Geranylgeraniol prevents the cytotoxic effects of mevastatin in THP-1 cells, without decreasing the beneficial effects on cholesterol synthesis

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**Background and purpose:** Statins, inhibitors of hydroxymethylglutaryl-CoA reductase, reduce the intracellular synthesis of cholesterol and prevent the onset of atherosclerosis. They also decrease the synthesis of isoprenoid molecules, such as the side chain of ubiquinone and geranylgeranyl pyrophosphate. As a consequence, statins impair mitochondrial metabolism and the activation of small monomeric GTPases (such as Rho and Ras), causing toxic effects. To date, a successful strategy to prevent statin toxicity is lacking.

**Experimental approach:** In human monocytic THP-1 cells, we measured the synthesis of cholesterol and isoprenoids, mitochondrial electron flow, the activity of RhoA and Rac, cell death and proliferation.

**Key results:** Mevastatin reduced the synthesis of cholesterol, geranylgeranyl pyrophosphate and ubiquinone, mitochondrial electron transport, activity of RhoA and Rac, and cell proliferation, accompanied by increased cell death. Geranylgeraniol, a cell-permeable analogue of geranylgeranyl pyrophosphate, reversed all these effects of mevastatin, without affecting its ability to reduce cholesterol synthesis. Notably, geranylgeraniol was more effective than the addition of exogenous ubiquinone, which rescued mitochondrial respiratory activity and reversed mevastatin cytotoxicity, but did not alter the decrease in cell proliferation. The same results were obtained in human liver HepG2 cells.

**Conclusions and implications:** Geranylgeraniol had a broader protective effect against the cytotoxicity of statins than exogenous ubiquinone. Therefore, geranylgeraniol may be a more useful and practical means of limiting the toxicities of statins, without reducing their efficacy as cholesterol lowering agents.

**Keywords:** statins; cholesterol; ubiquinone; geranylgeraniol; G-proteins

**Abbreviations:** CoQ10, coenzyme Q10; FPP, farnesyl pyrophosphate; GGO, all-trans-geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; HMGCoAR, hydroxymethylglutaryl-CoA reductase

**Introduction**

Statins, which inhibit hydroxymethylglutaryl-CoA reductase (HMGCoAR; EC 1.1.1.88), are presently the most effective drugs for decreasing the intracellular synthesis of cholesterol and circulating cholesterol levels, thus preventing the onset of atherosclerosis and cardiovascular diseases (Liao, 2005). While they decrease HMGCoAR activity, they also decrease the synthesis of isoprenoid side products of cholesterol synthesis, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Liao and Laufs, 2005). Therefore, statins may impair the isoprenylation of small monomeric GTPases (EC 3.6.5.2), like Ras and Rho, which must be iso-
statins have been proposed as potential adjuvant drugs in anticancer therapy (Sassano and Platanias, 2008). On the other hand, patients treated with statins may be subject to severe side effects, like rhadomyolysis and liver cytolysis (Levy and Kohlihaas, 2006; Marcoff and Thompson, 2007), because of the pro-apoptotic and cytotoxic effects of statins. The molecular basis of this cytoxicity is still controversial, as it may be caused either by a reduction in activity of the small monomeric GTPases or by the impairment of mitochondrial metabolism (Marcoff and Thompson, 2007).

Indeed, by decreasing the levels of isoprenoids within the cell, statins also reduce the synthesis of the side chain of ubiquinone (also called coenzyme Q10; CoQ10), an antioxidant molecule that shuttles electrons between NADH dehydrogenase (ubiquinone) (complex I; EC 1.6.5.3) and ubiquinol-cytochrome c reductase (complex III; EC 1.10.2.2) in mitochondria (Tecelebrhan et al., 1993). It has been suggested that lower intracellular levels of ubiquinone may impair the energy metabolism of muscles and eventually lead to muscle damage (Marcoff and Thompson, 2007). On the basis of this hypothesis, several clinical trials have been designed, wherein therapy with statins has been supplemented with ubiquinone (Tecelebrhan et al., 1993). The contradictory results obtained in most trials suggest that statin toxicity may not be fully prevented by the restoration of normal ubiquinone levels (Johnson et al., 2004; Levy and Kohlihaas, 2006; Marcoff and Thompson, 2007).

