



Effects of pravastatin on the in vitro phagocytic function and hydrogen peroxide production by monocytes of healthy individuals

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Abstract

Macrophages play a part in pathogenesis of atherosclerosis, oxidizing LDL-cholesterol and transforming themselves in foam cells and producing free radicals of oxygen that may also oxidize LDL-cholesterol. HMG-CoA reductase inhibitors are very efficient in long-term control of atherogenesis acting by different mechanisms not fully established. Thus, we investigated the in vitro influence of pravastatin on phagocytosis and hydrogen peroxide production by monocytes of healthy individuals. Phagocytosis of *Saccharomyces erevisiae* by peripheral blood monocytes of 20 healthy individuals was assessed in the absence or presence of pravastatin. Hydrogen peroxide production was assessed based on the horseradish peroxidase-dependent oxidation of phenol red method. Pravastatin had no influence on phagocytosis through scavenger receptors, while it decreased by 20% the mean \pm SD phagocytic index of monocytes through complement receptors, from 141 ± 77 to 113 ± 56 ($p=0.017$), due to a decrease in the number of particles ingested by monocytes, from 2.1 ± 0.5 to 1.7 ± 0.3 ($p=0.003$). This statin also decreased the baseline production of hydrogen peroxide, by 7.7%, from 0.098 ± 0.013 to 0.091 ± 0.013 (OD by 2×10^5 monocytes per hour) ($p=0.025$). Pravastatin was able to decrease the phagocytosis through complement receptors and caused a decrease in the production of hydrogen peroxide by monocytes. It is possible this statin may directly inhibit the development of atherosclerotic plaque and its instability dependent on phagocytosis and the presence of reactive species of oxygen. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Atherosclerosis is now considered a complex chronic inflammatory vascular disease developing in response to injury in the vessel inner layers due to initial endothelial adherence of oxidized low-density lipoprotein (LDL) as a matrix for the progressive ath-

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erosclerotic plaque development [1]. Monocytes and macrophages are the central immune cells of the complex pathogenesis of the atherosclerotic disease [2] and actively participate in all stages of atherogenesis influencing several steps of plaque formation, since the constitution of the fatty streak to the advanced and complicated vascular lesion of atherosclerosis [3].

Macrophages produce oxygen radicals that may oxidize LDL-cholesterol molecules [2]. There are several receptors for oxidized low-density lipoprotein, but uptake of this molecule by macrophages through scavenger receptor leads to massive lipid accumulation inside the phagocyte because this receptor on the surface of these immune cells binds oxidized-LDL, but not native LDL, in unregulated manner [4], leading to development of cholesterol ester-engorged cells that have the appearance of foam cells in the fatty streaks, the precursors of atherosclerotic lesions [3].

Statins are drugs that effectively decrease serum total cholesterol and LDL-cholesterol levels, competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyses the rate-limiting step of the cholesterol synthesis pathway in the liver and other tissues. Pravastatin improves the clinical outcome and increases the survival of patients with atherosclerosis [5,6]. However, clinical and experimental data suggest that the benefits of statins might extend beyond their lipid-lowering effects, including modulation of endothelial function, inflammation, coagulation, and plaque stability [7].

It has been suggested that fluvastatin has antioxidant properties that might prevent toxic effects of oxidized-LDL and may decrease vascular superoxide generation and atheromatous plaque formation in cholesterol-fed rabbits [8]. Furthermore, pravastatin could inhibit superoxide generation by neutrophils [9]. However, the influence of statins on hydrogen peroxide production by human phagocytes is not known. The relatively low reactivity of hydrogen peroxide allows it to pass intact through cell membranes at a distance under conditions in which the more reactive oxygen products are readily scavenged [10]. Therefore, it is possible this molecule plays an important role in atherogenesis.

