Geranylgeraniol prevents statins side effects

GERANYLGERANIOL PREVENTS THE SIDE EFFECTS OF MEVASTATIN, WITHOUT REDUCING THE ANTICHOLESTEROLEMIC EFFICACY
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Statins, which inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase, effectively reduce the intracellular synthesis of cholesterol and prevent the onset of atherosclerosis. However, they also decrease the synthesis of isoprenoid molecules, like geranylgeranyl pyrophosphate, thus impairing the activation of small G proteins (such as Rho, Rac and Cdc42) crucial to cytoskeleton remodelling, cell adhesion, motility, proliferation and apoptosis. By this way, statins may exert both beneficial and toxic effects on human tissues. In human monocytic THP-1 cells mevastatin reduced the synthesis of cholesterol, geranylgeranyl pyrophosphate and ubiquinone, the mitochondrial electrons transport, the activity of RhoA and Rac, the assembly of NADPH oxidase, the production of superoxide, and impaired the F-actin fibers organization and the surface expression of adhesion molecules. Geranylgeraniol (GGO), a cell-permeant analogue of geranylgeranyl pyrophosphate, reverted all the effects of mevastatin, without affecting its ability to reduce the cholesterol synthesis. Moreover mevastatin was cytotoxic for THP-1 cells and reduced their rate of proliferation: these effects were mimicked by the geranylgeranyltransferase inhibitor GGTI-286 and by the RhoA kinase inhibitor Y27632. GGO prevented both the increased cell death and the decreased proliferation elicited by mevastatin, and was more effective than the addition of exogenous ubiquinone, which rescued the mitochondrial respiratory activity and reversed the statin’s cytotoxicity, but had no effect on the decreased cell proliferation. Our results suggest that GGO may be associated to the therapy with statins, in order to prevent their side effects without reducing their efficacy as anticholesterolemic drugs.

Atherosclerosis is the main risk factor for cardiovascular diseases and is often associated with hypercholesterolaemia; in several clinical trials, the incidence of atherosclerotic lesions has been strongly reduced by lowering the blood cholesterol levels (1). Statins, which inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), are actually the most effective drugs to reduce the intracellular synthesis of cholesterol and the circulating cholesterol levels. In reducing the HMGCoAR activity, they also decrease the synthesis of isoprenoid side products of the cholesterol synthesis, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (2). As a consequence, statins may impair the isoprenylation of small G-proteins, like Ras and Rho, which must be isoprenylated to bind and hydrolyze GTP and to activate their downstream effectors (3). Rho proteins, which include Rho A-G, Rac 1-3 and Cdc42 members, play an extensive role in cytoskeleton remodelling, cell adhesion, motility and proliferation. Most of these functions are mediated by downstream serine/threonine kinases, such as RhoA-dependent kinase (RhoA kinase), protein kinase N, phosphatidic acid kinase, mitogen-activated kinases, c-Jun-N terminal kinase, etc… (4). Focusing on activated leukocytes, a peculiar process controlled by the active p21 Rac is the recruitment of the scaffold proteins p67phox and p47phox; after this assembly, the trimer translocates to the plasma membrane, where it binds the subunits gp91phox/p22phox forming the fully active NADPH oxidase complex, which triggers the production of superoxide anion radical (O₂•⁻) (5).
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By preventing the isoprenoids synthesis and the consequent activation of Ras/Rho proteins, statins may have beneficial pleiotropic effects on the cardiovascular apparatus, for instance they improve endothelial function, decrease oxidative stress and inflammation, inhibit the thrombogenic response (2), and exert clinical benefits in colon, lung, kidney, bone, central nervous system diseases, as well as in diabetes (1). Recently statins have been proposed as potential adjuvant drugs in anticancer therapy (6). On the other hand, patients treated with statins may complain severe side effects, like rhabdomyolysis and liver cytolysis (7,8), owing to the pro-apoptotic and cytotoxic effects of statins. The molecular bases of cytotoxicity are still controversial and may rely either on the reduction of G-proteins’ activity or on the impairment of mitochondrial metabolism (8). Indeed, by decreasing the isoprenoids levels within cells, statins also reduce the synthesis of the side chain of ubiquinone/coenzyme Q10 (CoQ10), which in mitochondria is an antioxidant molecule and an electrons shuttle between NADH-ubiquinone oxidoreductase (Complex I) and ubiquinol-cytochrome c oxidoreductase (Complex III) (9). It has been suggested that a decreased amount of ubiquinone may impair the energy metabolism of muscle and eventually lead to muscle damage (8). On the basis of this hypothesis, several clinical trials have been designed, wherein therapy with statins has been supplemented with CoQ10 (8). However, the contradictory results obtained in most trials suggest that the statins’ toxicity is not fully prevented by restoring the ubiquinone levels (7,8,10). Therefore, an effective strategy aimed at preventing the statins-dependent impairment in cell metabolism, viability and proliferation, without reducing the anticholesterolemic effect, is not yet available. All-trans-geranylgeraniol (GGO) is a 20-carbon cell-permeant isoprenoid molecule, which may be phosphorylated within cells, yielding GGPP. In its turn, GGPP may be easily recognized as a substrate by several isoprenyl-transferases and actively involved in the “salvage pathways” of isoprenoids molecules (11).

