Clin Exp Immunol 2003; **131**:264–267

Atorvastatin suppresses interferon-γ-induced neopterin formation and tryptophan degradation in human peripheral blood mononuclear cells and in monocytic cell lines

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(Accepted for publication 20 September 2002)

SUMMARY

Inhibitors of 3-hydroxy-3methylglutaryl-co-enzyme A (HMG-CoA) reductase, so-called statins, are used in medical practice because of their lipid-lowering effect and to reduce the risk of coronary heart disease. Recent findings indicate that statins also have anti-inflammatory properties and can modulate the immune response. *In vitro*, we investigated the effect of atorvastatin on the T cell/macrophage system in peripheral blood mononuclear cells (PBMC) and in the human monocytic cell lines THP-1 and MonoMac6. We monitored neopterin production and tryptophan degradation in PBMC after treatment with 10 μM and 100 μM atorvastatin in the presence or absence of 100 U/ml IFN-γ, 10 μg/ml phytohaemagglutinin (PHA) or 10 μg/ml concanavalin A (ConA) and in monocytic cell lines THP-1 and MonoMac6 with or without stimulation with 100 U/ml IFN-γ or 10 ng/ml to 1 μg/ml lipopolysaccharide (LPS). In stimulated PBMC 100 μM atorvastatin inhibited neopterin formation and tryptophan degradation completely, whereas 10 μM atorvastatin was only partially effective. Also in monocytic cell lines THP-1 and MonoMac6, atorvastatin was able to suppress IFN-γ- and LPS-induced formation of neopterin and degradation of tryptophan. Our data from PBMC agree well with previous investigations that statins inhibit T cell activation within the cellular immune response. In addition we demonstrate that atorvastatin directly inhibits IFN-γ-mediated pathways in monocytic cells, suggesting that both immunoreactivity of T cells and of monocyte-derived macrophages are down-regulated by this statin.

Keywords IDO MonoMac6 neopterin statins THP-1

INTRODUCTION

Statins, 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase inhibitors, are used for treatment of hypercholesterolaemia because of their lipid-lowering effects and the resulting decrease in intracellular cholesterol [1,2]. A reduction of cholesterol may influence numerous pathogenic mechanisms such as atherogenesis or thrombosis, and large-scale intervention trials have shown that statin treatment reduces the risk of cardiovascular diseases [3]. In addition to their lipid-lowering properties, statins are able to regulate molecules in vascular biology [4].

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Statins also appear to play a role in immunomodulation as they show an immunosuppressive activity, e.g. statins are able to repress the induction of major histocompatibility complex class II (MHC-II) expression induced by interferon-γ (IFN-γ) [5,6]. In turn, the release of proinflammatory cytokines may also be decreased.

Neopterin, a metabolite of guanosinetriphosphate, is produced in humans and primates by monocyte-derived macrophages (MdM) upon stimulation with IFN-γ. It therefore represents a marker of activated cellular immune system [7]. IFN-γ also induces indoleamine 2,3-dioxygenase (IDO) to convert tryptophan into N-formylkynurenine, which deformylates to kynurenine [8]. Parallel induction of neopterin formation and tryptophan degradation has been demonstrated earlier in monocytic cells and in PBMC [9,10]. The kynurenine to tryptophan ratio (kyn/trp) is usually high during diseases which go along with cellular immune activation due to an increased IDO activity.

Thereby, kyn/trp correlates closely with neopterin concentrations in various diseases [11,12].

Among statins, atorvastatin was shown to be probably the best modulator of MHC expression [5]. In this study, we investigated the influence of atorvastatin on effector cells of the cellular immune system by determination of neopterin formation and tryptophan degradation in stimulated PBMC and in monocytic cell lines.

MATERIALS AND METHODS

Cell culture

PBMC were isolated from whole blood obtained from healthy voluntary blood donors by density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Cells were maintained in RPMI-1640 (PAA Laboratories, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 2 mM L-glutamine (Serva, Heidelberg, Germany) and 50 μg/ml gentamycin (Bio-Whittaker, Walkersville, MD, USA). THP-1 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and MonoMac6 (kindly provided from the Institute of Hygiene, University of Innsbruck) were cultured in completed medium as described above with 0·36 μM mercaptoethanol. Cells were from early passages and were kept for <1·5 months, STR profiling showed identity with original ATTC clones. All cells regularly tested negative for mycoplasma.

For stimulation cells were seeded at a density of 3×10^6/ml and stimulated with IFN-γ (Bioferon, Laupheim, Germany), concanavalin A (ConA, Sigma, Vienna, Austria), phytohaemagglutinin (PHA, Sigma) or lipopolysaccharides (LPS, Sigma) with or without atorvastatin in co-incubation experiments (Sortis®, Atorvastatin-Calcium, Gödecke AG, Berlin, Germany and Pfizer, Ann Arbor, MI, USA). Geranylgeranylpyrophosphate (GGPP) and Farnesylpyrophosphate (FPP) were from Sigma.