Uptake through scavenger receptors on the surface of monocytes is the main way oxidized LDL-cholesterol gains access to the cytoplasm of this cell in order to transform it into a foam cell. The influence of statins on scavenger receptor expression in the membrane of

phagocytes is still controversial. While atorvastatin enhances cellular uptake of oxidized LDL and the expression of CD36 in adipocytes from hypercholesterolemic rabbits [11], this drug decreases cellular scavenger receptor gene expression in monocytes of hypercholesterolemic individuals [12]. Lovastatin also reduces the expression of scavenger receptor in human lineage of human monocytic cells [13] and pravastatin down-regulates the scavenger receptors in human macrophages cell line [14], suggesting that the decrease in scavenger receptors is a possible mechanism by which these drugs could reduce oxidized LDL-cholesterol uptake. However, it remains still unclear if these influences of statins on phagocytes result in some modification of phagocytosis by monocytes. Phagocytosis of ox-LDL cholesterol and oxygen peroxide production by means of oxidizing LDL are the first steps for atherosclerotic plaque formation and the influence of these drugs on such functional aspects of monocytes is not fully established. Therefore, in the present work we aimed to evaluate the *in vitro* influence of pravastatin on the phagocytic function and hydrogen peroxide production by peripheral blood monocytes of healthy individuals, in order to uncover possible effects of HMG-CoA reductase inhibitors on phagocytes in the context of the pleiotropic action of this drug class.

2. Subjects and methods

2.1. Subjects

Blood samples were obtained from 20 healthy adult volunteer subjects, being 10 males and 10 females, aged 23 to 50 years (mean \pm SD: 38.1 \pm 7.2 years old), showing body mass index (BMI) \leq 30 kg/m² (mean \pm SD: 22.3 \pm 4.1 kg/m²). Each individual had at least four out of five normal levels of the serum lipids evaluated, according to the guidelines of the ATP III and of the Brazilian Society of Cardiology [15]—a value of \leq 239 mg/dL for total cholesterol, \leq 159 mg/dL for LDL-cholesterol, \leq 199 mg/dL for triglycerides, \leq 40 mg/dL for VLDL-cholesterol and \geq 40 mg/dL for HDL-cholesterol. The mean \pm SD of total cholesterol was 181.5 \pm 17.7 mg/dL, LDL-cholesterol 110.7 \pm 11.8 mg/dL, HDL-cholesterol 50.2 \pm 12 mg/dL, VLDL-cholesterol 20.5 \pm 6.5 mg/dL and triglycerides 103.3 \pm 32 mg/dL. All possible influencing factors on the immunological function were ruled out for each subject and none had habit of smoking nor were using any drug and the majority performed only occasional physical activity.

The ethical rules of the Helsinki Declaration and those from the Brazilian National Council of Health for experimentation in human beings were strictly followed. The Human Research Ethical Committee of the School of Medicine of the University of Brasilia approved the experimental protocol (process number 005/01) and each volunteer gave written informed consent for blood donation. This study was exclusively designed and conducted by the authors that have no conflict of interest. The pure sodium pravastatin powder was provided by Bristol-Meyer-Squibb of Brazil.

2.2. Phagocytosis test

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation through a cushion of Percoll (Pharmacia, Uppsala, Sweden), density 1.077, at $750 \times g$ for 10 min at 4°C , followed by two washings at 400 and $200 \times g$, and suspended into cold RPMI 1640 (Sigma, St. Louis, MO), pH 7.2, supplemented with 20 mM Hepes (Sigma), 2 mM glutamine (Sigma), and 2.5 mg/dL gentamicin. Viability was assessed with 0.05% nigrosin solution in 0.15 M phosphate-buffered saline (PBS), pH 7.2, and was always higher than 97%. Phagocytosis of *Saccharomyces cerevisiae* was individually tested in duplicate for each individual by a technique adapted as previously described [16]. Briefly, 10^6 PBMC placed on 13-mm-diameter glass coverslips in 24-well plastic plates were incubated in the presence or absence of $40 \mu\text{g/L}$ of pravastatin [17], for 2 h, in a wet chamber, at 37°C , in the presence of 5% CO_2 in air. Afterwards, the coverslips were rinsed with PBS and adherent cells ($>98\%$ monocytes, average of $26,000 \pm 4200$ cells/coverslip) were incubated with 10^6 *S. cerevisiae* per well and suspended on RPMI 1640 (Sigma) supplemented with 10% inactivated fetal calf serum (Gibco) with the same concentration of pravastatin described above in a wet chamber for 30 min at 37°C in 5% CO_2 in air. Coverslips were then rinsed with PBS at 37°C to eliminate non-phagocytosed *S. cerevisiae*, fixed with absolute methanol, and stained with 10% buffered Giemsa solution. The number of *S. cerevisiae* that were attached to or that were ingested by 200 monocytes in individual preparations, in duplicate for each sample, was assessed by optic microscopy and the source of the preparation was revealed only at the end of the evaluation. Microscopic fields were randomly selected from throughout the slide and all monocytes in each particular microscopic field were examined. Similar results were observed in each duplicate observation. The phagocytic index was calculated as the average number of attached plus ingested *S. cerevisiae* per phagocytosing monocytes, multiplied by the percentage of these cells engaged in phagocytosis [16]. It was previously observed that 93.4% of the yeasts were ingested by phagocytes when the preparations were treated with 1% tannic acid,