In this work we have analyzed the metabolic effects of statins using the in vitro model of THP-1 human monocytic cell line. We have investigated the statin-mediated dysfunctions of the monocyte responses, as far as the ubiquinone-dependent respiratory chain activity and the Rho/Rac G-proteins are concerned. Indeed monocytes have an active part in atheroma genesis and cardiovascular diseases and it is already known that their pathophysiological activities may be widely affected by circulating statins (2). In our work we have additionally investigated whether GGO may effectively prevent the statins side effects in THP-1 cells. In fact, peripheral blood monocytes are easy to collect when such side effects are to be monitored in patients.

Experimental procedures

Materials- Foetal bovine serum and culture medium were supplied by BioWhittaker (Walkersville, MD); plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ); mevastatin, simvastatin, GGTI-286 and Y27632 were from Calbiochem (San Diego, CA). Electrophoresis reagents were obtained from Biorad Laboratories (Hercules, CA). The protein content of cell monolayers and lysates was assessed with the BCA Kit from Sigma-Aldrich Co (St Louis, MO). When not otherwise specified, the other reagents were obtained from Sigma-Aldrich Co.

Cells- Human monocytic leukaemia THP-1 cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, 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 molecules synthesis- The synthesis of isoprenoid molecules was checked as intracellular accumulation of cholesterol, ubiquinone and GGPP, and measured as previously described (12). Cells were incubated for 24 h with 1 µCi/ml of [3H]acetate (3600 mCi/mmol; Amersham Bioscience, Piscataway, NJ), then washed twice with PBS, re-suspended in 200 µl of PBS and transferred in glass microcentrifuge tubes. 1.5 ml of a 1:2 methanol/hexane solution was added, and the cellular suspensions were vortexed for 1 h, then centrifuged at 2,000 x g for 5 min. The upper phase was transferred in a new set of glass microcentrifuge tubes, while the lower phase was re-suspended in 1 ml of hexane, vortexed overnight, and centrifuged at 2,000 x 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/exane as mobile phase. Standard solutions of 10 µg/ml cholesterol, ubiquinone or GGPP were employed. After the separation, the gel was
maintained in an I₂-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, according to the calibration curves prepared previously.

Urobilin loading and spectrophotometric measurement of intracellular ubinone. To load cells with ubiquinone, a 10 mM aqueous solution of β-methyl-cyclodextrin, 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-cyclodextrin-ubinone complexes were re-suspended 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-cyclodextrin-ubiquinone complexes was measured as described previously (13): cells were washed in PBS, re-suspended in 1 ml PBS, and a 50 µl aliquot was sonicated and used for the measurement of protein content. The remaining part was centrifuged (13,000 x g for 1 min) and the pellet was rinsed with 1 ml methanol and 0.5 ml hexane, shaken for 1 min and centrifuged at 2,000 x g for 5 min. The upper hexane phase was collected and transferred into a glass tube. Another 0.5 ml hexane was added to the sample and the procedure was repeated. The new hexane phase was added to the previous one and the solvent was let to evaporate overnight at room temperature. Finally, the cellular extracts were re-suspended in 0.5 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 in 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 (14).

R G proteins activation assay. The GTP binding, which is considered an index of Rac and RhoA activation (15), was measured using the G-LISATM Rac 1/2/3 Activation Assay Biochem Kit or the G-LISATM RhoA Activation Assay Biochem Kit (Cytoskeleton Inc, Denver, CO), respectively. 1 x 10⁶ cells, incubated in the experimental conditions indicated under Results, were washed with ice-cold PBS, lysed in 0.2 ml lysis buffer of the kit and centrifuged at 13,000 x g for 5 min. 10 µl of supernatants were taken off to measure the protein content, whereas the remaining part was diluted at a protein concentration of 10 µg/µl and stored at -80°C until the use. To verify 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 1:1,000 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, re-suspended 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 in 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 (14).
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Upstate Millipore, Billerica, MA), as previously described (16).

**NADPH oxidase expression and activity**- To assess the NADPH oxidase expression, 2 x 10^6 cells were incubated with 200 ng/ml phorbol 12-myristate 13-acetate (PMA) for 10 min, then washed twice in ice-cold PBS, rinsed with 1 ml of PBS containing 0.9 mM CaCl_2, 0.5 mM MgCl_2, 7.5 mM glucose, sonicated and centrifugated at 100,000 x g for 1 h, using a Optima L-90K Beckman Coulter Ultracentrifuge (Beckman Coulter Inc, Fullerton, CA). Both the supernatant (cytosolic fraction) and the pellet (membrane fraction) were collected, diluted 1:2 in Laemli buffer, and subjected to 10% SDS-PAGE and Western blotting analysis, using the following antibodies (from Santa Cruz Biotechnology Inc, Santa Cruz, CA): anti-p47^phox^ (from rabbit, diluted 1:500 in PBS-BSA 1%); anti-p67^phox^ (from mouse, diluted 1:250 in PBS-BSA 1%). The activity of NADPH oxidase was assessed as reported previously (17): 2 x 10^6 cells were washed twice in ice-cold PBS, transferred into luminometer tubes and incubated for 10 min at 37°C with 20 µM isoluminol and 8 U horseradish peroxidase, then stimulated with 200 ng/ml PMA. The chemiluminescence was recorded for 20 min using a Magic Lite Analyzer (GMI Inc, Ramsey, MI) and expressed as nmol O_2^-·min^−1·10^6 cells^−1. In each experiment, an aliquot of PMA-stimulated cells was incubated with 0.3 mg superoxide dismutase (from bovine erythrocyte) and the chemiluminescence was measured as described above; under these experimental conditions, chemiluminescence was reduced to at least 95% (data not shown).