Therefore, an effective strategy aimed at preventing the statin-dependent impairment in cell metabolism, viability and proliferation, without a reduction of their anti-cholesterolamaic effect, is not yet available. All-trans-geranylgeraniol (GGO) is a 20-carbon, cell-permeable isoprenoid molecule, which may be phosphorylated within cells, to yield GGPP. In turn, GGPP may be a substrate for several prenyltransferases (such as protein geranylgeranyltransferase type I, EC 2.5.1.59 and protein geranylgeranyltransferase type II, EC 2.5.1.60) and may be actively involved in the ‘salvage pathways’ of isoprenoid molecules (Crick et al., 1997).

Using the THP-1 human monocytic cell line as an in vitro model, we set out to investigate how GGO could counteract the metabolic effects of statins on cholesterol synthesis, ubiquinone-dependent respiratory chain activity, RhoA/Rho kinase activity, cell proliferation and toxicity. We have also analysed whether GGO might represent an alternative to ubiquinone for the prevention of the toxic effects of the statins.

### Methods

#### Cells

Human monocytic leukaemia (THP-1) cells and human liver (HepG2) cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Cells were maintained in a humidified atmosphere at 37°C, 5% CO2 and 20% O2.

#### Measurement of cholesterol and isoprenoid synthesis

The synthesis of isoprenoids was assessed by the intracellular accumulation of cholesterol, ubiquinone and GGPP, and measured as previously described (Seifert and Lucas, 1988), with minor modifications. Cells were incubated for 24 h with 1 μCi·mL⁻¹ of [³H]acetate (3600 mCi·mmol⁻¹; Amersham BioScience, Piscataway, NJ, USA), washed twice with phosphate-buffered saline (PBS), resuspended in 200 μL of PBS and transferred in glass microcentrifuge tubes. A 1:2 methanol/hexane solution (1.5 mL) was added, and the cellular suspensions were vortexed for 1 h, then centrifuged at 2000 × g for 5 min. The upper phase was transferred in a new set of glass microcentrifuge tubes, while the lower phase was resuspended in 1 mL of hexane, vortexed overnight and centrifuged at 2000 × g for 5 min: the new upper phase was added to the previously isolated phase. After a 24-h evaporation, samples were dissolved in 30 μL of chloroform and resolved by thin layer chromatography on LK6D Whatmann silica gel (Merck, Darmstadt, Germany), using 1:1 diethyl ether/hexane as mobile phase. Standard solutions of 10 μg·mL⁻¹ cholesterol, ubiquinone or GGPP were employed. After the separation, the gel was maintained in an iodine-saturated atmosphere for 2 h, and the spots corresponding to cholesterol, ubiquinone and GGPP were isolated. The radioactivity of each spot was measured by liquid scintillation counting and expressed as fmol·(10⁶ cells)⁻¹, using calibration curves prepared previously.

#### Ubiquinone loading and spectrophotometric measurement of intracellular ubiquinone

To load cells with ubiquinone, a 10 mM aqueous solution of β-methyl-cyclohextrin, heated at 80°C, was incubated with a 1 mM solution of ubiquinone, dissolved in chloroform. After the evaporation of the solvent at room temperature, β-methyl-cyclohextrin–ubiquinone complexes were resuspended in sterile water at a final concentration of 1 mM and stored at 4°C until the use. The intracellular level of ubiquinone after the loading of β-methyl-cyclohextrin–ubiquinone complexes was measured as described previously (Karpinska et al., 1998), using a Lambda 3 spectrophotometer (PerkinElmer, Waltham, MA, USA). The absorbance was read at 390 nm and converted to nmol ubiquinone·(mg cell protein)⁻¹, using a calibration curve prepared previously.