with only 6.6% of them appearing to be attached to these cells [18,19]. To assess phagocytosis through complement receptors, *S. cerevisiae* were firstly incubated for 30 min at 37°C with the fresh serum of the donor. By previous standardization, it was observed that the ingestion of the particles sensitized by fresh serum occur preferentially through complement receptors, with about 300% decreases in the phagocytic index of monocytes by using sensitized yeasts before and after inactivation of complement at 56°C [20].

Pravastatin drug was used in the concentration of $40 \mu\text{g/L}$ considering that pharmacokinetic studies had shown that the upper limit range of therapeutic concentration of the drug was 38.4 to $39.8 \mu\text{g/L}$ [17]. In addition, it was chosen to expose phagocytes to this drug for 2 h and 30 min considering that, after a single therapeutic dose administered to healthy subjects, this level decreases to lower than $5 \mu\text{g/L}$ after this time [17]. Baker's yeast (*S. cerevisiae*) was prepared according to a technique previously described [21]. When *S. cerevisiae* are prepared by this technique and incubated with human complement from human fresh serum it retains considerable C3 activity on its surface [22].

2.3. Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production by PBMC was assessed according to the method of horseradish peroxidase-dependent oxidation of phenol red, described by Pick and Keisari [23]. In brief, PBMC were obtained from each individual, as described above, and triplicate samples of 2×10^5 cells per well in RPMI 1640 were placed in 96-well plastic microplates (Corning, NY, USA). After 1 h incubation with a solution containing 5.5 mM dextrose, 0.5 mM phenol red and 19 U/ mL of horseradish peroxidase type I RZ 1.0 (Sigma) with or without $2 \mu\text{M}$ of phorbol 12-myristate 13-acetate 4-*O* methyl ether (Sigma) in the presence or absence of $40 \mu\text{g/L}$ pravastatin sodium, the reaction was stopped by adding $10 \mu\text{L}$ 1 N NaOH per well and absorbance was read at 620 nm with a Multiskan Titertek microplate reader. The mean value of the triplicate was considered for each individual and expressed as optical density (OD) for 2×10^5 PBMC h^{-1} . It was observed that results are similar in the triplicate observation.

2.4. Statistical analysis

Overall distribution of data was normal as tested by the Kolmogorov–Smirnov test and the paired *t*-test was employed to compare samples in the absence or presence of the drug. Descriptive values were expressed as mean \pm SD. Differences with a two-tailed value of $p < 0.05$ were considered statistically significant. The Prism[®] software package (GraphPad, USA, 1997) was employed for analysis and graphical design of the data.