**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 (16). 1 x 10^6 cells were washed twice with ice-cold PBS, lysed in 0.2 ml lysis buffer provided by the kit and sonicated. The protein content of the lysates was measured and the samples were diluted 1:4 in the kinase buffer provided with the kit, containing 20 mM ATP, and incubated for 60 min at 30°C in 96-wells plates, precoated with the recombinant C-terminus of the myosin binding subunit (MBS) of myosin phosphatase. Wells were washed with 2% Tween-20, and 100 µl of the horseradish peroxidase-conjugated anti-phospho(Thr 696)-MBS antibody were added. After a 60 min incubation at room temperature, samples were washed again, and 100 µl of the chromogenic substrate tetra-methylbenzidine were added. After a 15 min incubation at room temperature, the reaction was stopped with 100 µl of 0.5 N H_2SO_4 and absorbance was read at 450 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments). For each set of experiments, a titration curve was set, using serial dilution of recombinant RhoA kinase (Rock2, MBL Inc, Woburn, MA) in kinase buffer. Data were expressed as nmol phospho-MBS min^−1·mg cell proteins^−1.

**Immunofluorescence staining**- 0.5 x 10^6 cells were washed twice in PBS and centrifuged at 30 x g for 10 min in a cytopsin device to let them adhere on polylysine-covered sterile glasses. Cells were fixed with 300 µl of 4% w/v paraformaldehyde for 20 minute at room temperature, washed twice in PBS and permeabilized with 0.5 ml of IGEPAL CA-630 1.5% for 5 min. After washing in PBS, samples were incubated for 45 min at room temperature with 0.2 ml of 1.5% v/v Normal Goat Serum, then washed and stained with 2 µl of anti-actin fluorescein isothiocyanate (FITC)-conjugated antibody (Santa Cruz Biotechnology Inc), diluted 1:50 in Normal Goat Serum, for 1 h at room temperature. After a further washing step, cells were incubated with 0.025 µl of 4',6-diamidino-2-phenylindole dihydrochloride, diluted in 50 µl of PBS for 3 min at room temperature in the dark. Finally, samples were washed five times with PBS, then the slides were mounted with 4 µl of Gel Mount Aqueous medium and examined with a Leica DC100 fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Control experiments included the incubation of cells with non-immune isotypic control antibodies followed by the appropriate labeled secondary antibodies. For each experimental point, a minimum of 5 microscopic fields were examined.

**FACS analysis**- 0.5 x 10^6 cells, incubated for 24 h under the experimental conditions described in Results, in absence or presence of 20 µg/ml lipopolysaccharide (LPS, from *Escherichia coli*), were washed twice with PBS, rinsed with 1 ml of 0.25% w/v PBS-BSA and centrifuged at 10,000 x g for 5 min. The pelleted cells were incubated for 45 min at 4°C in 100 µl of 0.25% PBS-BSA, containing 2 µl of an anti-intercellular adhesion molecule-1 (ICAM-1, CD54), phcoerythrin (PE)-conjugated antibody, or of an anti-lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18), FITC-conjugated antibody (from Santa Cruz Biotechnology Inc.), then washed twice and re-suspended in 0.5 ml of 0.25% PBS-BSA. The fluorescence of each sample was recorded using a FACSCalibur system (Becton Dickinson). For each analysis 10,000 events were collected; the green
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fluorescence (of FITC) was detected using a 530 nm band pass filter, while the red fluorescence (of PE) was detected with a 640 nm longpass filter. The percentage of fluorescent cells was calculated by the Cell Quest software (Becton Dickinson). Control experiments included incubation of cells with non-immune isotypic control antibodies followed by the appropriate labeled secondary antibodies.

**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 x g for 15 min to pellet cellular debris, whereas cells were washed with fresh medium, re-suspended at 1 x 10^6 cells/ml in 0.2 ml of 82.3 mM triethanolamine phosphate hydrochloride (TRAP, pH 7.6) and sonicated. Lactate dehydrogenase (LDH) activity was measured in the extracellular medium and in the cell lysate, as previously described (16). 50 µl of supernatant from extracellular medium or 5 µl of cell lysate were incubated at 37°C with 82.3 mM TRAP and 5 mM NADH. The reaction was started by adding 20 mM pyruvic acid and was followed for 6 min, measuring the absorbance at 340 nm by a Packard EL340 microplate reader (Bio-Tek Instruments). The reaction kinetics was linear throughout the time of measurement. 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, cytosolic 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 cytosolic release of cytochrome c, taken as an index of apoptosis (18).

[^H]thymidine incorporation- 0.5 x 10⁶ cells were grown for 24 h under the experimental conditions reported in Results, in a culture medium containing 1 µCi/ml[^H]thymidine (62 Ci/mmol; Amersham Bioscience). Cells were washed with ice-cold PBS, re-suspended in 200 µl of PBS, and transferred in polyethylene vials. The radioactivity was measured by liquid scintillation.[^H]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.