#### Isolation of mitochondria and measurement of Complex I–III activity

To isolate mitochondrial fractions, 5 × 10⁶ cells were washed twice in ice-cold PBS, then lysed in 0.5 mL buffer A (50 mM Tris, 100 mM KCl, 5 mM MgCl₂, 1.8 mM ATP, 1 mM EDTA, pH 7.2), supplemented with protease inhibitor cocktail III (Calbiochem), 1 mM PMSF and 250 mM NaF. Samples were clarified by centrifuging at 6500 × g for 3 min at 4°C, and the supernatant was collected and centrifuged at 13 000 × g for 5 min at 4°C. This supernatant was discarded and the pellet containing mitochondria was washed in 0.5 mL buffer A and resuspended in 0.25 mL buffer B (250 mM sucrose, 15 mM K₂HPO₄, 2 mM MgCl₂, 0.5 mM EDTA, 5% w/v BSA). A 50 μL aliquot was sonicated and used for the measurement of
protein content; the remaining part was diluted to a protein concentration of 10 μg·μL⁻¹ and stored at −80°C until the use. To confirm the presence of mitochondrial proteins in the extracts, 10 μg of each sample were subjected to 15% SDS-PAGE and probed with an anti-cytochrome c antibody (diluted 1:1000 in PBS-BSA 1%, from Becton Dickinson; data not shown). The activity of Complex I–III was measured on 10 μL of non-sonicated mitochondrial samples, resuspended in 0.59 mL buffer C (5 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA) and transferred into a quartz spectrophotometer cuvette. Then 0.38 mL buffer D (25% w/v saponin, 50 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA, 0.12 mM cytochrome c oxidized form, 0.2 mM NaN₃) was added for 5 min at room temperature. The reaction was started with 0.15 mM NADH and was followed for 5 min, reading the absorbance at 550 nm by a Lambda 3 spectrophotometer (PerkinElmer). Under these experimental conditions, the rate of cytochrome c reduction, expressed as nmol cyt c reduced·min⁻¹·(mg cell protein)⁻¹, was dependent on the activity of both Complex I and Complex III. Thereafter, 0.05 mM rotenone was added to the cuvette, and the reaction was followed for further 5 min. In this phase, the rate of cytochrome c reduction was dependent on ubiquinone and Complex III activity only and was taken as an index of the amount of active ubiquinone in mitochondria (Wibom et al., 2002).

Activation of small monomeric GTPases

Binding of GTP, which is considered an index of Rac and RhoA activation (Laufs and Liao, 2000), was measured using the G-LISA™ Rac 1/2/3 Activation Assay Biochem Kit or the G-LISA™ RhoA Activation Assay Biochem Kit (Cytoskeleton Inc., Denver, CO, USA) respectively. Cells (1 × 10⁶) were grown for 24 h under the experimental conditions indicated under Results were washed with ice-cold PBS, lysed in 0.2 mL lysis buffer of the G-LISA™ Biochem Kit. Data were expressed as nmol GTP-bound·min⁻¹·(mg cell protein)⁻¹.

Rho kinase activity

Rho kinase activity was measured using the CycLex Rho Kinase Assay Kit (CycLex Co., Nagano, Japan), a single site binding immunoassay, as previously reported (Riganti et al., 2006), using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). For each set of experiments, a titration curve was prepared, using serial dilution of recombinant Rho kinase (ROCK2, MBL Inc., Woburn, MA, USA) in the kinase buffer of the kit. Data were expressed as nmol phospho-MBS (myosin binding subunit of myosin phosphatase)-min⁻¹·(mg cell protein)⁻¹.

Cytotoxicity assays

After a 24 h incubation under the different experimental conditions described in the Results section, the extracellular medium was collected and centrifuged at 12 000× g for 15 min to pellet cellular debris. The cells were washed with fresh medium, resuspended at 1 × 10⁶ cells·mL⁻¹ in 0.2 mL of 82.3 mM triethanolamine phosphate hydrochloride (pH 7.6) and sonicated. L-lactate dehydrogenase (LDH; EC 1.1.1.27) activity was measured in the extracellular medium and in the cell lysate, as previously described (Riganti et al., 2006). Both intracellular and extracellular enzyme activities were expressed as μmol NADH oxidized·min⁻¹·(10⁶ cells)⁻¹ and the extracellular LDH activity was calculated as percentage of the total LDH activity. In the same experimental conditions, cytosol and mitochondrial fractions were isolated as reported above; 10 μg of proteins from each fraction were diluted 1:2 in Laemmli buffer and subjected to a Western blotting analysis to assess the cytosol release of cytochrome c, taken as an index of apoptosis (Russo et al., 2005).

[³H]thymidine incorporation

Cells(0.5 × 10⁶) were grown for 24 h under the experimental conditions reported in Results, in a culture medium containing 1 μCi·mL⁻¹ [³H]thymidine (62 Ci·mmol⁻¹; Amersham Bioscience). Cells were washed with ice-cold PBS, resuspended in 200 μL of PBS and transferred in polyethylene vials. The radioactivity was measured by liquid scintillation. [³H]thymidine incorporated in each sample was expressed as pmol·(10⁶ cells)⁻¹, according to the titration curve prepared previously.

Statistical analysis

All data in text and figures are provided as means ± SE. The results were analysed by a one-way analysis of variance (ANOVA). P < 0.05 was considered significant.