3. Results

Pravastatin had no influence on phagocytic capacity of monocytes of healthy subjects when assessed through scavenger receptors, as illustrated in the Fig. 1. The mean \pm SD of phagocytic index of monocytes in the absence of pravastatin

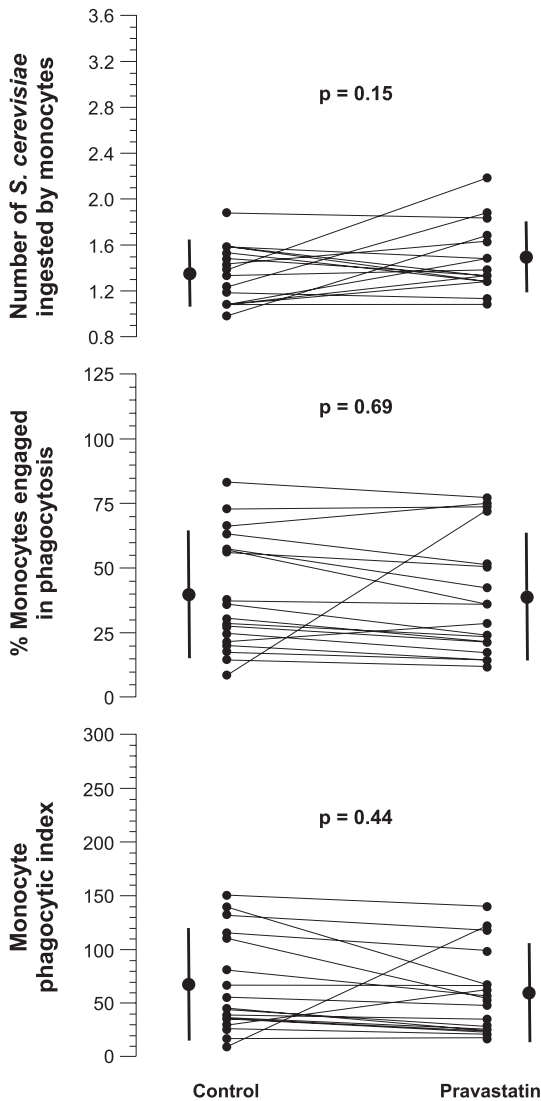


Fig. 1. In vitro influence of pravastatin on phagocytic capacity of monocytes through scavenger receptor from 20 healthy adult individuals. Individual values before and after treatment with pravastatin are shown for the number of *S. cerevisiae* (10^6 per well) ingested by monocytes (top), proportion of monocytes engaged in phagocytosis (middle), and the respective phagocytic index of these cells (bottom). Mean \pm SD is also shown for each condition. The effects of the drug were tested by *t*-paired test.