**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 labelled with 1 µCi[^H]acetate for further 24 h: as shown in Figure 1A, both statins decreased dose-dependently the synthesis of cholesterol, ubiquinone and GGPP. In a further set of time-dependence experiments, THP-1 cells were pre-incubated with a 10 µM concentration of each statin for 6, 24 or 48 h, then washed and radiolabelled for further 24 h: both mevastatin and simvastatin reduced the synthesis of cholesterol, GGPP and ubiquinone as a function of time (Figure 1B). A 24 h incubation with 10 µM mevastatin was sufficient to reduce significantly the synthesis of cholesterol and isoprenoid molecules and gave superimposable results with those obtained with simvastatin. Therefore 10 µM mevastatin (24 h) was chosen as experimental condition in all the following assays.

**GGO prevents the decrease of ubiquinone synthesis and of the electron flow in mitochondria induced by mevastatin.** The decrease of cholesterol and ubiquinone induced by mevastatin in THP-1 cells was completely abolished by the presence of mevalonic acid (Figure 2A). When GGO was incubated in place of mevalonic acid, a different pattern of response was observed: GGO alone increased the synthesis of ubiquinone, without changing the synthesis of cholesterol, and, when incubated with mevastatin, GGO did not reverse the inhibition of cholesterol synthesis, but it prevented the decrease of ubiquinone (Figure 2A). Mevastatin inhibited also the ubiquinone-dependent mitochondrial electron transport, measured as the rate of reduction of cytochrome c (Figure 2B). This effect was clear both in the absence of rotenone, when the electrons flow was dependent on Complex I plus ubiquinone activity, and in the presence of rotenone, when the electrons transport could rely on ubiquinone activity only. Both mevalonic acid and GGO prevented the effects of mevastatin, in the absence or presence of rotenone (Figure 2B). GGO alone, which was able to increase the synthesis of ubiquinone (Figure 2A), also enhanced the electrons flow after the addition of rotenone, whereas mevalonic acid per se had no effect (Figure 2B). The rate of cytochrome c reduction was then measured 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 prot⁻¹ to 14.55 ±2.58 nmol ubiquinone mg cell prot⁻¹ (n = 3, p < 0.005). In ubiquinone-loaded cells the rate of cytochrome c reduction in the presence of rotenone increased and did not change significantly even when cells were incubated with mevastatin (Figure 2B). Notably, the effect of GGO was superimposable to that obtained by exogenous ubiquinone. GGPP was as effective as GGO in preventing the mevastatin’s effects, while, on the opposite, farnesol or FPP did not reverse the statin’s effects on ubiquinone synthesis and cytochrome c reduction (data not shown).

...Geranylgeraniol restores the activity of Rac and RhoA, which is inhibited by mevastatin in THP-1 cells. Mevastatin decreased the binding of GTP to Rac and RhoA proteins (measured as an index of their activation) in THP-1 cells, with the same efficacy of the geranylgeranyltransferase inhibitor GGTTI-286 (Figure 3). On the opposite, mevalonic acid and GGO, which per se did not modify the GTP-binding versus control cells, prevented the mevastatin’s effects on Rac and RhoA activation (Figure 3). Superimposable results were obtained when GGPP was incubated in place of GGO (data not shown). 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 and this effect was reverted by mevalonic acid and farnesol, but not by GGO (data not shown).

Mevastatin elicits an inhibition of NADPH oxidase activity in THP-1 cells, that is reversed by geranylgeraniol. Rac controls the NADPH oxidase activity in many cell types (5). In THP-1 cells stimulated for 10 min with PMA, mevastatin inhibited also the translocation of p47(phox) and p67(phox) on the cell membrane (Figure 4A) and the subsequent NADPH oxidase activation (Figure 4C), producing a similar effect to that evoked by GGTTI-286. When mevastatin was co-incubated with either GGO or mevalonic acid, the membrane levels of p47(phox) and p67(phox) (Figure 4A) and the NADPH activity, measured as O₂⁻ synthesis by the whole cells (Figure 4C) were not changed versus controls. The cytosolic amount of p47(phox) and p67(phox) did not change under any experimental condition (Figure 4B).

Geranylgeraniol prevents the mevastatin’s effects on RhoA kinase, actin cytoskeleton and surface adhesion molecules on THP-1 cells. The inhibition of RhoA activity and of its effector RhoA kinase may impair several cell functions, such as cytoskeleton remodelling, adhesion, motility and proliferation (3,4). Mevastatin prevented the RhoA-kinase activation in THP-1 cells, mimicking the effect of the RhoA kinase inhibitor Y27632 (Figure 5A). GGO and mevalonic acid, which per se did not exert any appreciable effects toward the control cells, completely reverted the inhibition of RhoA kinase activity induced by the statin (Figure 5A). In immunofluorescence staining for F-actin, untreated THP-1 cells showed a round monocytes-like shape, with an homogeneous intensity of fluorescence in the cytosol (Figure 5B); such a morphology, which did not change in the presence of mevalonic acid and GGO, was disrupted by mevastatin and, at a minor extent, by Y27632. Again, when mevalonic acid or GGO were added to mevastatin, the F-actin fluorescence patterns were superimposable to the ones of control cells (Figure 5B). The diminished activity of RhoA kinase and the loss of actin fibers organization has been related to an impaired cell-cell or cell-matrix adhesion (19,20). THP-1 cells exhibited a low surface level of ICAM-1 and LFA-1, but the amount of both proteins on the cell surface was greatly enhanced by LPS (Figure 5C). Mevastatin, as well as Y27632, significantly decreased ICAM-1 and LFA-1 on the membranes of LPS-stimulated cells, whereas mevalonic acid and GGO reverted the mevastatin effect (Figure 5C). In the absence of LPS none of these agents modified the amount of ICAM-1 and LFA-1 proteins on the cells surface (data not shown).