Materials

Fetal bovine serum and culture medium were supplied by BioWhittaker (Walkersville, MD, USA); plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA); mevastatin, simvastatin, GGTI-286, Y27632 and camptothecin were from Calbiochem (San Diego, CA, USA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The protein content of cell monolayers and lysates was assessed with the BCA Kit from Sigma-Aldrich Co (St. Louis, MO, USA). When not otherwise specified, the other reagents were obtained from Sigma-Aldrich Co. Drug/molecular target nomenclature follows Alexander et al. (2008).

Results

Statins decrease the synthesis of cholesterol, ubiquinone and GGPP in THP-1 cells

THP-1 cells were incubated for 24 h with different concentrations of either mevastatin or simvastatin, then washed and
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The decrease of cholesterol and ubiquinone induced by mevastatin in THP-1 cells was completely prevented by mevalonic acid (Figure 2A). When GGO was used instead of mevalonic acid, a different pattern of response was observed. Incubation of THP-1 cells with GGO alone increased the synthesis of ubiquinone, with no change in the synthesis of cholesterol. When incubated with mevastatin, GGO did not reverse the inhibition of cholesterol synthesis, but still prevented the decrease in ubiquinone (Figure 2A).

Mevastatin inhibited also the ubiquinone-dependent mitochondrial electron transport, as measured by the rate of reduction of cytochrome c (Figure 2B). This effect was clear both in the absence of rotenone, when the electron flow was dependent on Complex I plus ubiquinone activity, and in the presence of rotenone, when the electron transport only relied on ubiquinone activity. Both mevalonic acid and GGO prevented
the effects of mevastatin, with or without rotenone (Figure 2B). GGO alone, which was able to increase the synthesis of ubiquinone (Figure 2A), also enhanced the electron flow after the addition of rotenone, whereas mevalonic acid alone had no effect (Figure 2B). We also measured the rate of cytochrome c reduction in THP-1 cells loaded with exogenous ubiquinone. After a 24 h incubation with 10 μM β-methyl-cyclodextrin–ubiquinone, the intracellular ubiquinone amount significantly increased from 1.93 ± 0.03 nmol ubiquinone-(mg cell protein)−1 to 14.55 ± 2.58 nmol ubiquinone-(mg cell protein)−1 (n = 3, P < 0.005). In ubiquinone-loaded cells, there was an increase in cytochrome c reduction in the presence of rotenone and no significant change even when cells were incubated with mevastatin (Figure 2B). Notably, the effect of GGO was the same as that obtained after exogenous ubiquinone. GGPP was as effective as GGO in preventing the effects of mevastatin (data not shown).

GGO restores the activity of RhoA/Rho kinase, inhibited by mevastatin
Mevastatin decreased the binding of GTP to RhoA (measured as an index of its activation) in THP-1 cells, with the same efficacy as the inhibitor of the protein geranylgeranyltransferases, GGTTI-286 (Figure 3A). Conversely, mevalonic acid and GGO, which by themselves did not modify the GTP-binding relative to control cells, prevented the effects of mevastatin on RhoA activation (Figure 3A). The same results were obtained when cells were incubated with GGPP instead of GGO (data not shown). Mevastatin and GGO produced the same effects, as on RhoA, when tested on Rac, another geranylgeranylated protein present in THP-1 cells (Figure 3A). To assess the specificity of GGO, we performed a pull-down assay for Ras-GTP: as expected, mevastatin reduced the level of GTP-bound Ras. This effect was reversed by mevalonic acid and farnesol, but not by GGO (Figure 3B).

As a consequence of the reduced activity of RhoA, mevastatin also prevented the activation of Rho kinase in THP-1 cells, mimicking the effect of the Rho kinase inhibitor Y27632 (Figure 3C). GGO and mevalonic acid, which by themselves did not exert any appreciable effect in the control cells, completely reversed the inhibition of Rho kinase activity induced by the statin (Figure 3C).