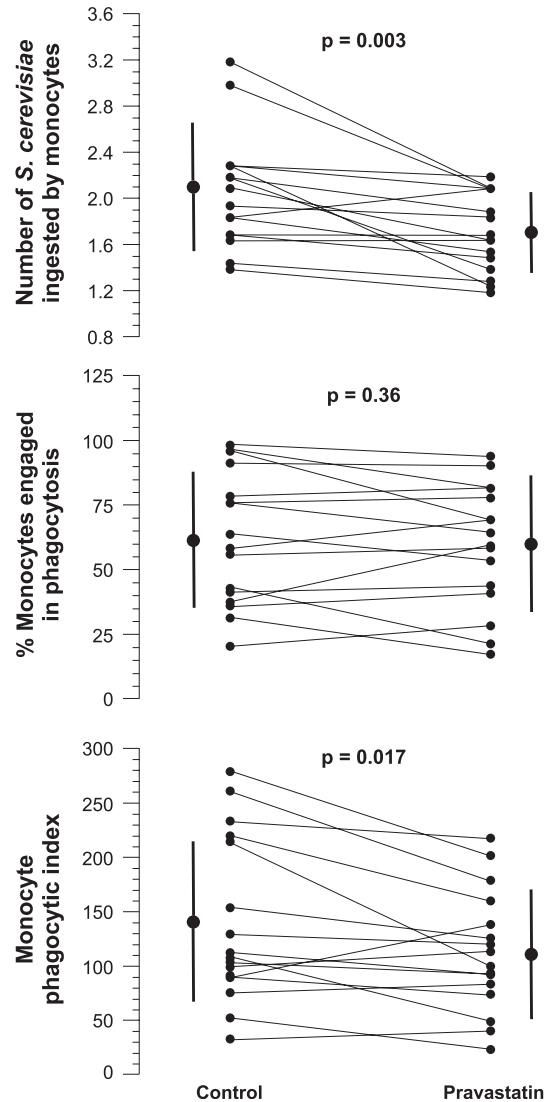


Fig. 2. In vitro influence of pravastatin on phagocytic capacity of monocytes through complement receptor from 20 healthy adult individuals. Individual values before and after treatment with pravastatin are shown for the number of *S. cerevisiae* (10^6 per well) ingested by monocytes (top), proportion of monocytes engaged in phagocytosis (middle), and the respective phagocytic index (bottom). Mean \pm SD is also shown for each condition. The effects of the drug were tested by *t*-paired test.

was 68.2 ± 45.8 and in the presence of therapeutic concentrations of the drug was 60.8 ± 38.7 ($p=0.44$) (Fig. 1, bottom). The same was observed for the percentage of monocytes engaged in phagocytosis, being $40.7 \pm 22.5\%$ in the absence of pravastatin and $39 \pm 23.0\%$ in its presence ($p=0.69$) (Fig. 1, middle), and for the number of *S. cerevisiae* ingested by mon-

ocyte, that were 1.36 ± 0.25 in the absence of pravastatin and 1.49 ± 0.29 in the presence of the drug ($p=0.15$) (Fig. 1, top).

Differently, when phagocytosis was tested using yeast sensitized with fresh human serum, mainly through complement receptors, pravastatin decreased the mean \pm SD value of phagocytic index from 141 ± 77 to 113 ± 56 ($p=0.017$) (Fig. 2, bottom) and the number of *S. cerevisiae* ingested by monocyte from 2.1 ± 0.5 to 1.7 ± 0.3 ($p=0.003$) (Fig. 2, top), while it had no influence on the percentage of monocytes engaged in phagocytosis that was $63 \pm 26\%$ before and $60 \pm 24\%$ after the drug ($p=0.36$) (Fig. 2, middle).

Pravastatin influenced differently the production of hydrogen peroxide when assessed in the presence or absence of concomitant stimulus with PMA. Pravastatin significantly decreased the mean \pm SD of baseline production of hydrogen peroxide, in the absence of stimulus with PMA, from 0.098 ± 0.013 to 0.091 ± 0.013 ($p=0.025$) (Fig. 3, top). However, no influence was observed in the production of hydrogen peroxide when stimulus with PMA was present together with pravastatin (from 0.099 ± 0.01 to 0.099 ± 0.02)

($p=0.62$) (Fig. 3, bottom). After stimulus with PMA, the individual values of H_2O_2 production of three individuals were atypically lower than the minor value observed in the group without stimulus with PMA. The hydrogen peroxide production of these three individuals was not influenced by incubation with pravastatin, similar to the other individuals of this group. As these atypical results could be due to individual variability of these three individuals we did not exclude them from Fig. 3 (bottom) nor from statistical analysis.

4. Discussion

The objective of the present work was to evaluate the in vitro influence of pravastatin on the phagocytic function and hydrogen peroxide production by peripheral blood monocytes of healthy adult individuals in order to contribute to a better understanding of the action of this therapy on the atherogenesis as related to the immunomodulation implicated.

Monocytes were used to test these functions because they are the cells that are exposed to pravastatin in circulating blood just before their migration to wall vessels. *S. cerevisiae* yeasts were chosen as the particle to be phagocytosed because it is taken up by monocytes mainly through scavenger receptors present on membrane surface of these cells [24], which is the same receptor that uptakes oxidized-LDL cholesterol by phagocytes.