Geranylgeraniol protects cells from the cytotoxic and anti-proliferative effects of mevastatin. Mevastatin significantly increased the release of LDH activity in the extracellular medium, but such cytotoxic effect was prevented when THP-1 cells were incubated with mevastatin plus either mevalonic acid or GGO (Figure 6). The statin induced a prompt release of cytochrome c from mitochondria into the cytosol, that was absent in untreated cells; again mevalonic acid and GGO inhibited this effect (data not shown). Beside inducing cytotoxicity, mevastatin also decreased the [³²P]thymidine incorporation into the cells, which was restored by mevalonic acid and GGO (Figure 6). In THP-1 cells loaded with exogenous ubiquinone, mevastatin did not increase the extracellular LDH activity, but still decreased the cell proliferation (Figure 6). Notably, when used alone, mevalonic acid, GGO and ubiquinone did not affect the LDH release or the [³²P]thymidine incorporation (Figure 6). GGPP had the same effects of GGO on these two parameters (data not shown).
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The mevastatin effect on cell toxicity and proliferation was independent from the inhibition of cholesterol synthesis. Indeed, the squalene synthase inhibitor zaragozig acid (10 mM for 24 h), which reduced cholesterol synthesis without changing the isoprenoids levels (cholesterol synthesis: 346.1 ± 24.66 fmol 10^6 cells^-1 in control cells versus 93.59 ± 15.65 fmol 10^6 cells^-1 in zaragozig acid-treated cells, n = 3, p < 0.001; ubiquinone synthesis: 74.40 ± 2.44 fmol 10^6 cells^-1 in control cells versus 95.00 ± 5.62 fmol 10^6 cells^-1 in zaragozig acid-treated cells, n = 3), was not cytotoxic (LDH release: 5.71 ± 0.37% in control cells versus 5.04 ± 0.52% in zaragozig acid-treated cells, n = 3) and did not reduce the proliferation of THP-1 cells ([^3]H]thymidine incorporation: 4.75 ± 0.17 pmol 10^6 cells^-1 in control cells versus 5.41 ± 0.51 pmol 10^6 cells^-1 in zaragozig acid-treated cells, n = 3). On the other hand, the geranylgeranyltransferase inhibitor GGTT-286 and the RhoA kinase inhibitor Y27632 enhanced the LDH release and reduced the [^3]H]thymidine incorporation in THP-1 cells (Figure 7), as mevastatin did. GGO did not reverse the increase of cytotoxicity and the decrease of proliferation when GGTT-286 and Y27632 were present (Figure 7).

DISCUSSION

The anticholesterolemic agents statins have significantly reduced the incidence of cardiovascular diseases and the progression of atherosclerotic lesions, and are amongst the drugs more widely prescribed (1). Recently, statins have been proposed as adjuvant therapy in diabetes, heart failure, rheumatologic diseases, osteoporosis, Alzheimer disease and multiple sclerosis (1). Moreover they have recently shown to enhance the efficacy of several antineoplastic drugs (6). These pleiotropic effects of statins are independent from the inhibition of cholesterol synthesis, but rely on the decreased production of isoprenoid molecules, such as FPP and GGPP, which are normally incorporated in the lipidic tail of Ras and Rho small GTPases and are essential for their activation (3). Preventing the activation of Ras and Rho G proteins, statins may modulate the processes under their control, such as cell proliferation and apoptosis, cytoskeleton remodelling, cell adhesion, motility and angiogenesis (3,4). The broad spectrum of cell signalling pathways affected by statins may account for their pleiotropic therapeutic benefits, but also for the statins side effects, like rhabdomyolysis and liver toxicity (7,8).