GGO protects cells from the cytotoxic and anti-proliferative effects of mevastatin
Mevastatin significantly increased the release of LDH activity into the extracellular medium, and such cytotoxic effects were prevented when mevalonic acid or GGO were co-incubated with mevastatin (Figure 4A). The statin also induced a release of cytochrome c from mitochondria into the cytosol, not seen in untreated cells; again mevalonic acid and GGO inhibited this effect (Figure 4B). Besides inducing cytotoxicity, mevastatin also decreased the incorporation of [3H]thymidine into the cells, again restored by mevalonic acid and GGO (Figure 4A). In THP-1 cells loaded with exogenous ubiquinone, mevastatin did not increase the extracellular LDH activity, but still decreased cell proliferation (Figure 4A). Moreover, ubiquinone greatly reduced, but did not completely prevent, the cytosolic release of cytochrome c elicited by mevastatin (Figure 4B). When used alone, mevalonic acid, GGO and ubiquinone did not affect LDH release or [3H]thymidine incorporation (Figure 4A), GGPP had the same effects of GGO on these two parameters (data not shown).

The effect of mevastatin on cell toxicity and proliferation was independent of the inhibition of cholesterol synthesis. Indeed, incubation of THP-1 cells with zaragozic acid (10 μM for 24 h), an inhibitor of squalene synthase (EC 2.5.1.21), which reduced cholesterol synthesis, without changing the levels of isoprenoids, was not cytotoxic and did not reduce the proliferation of THP-1 cells (Figure S2).

The toxic effects of the statin were reproduced by inhibiting the RhoA/Rho kinase pathway: the protein geranylgeranyl-
transf erase s inhibitor GGTI-286 and the Rho kinase inhibitor Y27632 increased LDH release and reduced [3H]thymidine incorporation in THP-1 cells. In the presence of FPP or farnesol, mevastatin was able to reduce the synthesis of cholesterol and ubiquinone (Figure 6A) and to lower the ubiquinone-dependent mitochondrial electron transport (Figure 6B) in THP-1 cells. Both statins reduced, as expected, the synthesis of cholesterol, ubiquinone and GGPP in human monocytic THP-1 cells. In THP-1 cells, mevalonic acid reversed the effect of mevastatin on cholesterol and ubiquinone levels, confirming that they were attributable to the inhibition of HMGCoAR. When we co-incubated mevastatin with all-trans-GGO, a cell-permeable precursor of GGPP (Crick et al., 1997), the inhibition of cholesterol synthesis, but with the decreased production of isoprenoid molecules, such as FPP and GGPP, which are a part of the lipid tail of Ras and Rho GTPases and are essential for their activation (Swanson and Hohl, 2006). By preventing the activation of Ras and Rho GTPases, statins may modulate the processes controlled by these proteins, such as cell proliferation and apoptosis, cytoskeleton remodelling, cell adhesion, motility and angiogenesis (Fritz and Kaina, 2006; Swanson and Hohl, 2006). The broad spectrum of cell signalling pathways affected by statins may account for their pleiotropic therapeutic benefits, but also for the undesired effects of the statins, such as rhabdomyolysis and liver toxicity (Levy and Kohlhaas, 2006; Marcoff and Thompson, 2007).

Discussion

Statins have significantly reduced the onset of cardiovascular diseases and the progression of atherosclerotic lesions and are widely prescribed for that purpose (Liao, 2005). Recently, statins have been proposed as adjuvant therapy in diabetes, heart failure, rheumatologic diseases, osteoporosis, Alzheimer’s disease and multiple sclerosis (Liao, 2005). Moreover, they have recently shown to enhance the efficacy of several antineoplastic drugs (Sassano and Piltanas, 2008). These pleiotropic effects of statins do not seem to correlate with
synthesis and the activity of ubiquinone was also restored, although the synthesis of cholesterol still remained low. Thus GGO seems to have promise in reversing the decrease of ubiquinone levels caused by statins, without reducing their inhibitory effect on cholesterol synthesis. Interestingly, GGO by itself elicited a significant increase of ubiquinone synthesis, whereas basal cholesterol levels were not affected. This reflect the fact that the ubiquinone synthesis is less finely regulated than cholesterol production, and that GGPP (which derives from GGO within the cell) can promote the synthesis of the ubiquinone side chain, by a simple mass action effect, in our cell line. This observation may appear in contrast to previous results showing that GGO was not incorporated into ubiquinone in rat glioma C6 cells (Crick et al., 1994). However, the metabolic pathways leading to the synthesis of ubiquinone side chain are highly variable in different cell types and species (Teclebrhan et al., 1993). The increase of ubiquinone synthesis was fairly specific for GGO and GGPP, as neither farnesol nor FPP had similar effects on ubiquinone levels and activity.