Our data showed that pravastatin, in the same concentration in which the drug is observed in blood after ingestion by subjects treated with it ($40 \mu\text{g/L}$) [17], had no influence on the in vitro phagocytic capacity of monocytes of healthy individuals through scavenger receptors after 2 h and 30 min incubation with these immune cells. Although some works have showed that statins may decrease or increase the expression of scavenger receptors on phagocytes, the overall phagocytic function of human monocytes was not influenced by in vitro treatment with pravastatin in the experimental conditions evaluated. Although decrease in scavenger receptors expression was previously observed [13,14] and mice with macrophage scavenger receptor deficiency exhibited 60% decrease in atherosclerotic lesions [25], the present treatment of human monocytes with pravastatin in the same conditions where these cells are submitted to this drug in vivo was unable to quantitatively influence phagocytosis. The possibility that some influence on scavenger receptors could be

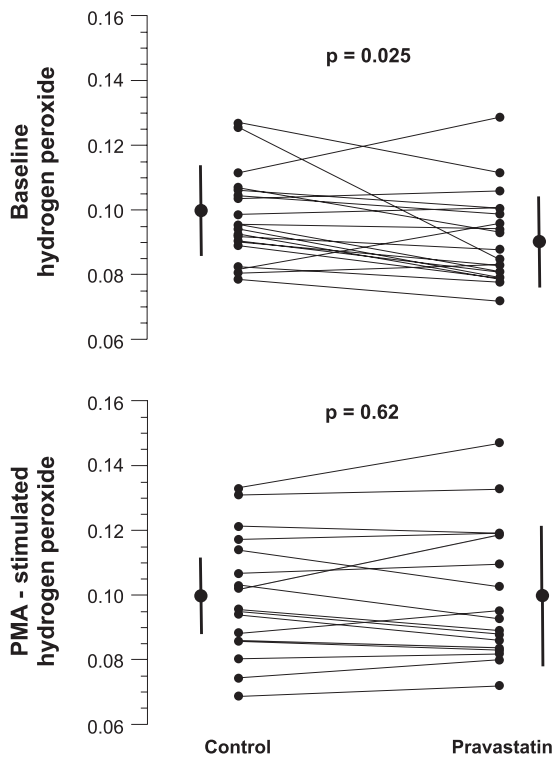


Fig. 3. In vitro influence of pravastatin on hydrogen peroxide production (OD) by 2×10^5 monocytes per well per hour, from 20 healthy adult individuals. Individual values are shown for the baseline non-stimulated (top) and PMA-stimulated H_2O_2 production (bottom). Mean \pm SD is also shown for each condition. The effects of the drug were tested by *t*-paired test.

observed using substantially fewer organisms to assess phagocytosis cannot be ruled out.

Differently, pravastatin decreased the phagocytic capacity of monocytes when assessed using *S. cerevisiae* sensitized with fresh serum, which has been suggested to occur preferentially through complement receptors [20,22]. The phagocytosis using yeast sensitized with human fresh serum may occur mainly through complement receptor, but the particle may also be ingested in a small amount through other scavenger receptors. The phagocytic index presently assessed using yeast sensitized with fresh human serum was 232% higher than that using yeast sensitized with inactivated fetal calf serum, through scavenger receptors. Therefore, the ingestion of the yeast that occurred effectively through complement receptors was the difference observed between the phagocytic index with and without fresh serum. As pravastatin cause no effect on phagocytosis through scavenger receptors, this decrease presently observed using human fresh serum was due to a decrease in phagocytosis through complement receptors. This decreased phagocytosis observed was mainly due to a decrease in the number of *S. cerevisiae* ingested by monocytes because there was no modification in the engagement of these cells on phagocytosis. As this decrease was caused by the lower ingestion of particles, a possible explanation for this is that pravastatin was able to decrease the expression of receptors for complement in the membrane of monocytes and this decrease influenced the function of the phagocytes. As phagocytosis through complement receptor is quantitatively important in the overall phagocytosis, it is possible that the depressed phagocytic function may quantitatively influence the amount of oxidized-LDL ingested by phagocytes.