In our work we first assessed the effects of two widely used lipophilic statins, simvastatin and mevastatin, on the synthesis of cholesterol, ubiquinone and GGPP in human monocyteic THP-1 cells. Both statins reduced, as expected, the synthesis of cholesterol in a dose- and time-dependent way. Also the accumulation of GGPP and ubiquinone were inhibited, since GGPP is an alternative metabolite of mevalonic acid, and the side chain of ubiquinone arises from the polymerization of isoprenoids moieties by a trans-isoprenyltransferase. In mammalian cells this enzyme recognizes different isoprenoids of the so called mevalonate cascade as substrates, including isopentenyl pyrophosphate, dimethylallyl pyrophosphate, trans-geranyl pyrophosphate, trans-FPP and trans-GGPP (9,21). In THP-1 cells the amount of cholesterol was higher than the one of GGPP and ubiquinone; this seems not unreasonable in fastly proliferating transformed cells. Mevalonic acid reverted the statins effect on cholesterol and ubiquinone levels, confirming that they were attributable to the inhibition of HMGCoAR. When THP-1 cells were incubated with mevastatin together with all-trans-GGO, a cell-permeant precursor of GGPP (11), the synthesis and the activity of ubiquinone was also restored, but in this case the synthesis of cholesterol still remained low. Thus GGO seemed a promising agent able to correct the decrease of ubiquinone levels elicited by statins, without reducing the inhibitory effect on cholesterol synthesis. Interestingly, GGO per se elicited a significant increase of ubiquinone synthesis, whereas basal cholesterol levels were not affected. This may be due to the fact that the ubiquinone synthesis is less finely regulated than cholesterol production, and that GGPP (which derives from GGO into the cell) can promote the synthesis of the ubiquinone side chain by simple mass action in our cell line. This observation may appear in contrast with a previous work, showing that GGO was not incorporated into ubiquinone in rat glioma C6 cells (22). However, the metabolic pathways leading to the synthesis of ubiquinone side chain are highly variable in different cell types (9). Moreover the pool of GGO/GGPP may be subjected to either trans-isoprenyltransferases and geranylgeranyl transferases activity; therefore, GGPP may be preferentially transferred toward ubiquinone or proteins, depending on the cell demand. Since in THP-1 cells GGO increased the synthesis of ubiquinone but not the GTP-binding activity of Rac and RhoA, we may suppose that it is incorporated preferably into the CoQ10 side chain than in the...
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G proteins. The increase of ubiquinone synthesis was rather specific for GGO and GGPP, since neither farnesol nor farnesyl pyrophosphate had effects on ubiquinone levels and activity. Ubiquinone, working as an electron shuttle between Complex I and Complex III in the mitochondria, plays a crucial role in the respiratory chain, which yields the synthesis of ATP. Indeed GGO increased the rate of reduction of cytochrome c in the presence of rotenone, meaning that it enhanced the electrons flow dependent on the amount of active ubiquinone (14). Moreover, GGO completely abrogated the decrease of electrons flow between Complex I and Complex III elicited by mevastatin. This result suggests that GGO prevented the inhibition of mevastatin on the mitochondrial metabolism. The addition of GGO was as effective as the cell loading with exogenous ubiquinone.

We then investigated whether other undesirable side effects of statins might be reverted by GGO, focusing our attention on human monocytic THP-1 cells. Indeed monocytes have an active part in atheroma genesis and cardiovascular diseases, and their pathophysiological activities may be widely affected by circulating statins (2). Moreover peripheral blood monocytes are easy to collect, when the side effects of statins are to be monitored in patients.

Isoprenylated small G-proteins are amongst the first target of statins: indeed mevastatin decreased the GTP binding to Ras, RhoA and Rac1, 2 and 3 in THP-1 cells, in a mevalonate-dependent way. GGO completely abolished the statins effect on RhoA and Rac. Geranylgeranylated proteins are involved in several functions in monocytes: indeed statins may affect the cell adhesion and migration (20,23), the release of extracellular enzyme and reactive oxygen species (20,24), the cholesterol uptake and efflux (25,26), and these effects have been prevented in monocytes by GGPP or GGO. Active Rac1 (27) and Rac2 (28) are necessary for the recruitment of p67phox and p47phox subunits of NADPH oxidase on the plasma membrane, an event that allows the activation of the enzyme. This enzyme is activated also by RhoA with indirect mechanisms (29).

Our results show that mevastatin inhibited the membrane translocation of p67phox and p47phox in PMA-activated THP-1 cells and decreased the O2•– production. This effect was likely due to the decreased geranylgeranylation of G proteins, because it was mimicked by GGTI-286 and reversed by GGO.

Focusing on RhoA and its effector RhoA kinase, their involvement has been described in cytoskeleton remodelling, stress fibers formation and focal adhesion assembly (4): in THP-1 cells, mevastatin, which decreased the activation of RhoA kinase, also induced a marked disorganization of F-actin filament, with a pronounced shape change. The specific inhibitor of RhoA kinase Y27632 had a similar, although less potent, effect than mevastatin. Indeed the cytoskeleton assembly is not under the exclusive control of Rho A/RhoA kinase in leukocytes, but involves also other small GTPases, such as Rac and Rap1 (30). Thereby, statins, exerting inhibition on several G-proteins, may cause more dramatic effects than Y27632; on the other hand, GGO, which may restore the activity of multiple geranylgeranyl-sensitive proteins, completely reverted the mevastatin’s effect.

When leukocytes are activated by infective or inflammatory stimuli, they usually increase their adhesiveness to endothelial cells, by exposing cell adhesion molecules and integrins on the surface (31). Most of these responses are mediated by the activation of RhoA and RhoA kinase (32). THP-1 cells increased the surface amount of ICAM-1 and LFA-1 proteins in response to LPS, an effect which was strongly reduced by mevastatin, as well as by Y27632. Again mevalonic acid and GGO completely abrogated the mevastatin’s effect. The O2•– production by NADPH oxidase and the increased adhesion to endothelial cells are key responses of monocytes against infective agents. Our data on PMA- and LPS-stimulated THP-1 cells suggest that statins could reduce the ability of monocytes to kill pathogens bacteria via ROS synthesis and to adhere to the endothelial wall, thus impairing their extravasation. GGO would preserve these important functions of monocytes during treatment with statins.