Sortis was dissolved in RPMI prior to experiments. Incubation period was chosen 24 h for THP-1 and MonoMac6, and 48 h for experiments with PBMC, because maximum neopterin production and tryptophan degradation in monocytic cell lines was reached within 24 h whereas it took 48 h in PBMC. Supernatants were harvested by centrifugation and frozen at −20°C until measured.

Determination of neopterin, tryptophan and kynurenine

Neopterin was determined by ELISA (Brahms, Berlin, Germany) according to the manufacturer's instructions with a detection limit of 2 nM. Tryptophan and kynurenine were determined by high performance liquid chromatography (HPLC) as described previously [13] using 3-nitro-L-tyrosine as external standard. Kyn/trp was calculated to estimate activity of IDO [13,14].

Statistical analysis

For comparison of grouped data Student's *t*-test was applied, and Spearman's rank correlation coefficients were calculated. *P*-values below 0·05 were considered to indicate significant differences.

RESULTS

Unstimulated PBMC produced an average of 3·6 ± 0·27 nM neopterin when kept in culture for 48 h, addition of atorvastatin to unstimulated cells had no effect on neopterin production (Table 1). Incubation of PBMC with IFN-γ (100 U/ml), ConA or PHA (10 μg/ml) led to an increased production of neopterin. Treatment of stimulated PBMC with 10 μM atorvastatin resulted in a significant down-regulation of neopterin formation; treatment with 100 μM atorvastatin completely blocked neopterin production (Table 1). Viability of cells remained unchanged.

Tryptophan degradation, as measured by a decrease of tryptophan and a parallel increase of kynurenine concentrations (kyn/trp), was increased in stimulated PBMC compared with unstimulated cells (Fig. 1). Atorvastatin inhibited stimulation-induced degradation of tryptophan completely at 100 μM concentration, and at 10 μM concentration it was already partially effective (Fig. 1). Overall, the influence of stimuli ± atorvastatin at different concentrations on tryptophan and kynurenine concentrations was almost predictable by the change of neopterin concentrations. A lowered degradation of tryptophan by atorvastatin was paralleled by decreasing neopterin concentrations and, similarly, a strong correlation existed between neopterin concentrations and kyn/trp in supernatants of PBMC ($r_s = 0{\cdot}902$, $P < 0{\cdot}001$).

Addition of FPP and GGPP to cells did not alter the inhibitory effect of atorvastatin on neopterin production and tryptophan degradation.

Table 1. Neopterin concentrations (nM; mean values ± s.e.m. of three experiments performed in triplicate) in supernatants of PBMC stimulated with IFN-γ, 1 concanavalin A (ConA) or phytohaemagglutinin (PHA) and treated with atorvastatin for 48 h

Treatment	Atorvastatin 0 μM	Atorvastatin 10 μM	Atorvastatin 100 μM
None	3·6 ± 0·3	4·0 ± 0·3	3·5 ± 0·4
IFN-γ 100 U/ml	12·6 ± 2·4*	5·5 ± 0·4†	4·0 ± 0·6†
ConA 10 μg/ml	28·2 ± 4·7*	14·0 ± 3·0‡	4·8 ± 1·6†
PHA 10 μg/ml	27·4 ± 2·2*	16·6 ± 2·0‡	4·7 ± 0·2†

*$P < 0{\cdot}001$ (compared with unstimulated cells), †$P < 0{\cdot}001$, ‡$P < 0{\cdot}01$ (compared with stimulated cells without atorvastatin).

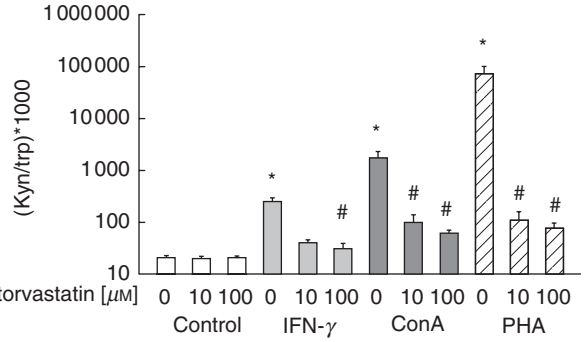

Fig. 1. Kynurenine to tryptophan ratios (kyn/trp) in supernatants of PBMC in the presence or absence of 100 U/ml IFN-γ, 10 ng/ml or 1 μg/ml lipopolysaccharide (LPS) and treated with atorvastatin for 48 h. Columns show mean values (± s.e.m.) of three experiments performed in triplicate. *$P < 0{\cdot}001$ (compared with unstimulated cells), #$P < 0{\cdot}001$ (compared with stimulated cells without atorvastatin); note logarithmic scale of the ordinate.