Ubiquinone, working as an electron shuttle between Complex I and III in the mitochondria, plays a crucial role in the respiratory chain, which yields the synthesis of ATP. GGO increased the rate of reduction of cytochrome c in the presence of rotenone, thus enhancing the electron flow dependent on the amount of active ubiquinone (Wibom et al., 2002), and prevented the decrease of electron flow caused by mevastatin, with the same efficacy as loading the cells with exogenous ubiquinone. This result suggests that GGO prevented, just as well as ubiquinone, the inhibition of mitochondrial metabolism exerted by mevastatin.

The pool of GGO/GGPP is a substrate for either the long-chain trans-prenyl diphosphate synthases or the protein geranylgeranyltransferases, which add GGPP to small monomeric GTPases like Rho and Rac. Therefore, GGPP may be transferred to ubiquinone or to proteins, depending on the cell demand and on the enzyme affinity. When used alone, GGO increased the synthesis of ubiquinone but not the GTP-binding activity of Rac and RhoA, suggesting that, in non-stimulated THP-1 cells, GGPP was incorporated preferentially.
into the ubiquinone side chain, rather than into the GTPases. However, when co-incubated with mevastatin, GGO prevented both the decrease of RhoA and Rac activity and the decrease of mitochondrial electron flow. This result suggested that the amount of GGPP derived from GGO is sufficient to sustain the activity of both the protein geranylgeranyltransferases and the ubiquinone synthesis, in the presence of statins. Geranylgeranylated proteins are involved in several functions in monocytes. For instance, statins affect cell chemotaxis (Pozo et al., 2006), secretion of extracellular enzymes (Wong et al., 2001) and cholesterol uptake and efflux (Ruiz-Velasco et al., 2004; Argmann et al., 2005), by inhibiting geranylgeranylation. It is possible that GGO may restore these particular activities in monocytes.

Another crucial issue we addressed was the effect of depre

nzylation of small monomeric GTPases on cell survival and proliferation as, in several cell types, statins reduce cell proliferation (Senokuchi et al., 2005) or induce apoptosis (Johnson et al., 2004). We confirmed these observations in THP-1 cells, where mevastatin reduced the incorporation of [3H]thymidine, increased the release of LDH in the extracellular medium and the release of cytochrome c from mitochondria to cytosol. It has been proposed that an adequate supplementation of ubiquinone would restore the mitochondrial respiration and prevent statin toxicity (Marcoff and Thompson, 2007). However, the results of clinical trials with statins and ubiquinone have been contradictory and have suggested that the administration of CoQ10 may result in detectable benefits only in small subgroups of patients taking statins (Levy and Kohlihaas, 2006; Marcoff and Thompson, 2007).

In rat and human myotubes, treated with cerivastatin, there was no correlation between the content of ubiquinone and the prevention of apoptosis (Johnson et al., 2004). In THP-1 cells, the only restoration of mitochondrial metabolism seemed important, but not sufficient, to fully prevent the toxic effects of statin. Indeed, in cells loaded with ubiquinone, mevastatin did not elicitch a significant release of LDH in the extracellular medium, but still induced a slight release of cytochrome c into the cytosol and blocked cell proliferation. The activity of geranylgeranylation-sensitive proteins, mainly the RhoA/Rho kinase pathway, seems to be the second critical factor regulating cell death or proliferation in our experimental model: GGO, which restored not only the mitochondrial electron flow, but also the activity of small monomeric GTPases, reversed both the cytotoxic and anti-proliferative effects of mevastatin. On the other hand, GGTL-286 or Y27632, which inhibited the protein geranylgeranyltransferases and Rho kinase, respectively, mimicked the toxic effects of statins and fully antagonized GGO. These results confirm that the activity of RhoA/Rho kinase was crucial in explaining statin-induced cell death, as recently shown in L6 rat fibroblasts (Itagaki et al., 2009). In our experimental model, the mechanism by which statins affect cell death and proliferation seems dependent on the levels of GGPP in a rather specific manner. In THP-1 cells treated with FPP or farnesol, which restored the activity of Ras without modifying geranylgeranylation, mevastatin was still able to increase the extracellular release of LDH, to raise the cytosolic amounts of cytochrome c and to reduce [3H]thymidine incorporation. However, we cannot exclude the possibility that, in cells other than human monocytes, the toxic effects of statins may be provoked by reduced farnesylation of proteins, making GGO ineffective in counteracting toxicity. For example, only inhibition of protein farnesyl transferase, but not of geranylgeranylation, reduced cell survival in human glioma cells (Pollack et al., 1999).