Another crucial issue we addressed to, was the mevastatin’s effect on cell survival or proliferation: indeed in several cell types statins reduce the cells proliferation (33) or induce apoptosis (10). 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 restoring the mitochondrial respiration by an adequate supplementation of CoQ10 would prevent the statins toxicity (8). 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
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benefits only in small subgroups of patients taking statins (7,8). In rat and human myotubes treated with cerivastatin there was no correlation between the content of ubiquinone and the prevention of apoptosis, whereas the statin toxicity was related to the deprenylation of Rap1 (10). In THP-1 cells the decrease of both electron transport and G proteins activation seemed to be critical for the mevastatin’s cytotoxicity: indeed ubiquinone and GGO abolished, whereas GGTTI-286 and Y27632 mimicked the extracellular LDH release elicited by mevastatin. Moreover, in the presence of GGTTI-286 and Y27632, GGO was not able to exert any cytoprotective effect. Such role of GGO is not generalized: 10 µM GGO (48 h) did not protect primary rat hepatocytes from the decrease of both electron transport and G proteins content. The activity of geranylgeranylation-sensitive proteins, mainly the activation of RhoA/RhoA kinase pathway, seemed critical to regulate the cell proliferation in our experimental model: GGO reversed the decrease of[^3H]thymidine incorporation induced by mevastatin, but it was fully antagonized when geranylgeranyltransferases or RhoA kinase were blocked by GGTTI-286 or Y27632 respectively. On the other hand, the amount of intracellular cholesterol was not crucial for cells proliferation or toxicity: indeed, when the synthesis of cholesterol was blunted by zaragozig acid, whereas the synthesis of isoprenoids was preserved, cells did not exhibit signs of death or decreased proliferation.

GGO, like mevalonic acid, did not stimulate cells proliferation per se, but only “buffered” the mevastatin’s effect. At the light of our in vitro results, a pro-mitogenic effect of GGO should be excluded.

In summary, our work suggests that GGO had a broader reversing action than CoQ10 on statins’ side effects: ubiquinone prevented the statin-induced impairment of mitochondrial respiration and was effective in cytoprotection, but did not recover the basal rate of cell proliferation. On the contrary, GGO, which is generated in an early step of mevalonate cascade and is involved in a wide range of signalling pathways, reversed all the mevastatin’s side effects, which are likely due to the decreased geranylgeranylation of cell targets. Moreover GGO did not reduce the mevastatin’s efficacy in lowering cholesterol synthesis. GGO is a cell-permeant and readily diffusible drug, and an oral supplementation with GGO was not toxic in animal models, whereas the synthesis of isoprenoids was crucial for cells proliferation. On the other hand, the amount of intracellular cholesterol was not crucial for cells proliferation or toxicity: indeed, when the synthesis of cholesterol was blunted by zaragozig acid, whereas the synthesis of isoprenoids was preserved, cells did not exhibit signs of death or decreased proliferation.

REFERENCES

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FOOTNOTES

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The abbreviations used are: HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGO, geranylgeraniol; CoQ10, ubiquinone/coenzyme Q10

FIGURE LEGENDS

**Fig. 1.** Effect of mevastatin and simvastatin on cholesterol, ubiquinone and GGPP synthesis in THP-1 cells. (A) The cells were incubated for 24 h in the absence (CTRL) or presence of different concentrations (1, 10 or 100 µM) of mevastatin (MVS) and simvastatin (SIM). (B) The cells were incubated for different time periods (6, 24, 48 h) in the absence (CTRL) or presence of 10 µM
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mevastatin (MVS) or simvastatin (SIM). After these incubation conditions, cells were washed and
grown for 24 h in culture medium containing bars was measured in duplicate as reported under
Materials and Methods. Data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.05.

Fig. 2. Effect of mevastatin, mevalonic acid and GGO on cholesterol and ubiquinone synthesis and
mitochondrial Complex I-III activity in THP-1 cells. Cells were cultured for 24 h in the absence
(CTRL) or presence of mevastatin (10 µM, MVS), mevalonic acid (100 µM, MA), GGO (10 µM,
GGTI), 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. After this incubation
time, cells were subjected to the following investigations. (A) Cells were labelled with [14C]acetate for
24 h, then the content of cholesterol (open bars) and ubiquinone (hatched bars) was measured in
duplicate as reported under Materials and Methods. Data are presented as means ± SE (n = 3). Vs
CTRL: * p < 0.05; vs MVS: ° p < 0.001. (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 under the Material and Methods section. In
ubiquinone-loaded cells, an aliquot of the cell lysate was used to measure the intracellular ubiquinone
amount by a spectrophotometric assay (see Material and Methods for details). The measurements were
performed in duplicate and data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.05; vs MVS: °
p < 0.05.

Fig. 3. Effect of mevastatin, mevalonic acid and GGO on Rac1/2/3 and RhoA binding to GTP in THP-
1 cells. Cells were cultured for 24 h in the absence (CTRL) or presence of mevastatin (10 µM, MVS),
mevalonic acid (100 µM, MA), GGO (10 µM, GGO), in different combinations. GGTI-286 (10 µM,
GGTI) was used as a specific inhibitor of geranylgeranyltransferase. The cells were lysed and
subjected to ELISA assay: the amount of Rac1/2/3-GTP (open bars) and of RhoA-GTP (hatched bars)
were measured in duplicate, as described under Materials and Methods. Data are presented as means ±
SE (n = 3). Vs CTRL: * p < 0.001; vs MVS: ° p < 0.001.

