginine. Values means ± SEM from two separate experiments containing three of four replicates in each experiment. *$p < 0.01$, significantly different from cells treated with calcium dobesilate alone.

dine, and with the soluble guanylate cyclase inhibitor methylene blue. These results suggested that NO and cGMP were partially implicated in the inhibition of SMC proliferation by calcium dobesilate. Prostaglandins are also known to inhibit DNA synthesis in SMC [1]. Furthermore, NO is also suggested to mediate prostaglandin production in SMC [19]. However, indomethacin, a cyclooxygenase inhibitor, did not modify the calcium dobesilate effect.

It has been shown that concentrations of NO donors required to inhibit DNA synthesis and proliferation of cultured SMC are in the range of 10^{-5}–10^{-3} M [20], which are 2- to 3-fold higher than concentrations required for smooth muscle relaxation. Calcium dobesilate at 10 μM increases endothelium-dependent relaxation in rabbit isolated aorta [10], whereas its antiproliferative effect was observed at 0.1–1 mM. Thus, NO production induced by calcium dobesilate in isolated SMC is not sufficient to inhibit the proliferation induced by serum mitogenic factors.

Calcium dobesilate provided a protective effect on cooper-induced LDL oxidation. This result is in agreement with recent data showing that calcium dobesilate protects human erythrocyte membranes and bovine aortic endothelial cells against the effects of oxygen-reactive species [11] and human peripheral blood mononuclear cells from oxidation and apoptosis [12]. Oxidized LDL possesses a number of potentially atherogenic activities. On arterial SMC, lightly oxidized LDL has a mitogenic effect in atherogenesis, and strongly oxidized LDL has a cytotoxic effect which is likely to play a role in the atherosclerotic lesion evolution leading to the plaque rupture [6]. The supply of antioxidants appears to have an inhibitory effect on the development of experimental atherosclerosis [21] and partially protects cultured SMC against the toxicity of oxidized LDL [22]. Thus, the antioxidant effect of calcium dobesilate could be an additional action on vascular complications.

Our *ex vivo* and *in vitro* results indicate that calcium dobesilate interferes with the accelerated proliferation of vascular SMC and with the oxidation of LDL. The first process is a prominent component in restenosis after percutaneous transluminal angioplasty. Thus, the ability of calcium dobesilate to inhibit SMC proliferation may be of potential clinical importance for an antiproliferative therapy. Some overall clinical benefit in patients undergoing balloon angioplasty of the coronary artery has been shown with NO donors. However, the benefit may be due to effects of NO other than direct inhibition of SMC proliferation [3]. In contrast, oxidative stress and the oxidation of LDL are majors mechanisms in the pathogenesis of atherosclerosis rather than SMC proliferation. Antiatherogenic therapy uses cholesterol-lowering hydroxymethylglutaryl coenzyme A reductase inhibitors (vastatins) and antioxidants. However, vastatins (lovastatin, simvastatin, fluvastatin) also inhibit SMC growth *in vitro* [23]. The ability of vastatins to inhibit SMC proliferation is directly related to their capacity to reduce cholesterol synthesis and it is observed at the reported clinical concentration (0.1 μM). Calcium dobesilate has been shown to inhibit *in vitro* SMC proliferation at higher concentrations than vastatins but also higher concentrations of calcium dobesilate are required in clinical therapy. Furthermore, lovastatin might also act as an antioxidant both *in vivo* and *in vitro* by decreasing plasma lipid peroxides and the oxidizability of LDL by leukocytes [21]. Thus, antiatherogenic effect of these agents may be, at least in part, mediated through a common antioxidant pathway.

In conclusion, our results demonstrated the antiproliferative effect of calcium dobesilate, both *ex vivo* and *in vitro*, and its antioxidant effect on cooper-induced LDL oxidation. It appears that calcium dobesilate may effectively interfere with multiple components of the atherosclerotic process. This, togehter with the observation that calcium dobesilate is not toxic, makes it a potentially useful compound for preventing vascular SMC proliferation. Since diseases associated with SMC hyperplasia are multifactorial, one would expect that a combination of several therapeutic modalities would lead to an optimal result. Our studies indicate that calcium dobesilate alone or in combination with other antiatherogenic compounds should be a candidate for prevention of arterial restenosis and accelerated atherosclerosis.

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