The protective role of GGO is not generalized to all cell types. Treatment with GGO (10 μM, 48 h) did not protect primary rat hepatocytes from cerivastatin-induced apoptosis and was toxic by itself (Johnson et al., 2004). An increased DNA fragmentation was observed also in U937 cells exposed to GGO at concentrations higher than 50 μM (Masuda et al., 2006). It is likely that above certain concentrations and incubation times, but not under our experimental conditions, GGO could be pro-apoptotic. The activation of RhoA can up-regulate cell proliferation (Fritz and Kaina, 2006) and thus GGO, which is expected to enhance RhoA activation, may theoretically also increase cell proliferation. However, in THP-1 cells, GGO did not increase RhoA and Rho kinase activity in the untreated cells and did not increase the incorporation of [3H]thymidine. GGO, like mevalonic acid, did not stimulate cells proliferation per se, but only ‘buffered’ the effect of mevastatin.

In our study we have analysed statins and GGO effects in THP-1 cells, as monocytes have an active part in the genesis of atheroma and cardiovascular diseases, two pathological conditions in which the cholesterol levels and the effect of cholesterol-lowering drugs play a significant role (Laufs and Liao, 2000; Liao, 2005; Liao and Laufs, 2005). Moreover, peripheral blood mononuclear cells are a cell type easy to collect from patients and therefore they may be used as a ‘standard’ to monitor the side effects of statins and the putative benefits derived from a possible GGO/ubiquinone supplementation in patients taking statins. GGO exerted identical results in the human hepatocyte HepG2 cells (Figures S3 and S4), showing that liver cells, another well-known target of statin toxicity (Levy and Kohlihaas, 2006; Marcoff and Thompson, 2007) might also be protected. Our work suggests that GGO has a broader antagonism than ubiquinone/CoQ10, of the toxic effects of statins, as ubiquinone prevented the statin-induced impairment of mitochondrial respiration and was effective in cytoprotection, but did not restore the basal rate of cell proliferation. On the other hand, GGO, which is generated in an early step of the mevalonate cascade and is involved in a wide range of signalling pathways, reversed all the toxic effects of mevastatin, which are likely due to a decreased geranylgeranylation. Importantly GGO did not change the effect of mevastatin on cholesterol synthesis. GGO is a cell-permeable and readily diffusible compound, and an oral supplementation with GGO was not toxic in animal models, where it was used as a chemopreventive agent (Burke et al., 1997). However, most of the beneficial effects of statins on the cardiovascular system are independent of their cholesterol-lowering activity, but are a consequence of the reduced isoprenylation of small GTPases. Indeed the inhibition of the RhoA/Rho kinase pathway reduced monocyte adhesion to vascular endothelium (Yoshida et al., 2001), as well as the pro-adhesive properties of endothelial cells (Bolick et al., 2005), thereby preventing two
critical steps in the genesis of atheroma. Moreover, a diminished Rho kinase activity is associated with an increased activity of the endothelial NO synthase (Arita et al., 2009) and to a reduced vascular tone (Oka et al., 2008). We cannot exclude the possibility that, by restoring the activity of Rho kinase, GGO may reduce part of the beneficial pleiotropic effects of statins. On the other hand, the reduced geranylgeranylation of RhoA is a crucial mechanism in their toxic effects (Itagaki et al., 2009), and we have demonstrated that GGO attenuated mevastatin cytotoxicity. Our results suggest that GGO supplementation might be proposed as an alternative to supplementation with CoQ10, in order to reduce toxic effects of statins, without affecting the cholesterol-lowering efficacy. However, further in vivo studies should be performed to ascertain dosage, safety and efficacy of GGO and to evaluate whether this compound, besides limiting the toxic effects of statins, does not also diminish their pleiotropic benefits for the cardiovascular system.

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Conflict of interest

None.

References


Arita R, Hata Y, Nakao S, Miura M, Kawahara S et al. (2009). Geranylgeraniol reduces part of the beneficial pleiotropic effects of GGO, and we have demonstrated that GGO attenuated mevastatin cytotoxicity. Our results suggest that GGO supplementation might be proposed as an alternative to supplementation with CoQ10, in order to reduce toxic effects of statins, without affecting the cholesterol-lowering efficacy. However, further in vivo studies should be performed to ascertain dosage, safety and efficacy of GGO and to evaluate whether this compound, besides limiting the toxic effects of statins, does not also diminish their pleiotropic benefits for the cardiovascular system.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Effect of simvastatin at low concentrations on cholesterol, ubiquinone and geranylgeranyl pyrophosphate (GGPP) synthesis in THP-1 cells. Cells were incubated in the absence (CTRL) or presence of 1 and 0.1 μM simvastatin (SM) for different time periods (24, 48, 72 and 96 h), then they were washed and grown for 24 h in culture medium containing [3H]acetate. The content of cholesterol (open bars), ubiquinone (hatched bars) and GGPP (solid bars) was measured in duplicate as indicated in the Methods section. Data are presented as means ± SE (n = 3). *P < 0.01 versus CTRL.