Fig. 4. Effect of mevastatin, mevalonic acid and GGO on NADPH oxidase assembly and activity in
THP-1 cells. Cells were grown for 24 h in the absence (CTRL) or presence of mevastatin (10 µM,
MVS), mevalonic acid (100 µM, MA), GGO (10 µM, GGO), GGTI-286 (10 µM, GGTI) in different
combinations, then they were subjected to the following investigations. A & B. Cells were stimulated
with 200 ng/ml PMA for 10 min, then washed and lysed: the membrane fractions (shown in panel A)
were separated from cytosolic extracts (shown in panel B) as described under Materials and Methods,
and both were checked for the expression p47phox and p67phox subunits by Western blotting. The figure
is representative of three experiments with similar results. C. The NADPH oxidase activity was
measured on the whole cells, stimulated with 200 ng/ml PMA for 10 min, by a chemiluminescence-
based assay, as reported under Materials and Methods. The measurements were performed in duplicate
and data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.05; vs MVS: ° p < 0.02. In the
absence of PMA, the levels of p47phox and p67phox on the plasma membrane were undetectable (data
not shown) and the NADPH oxidase activity was significantly lower than in the presence of PMA
(21.48 ± 4.51 nmol O2•−min−1 106 cells−1 versus 246.80 ± 18.71 nmol O2•−min−1 106 cells−1; n = 3, p <
0.001). None of the drugs changed significantly the NADPH oxidase activity in THP-1 cells not
stimulated with PMA (data not shown).

Fig. 5. Effect of mevastatin, mevalonic acid and GGO on RhoA kinase activity, F-actin fibers
organization and surface adhesion molecules in THP-1 cells. Cells were cultured for 24 h in the
absence (CTRL) or presence of mevastatin (10 µM, MVS), mevalonic acid (100 µM, MA), GGO (10
µM, GGO), in different combinations. Y27632 (10 µM, Y276) was used as a specific inhibitor of
RhoA kinase. Then cells were subjected to the following investigations. A. Cells were lysed and the
activity of RhoA kinase was detected by an ELISA assay. Measurements were performed in duplicate,
as described under Materials and Methods. Data are presented as means ± SE (n = 3). Vs CTRL: * p <
0.001; vs MVS: ° p < 0.001. B. Cells were fixed and analysed in immunofluorescence with a FITC-
conjugated anti-actin antibody (for details see the Materials and Methods section). The figure
is representative of three experiments with superimposable results. C. Cells were stained with an anti-
ICAM-1 (PE-conjugated) antibody and with an anti-LFA-1 (FITC-conjugated) antibody and the
fluorescence was assessed by FACS analysis, using the Cell Quest software, as described under
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Materials and Methods. When indicated, LPS (20 µg/ml for 24 h, LPS) was co-incubated with the other stimuli. The percentage of cells positive in LPS-stimulated cells treated with a non-immune isotypic control antibody was: 1.00 ± 0.05 % (for ICAM-1) and 0.36 ± 0.11 % (for LFA-1).

Measurements were performed in duplicate and data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.05; vs MVS: ° p < 0.05.

Fig. 6. Effect of mevastatin, mevalonic acid and GGO on cytotoxicity and proliferation in THP-1 cells. Cells were cultured for 24 h in the absence (CTRL) or presence of mevastatin (10 µM, MVS), mevalonic acid (100 µM, MA), 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. The LDH activity in the extracellular medium (open bars) and the [3H]thymidine incorporation (hatched bars) were assessed in duplicate as indicated in the Materials and Methods section. Data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.001; vs MVS: ° p < 0.005.

Fig. 7. Effect of GGO on the cytotoxicity and the antiproliferative effects of the inhibitors of geranylgeranyltransferase and RhoA kinase in THP-1 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, Y276), in different combinations. The LDH activity (open bars) in the extracellular medium and the [3H]thymidine incorporation (hatched bars) were measured in duplicate as described under Materials and Methods. Data are presented as means ± SE (n = 3). Vs CTRL: * p < 0.05; vs GGO: ° p < 0.05.
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Figure 1

A

B
Figure 2

A

Cholesterol (fmol/10^6 cells)

- chol  - ub

CTRL  MVS  MA  MA +MVS  GGO  GGO +MVS

Ubiquinone (fmol/10^6 cells)

*  *

B

Respiratory activity (nmol red cyt c min^-1 mg prot^-1)

- rot  + rot

CTRL  MVS  MA  MA +MVS  GGO  GGO +MVS  UB  UB +MVS

*  *
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Figure 3
Geranylgeraniol prevents statins side effects

Figure 4

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- p47phox (47 kDa)
- p67phox (67 kDa)

B

- p47phox (47 kDa)
- p67phox (67 kDa)

C

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Figure 5

A

![Graph showing RhoA kinase activity](image)

B

CTRL | MVS | MA | MA +MVS | GGO | GGO +MVS | Y276

![Images of cell cultures](image)

C

![Graph showing ICAM-1 and LFA-1 fluorescence](image)
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Figure 6

![Graph showing LDH release and [H]-thymidine incorporation with different treatments.]

- **CTRL**: Control
- **MVS**: Model of vascular stenosis
- **MA**: Model of atherosclerosis
- **MA + MVS**: Model of atherosclerosis + model of vascular stenosis
- **GGO**: Geranylgeraniol
- **GGO + MVS**: Geranylgeraniol + model of vascular stenosis
- **UB**: Untreated
- **UB + MVS**: Untreated + model of vascular stenosis

Legend:
- **LDH**: Light Dependence of Hemolysis
- **THYMID**: Thymidine Incorporation

* indicates significant difference compared to control.
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Figure 7