We also observed that pravastatin caused a small decrease in hydrogen peroxide baseline production by monocytes while it did not influence the production of this oxygen radical when monocytes were concomitantly stimulated with PMA. As hydrogen peroxide molecule has low reactivity, pass through cell membrane and complex biologic fluids and is readily converted to reactive hydroxyl radical able to oxidize LDL molecule [10], its decrease by pravastatin may play a role in preventing atherosclerotic plaque formation. Furthermore, this drug might also contribute to stability of plaque by decreasing the

formation of lipid peroxidation products, since these latter molecules may cause instability of the plaque, thus preventing vessel wall cell lesions and inflammation [1]. In addition, the decreased production of hydrogen peroxide could impair plaque formation and development by decreasing the amount of ox-LDL molecules available to phagocytosis by macrophages. Furthermore, pravastatin and other statins per se effectively decrease serum cholesterol levels improving survival of individuals with atherosclerosis [26].

It must be considered whether the small decrease of hydrogen peroxide production presently observed has some biological effect. Despite of such small decrease, these molecules are able to act in very small amount and are highly reactive and LDL cholesterol is extremely susceptible to oxidative damage [27]. Thus, it is possible that, even if small, the decrease in hydrogen peroxide may show some biological influence on oxidation of LDL-cholesterol and on stability of atherosclerotic plaque. Furthermore, it has been shown that the antioxidant properties of statins [8] and simvastatin may also inhibit the production of hydrogen peroxide in the rat model [28].

As pravastatin decreased the phagocytosis through complement receptors and decreased the basal hydrogen peroxide production, it is important to consider the possible consequences of these effects during infections because phagocytosis and H_2O_2 production are the first mechanisms of defense against several pathogens. As PMA stimulates phagocytes and infections also stimulate phagocytes, we may suppose that this response could be similar to the response observed during an infection. In this work pravastatin did not interfere with microbicidal hydrogen peroxide production when phagocytes were concomitantly stimulated with PMA, suggesting that it did not impair innate immune defense against pathogens. Furthermore, it was shown in the sepsis murine model that statins improved the survival of the treated mice [29], suggesting that this drug did not increase the susceptibility to infectious diseases.

A limitation of our study is the fact that the monocytes were derived from healthy subjects and not from subjects with atherosclerosis in order to evaluate directly the effects of pravastatin on the phagocytic function and hydrogen peroxide production by the phagocytes of diseased subjects. However, there are

several experimental concerns to study individuals with atherosclerosis in order to evaluate the influence of such drug without bias, such as the proper influence on phagocyte functions of cytokines and substances produced in consequence of the background disease.

In conclusion, our data showed for the first time that pravastatin decreased the *in vitro* phagocytic function of monocytes of healthy subjects through complement receptors, while this drug had no influence on phagocytosis through scavenger receptors. Furthermore, pravastatin was able to cause a small decrease in the baseline production of hydrogen peroxide by monocytes. As macrophages actively participate in atherogenesis influencing the plaque formation through phagocytosis of oxidized-LDL cholesterol [2], it is a possibility that pravastatin inhibiting phagocytosis may take a part also in diminishing the formation of the plaque of atheroma. In addition, since collagen is the main component of fibrous caps of atheroma responsible for their tensile strength and macrophages are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes and oxidative radicals, it is possible that pravastatin may also influence the stability of the plaque by decreasing both phagocytosis and hydrogen peroxide production. Hydrogen peroxide may weaken the fibrous cap predisposing its rupture and matrix degradation appears more active in macrophage-rich areas [30] so that pravastatin decreases phagocytosis and hydrogen peroxide production might influence the matrix degradation improving the stability within the plaque. Taking these facts together, it is possible that this statin may prevent both plaque formation and destabilization, probably inhibiting the atherogenesis and its consequences.

Our findings can contribute to broaden the understanding of the relationship between phagocytes, phagocytic functions and pravastatin. These drugs may decrease the phagocytic capacity and hydrogen peroxide production by monocytes, which represent the first step in atherosclerotic plaque formation. Although *in vivo* studies are necessary, the knowledge of these effects of pravastatin observed by us might suggest that besides decreasing lipid levels, this drug may also prevent the plaque formation and its therapeutic utilization may improve the outcome of individuals treated with it, influencing favorably the phagocytosis and hydrogen peroxide production.

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