**Figure S2** Effects of the squalene synthase inhibitor zaragozic acid on cholesterol and ubiquinone synthesis, cell toxicity and proliferation. THP-1 cells were incubated for 24 h in the absence (CTRL) or presence of zaragozic acid (10 μM, ZA), then subjected to the following investigations. (A,B) Cells were radiolabelled for 24 h with [3H]acetate, then the content of cholesterol (A) and ubiquinone (B) was measured in duplicate as reported under Methods. Data are presented as means ± SE (n = 3). *P < 0.001 versus CTRL. (C,D). The L-lactate dehydrogenase (LDH) activity in the extracellular medium (C) and the [3H]thymidine incorporation (D) were assessed in duplicate as indicated in the Methods section. Data are presented as means ± SE (n = 3).

**Figure S3** Effect of mevastatin, farnesol and geranylgeraniol on cholesterol and ubiquinone synthesis, mitochondrial Complex I-II activity, RhoA-GTP binding, cytotoxicity and proliferation in HepG2 cells. Human liver cancer HepG2 cells were incubated for 24 h in the absence (CTRL) or presence of mevastatin (10 μM, MVS), farnesol (10 μM, FO) and GGO (10 μM, GGO), in different combinations. When indicated, a 10 μM solution of β-methyl-cyclodextrin-ubiquinone (UB) was added in the culture medium to load cells with ubiquinone; under this experimental condition the amounts of intracellular ubiquinone, measured with a spectrophotometric assay as reported in the Methods section, was significantly increased (HepG2 control cells: 0.82 ± 0.02 nmol ubiquinone·(mg cell protein))⁻¹; ubiquinone-loaded cells: 18.07 ± 2.1 nmol ubiquinone·(mg cell protein))⁻¹; n = 3; P < 0.002). The following investigations were performed. (A) Cells were radiolabelled for 24 h with [3H]acetate, then the content of cholesterol (open bars) and ubiquinone (hatched bars) was measured in duplicate as reported under Methods. Data are presented as means ± SE (n = 3). *P < 0.002 versus CTRL; °P < 0.001 versus MVS. (B) Cells were lysed, and the mitochondrial fractions were isolated, then the rate of reduction of cytochrome c in the absence (open bars) or presence (hatched bars) of 0.05 mM rotenone was detected, as described in the Methods section. The measurements were performed in duplicate, and data are presented as means ± SE (n = 3). *P < 0.05 versus CTRL; °P < 0.002 versus MVS. (C) The cells were lysed and subjected to an ELISA assay to detect the amount of RhoA-GTP, as described under Methods. GGTI-286 (10 μM, GGTI) was used as a specific inhibitor of protein geranylgeranyltransferases. The measurement were performed in duplicate, and data are presented as means ± SE (n = 3). *P < 0.001 versus CTRL; °P < 0.001 versus MVS. (D) The L-lactate dehydrogenase (LDH) activity in the extracellular medium (open bars) and the [3H]thymidine incorporation (hatched bars) were assessed in duplicate as indicated in the Methods section. Data are presented as means ± SE (n = 3). *P < 0.05 versus CTRL; °P < 0.005 versus MVS.

**Figure S4** Effect of GGO on the cytotoxicity and the anti-proliferative effects exerted by the inhibitors of protein geranylgeranyltransferases and Rho kinase in HepG2 cells. Cells were cultured for 24 h in the absence (CTRL) or presence of GGO (10 μM, GGO), GGTI-286 (10 μM, GGTI) and Y27632 (10 μM, Y27632), in different combinations. The extracellular L-lactate dehydrogenase (LDH) activity (open bars) and the [3H]thymidine incorporation (hatched bars) were measured in duplicate as described under Methods. Data are presented as means ± SE (n = 3). *P < 0.02 versus CTRL; °P < 0.02 versus GGO.

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