Table 2. Neopterin concentrations (nM; mean values ± s.e.m. of three experiments performed in triplicate) in supernatants of THP-1 stimulated with IFN-γ, lipopolysaccharide (LPS) at different concentrations and mixtures and treated with atorvastatin for 24 h

Treatment	Atorvastatin 0 μM	Atorvastatin 10 μM	Atorvastatin 100 μM
None	2·3 ± 0·1	2·3 ± 0·2	2·3 ± 0·2
IFN-γ 100 U/ml	4·8 ± 1·2*	4·3 ± 0·8	2·6 ± 0·2‡
LPS 1 μg/ml	3·9 ± 0·5*	4·0 ± 0·6	2·4 ± 0·1‡
LPS 10 μg/ml	3·2 ± 0·5*	3·4 ± 0·5	2·5 ± 0·4‡
IFN-γ + LPS 1 μg/ml	29·2 ± 7·2*	23·4 ± 6·2§	4·1 ± 1·5†
IFN-γ + LPS 10 μg/ml	22·1 ± 8·8*	19·9 ± 7·6	3·2 ± 0·7†

*$P < 0{\cdot}001$ (compared with unstimulated cells), †$P < 0{\cdot}001$ and ‡$P < 0{\cdot}01$, §$P < 0{\cdot}05$ (compared with stimulated cells without atorvastatin).

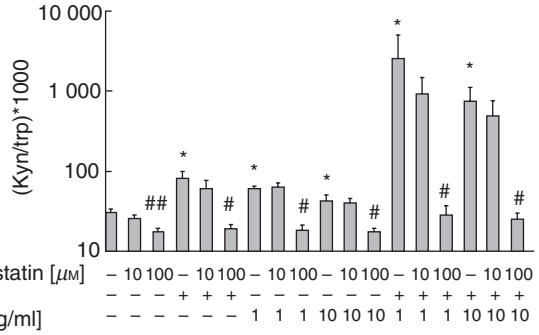

Fig. 2. Kynurenine to tryptophan ratios (kyn/trp) in supernatants of monocytic THP-1 cells in the presence or absence of 100 U/ml IFN-γ, 10 ng/ml or 1 μg/ml lipopolysaccharide (LPS) and treated with atorvastatin for 24 h. Columns show mean values (± s.e.m.) of three experiments performed in triplicate. *$P < 0{\cdot}001$ (compared with unstimulated cells), #$P < 0{\cdot}001$ (compared with stimulated cells without atorvastatin), ##$P < 0{\cdot}05$ (compared with stimulated cells without atorvastatin); note logarithmic scale of the ordinate.

In THP-1 cells, significantly enhanced neopterin production (Table 2) as well as tryptophan degradation (Fig. 2) was observed upon treatment with IFN-γ and LPS. The combination of stimuli led to a synergistic effect which was greater than the sum obtained by single stimuli. Addition of 10 μM atorvastatin induced a small decrease of neopterin production and tryptophan degradation, and 100 μM atorvastatin blocked these two activation-induced biochemical pathways. Again, any influence of atorvastatin to decrease tryptophan degradation in stimulated cells was paralleled by decreasing neopterin concentrations, and again a strong correlation existed between neopterin concentrations and kyn/trp in supernatants of THP-1 cells ($r_s = 0{\cdot}886$, $P < 0{\cdot}001$).

Also in MonoMac6 cells, atorvastatin was able to block IFN-γ- and LPS-induced neopterin and tryptophan degradation in a dose-dependent manner (data not shown) similar to that seen in THP-1 cells.

DISCUSSION

Atorvastatin decreases neopterin production and tryptophan degradation in PBMC stimulated with IFN-γ, ConA and PHA and also in the monocytic cell lines THP-1 and MonoMac6 stimulated with IFN-γ and/or LPS in a concentration-dependent manner. Neopterin production and tryptophan degradation were inhibited almost completely by addition of 100 μM atorvastatin, but 10 μM atorvastatin was also effective, this lower concentration being in the same range as the dose achieved during treatment in patients. The same results were obtained when using pure atorvastatin instead of Sortis® tablets (data not shown).

The effect of atorvastatin to reduce neopterin formation and tryptophan degradation in parallel was observed in PBMC but also in the established monocytic cell lines THP-1 and MonoMac6. In PBMC, treatment with atorvastatin most probably influenced macrophage activity by down-regulating T cell activation [5] and thus, production of IFN-γ. The data fit well to available information on statins to slow down T cell response *in vitro* [6]. In monocytic cell lines, a direct influence of atorvastatin on human monocytic cells stimulated with IFN-γ was observed. It appears that a higher dose of atorvastatin was needed to achieve similar effects in monocytic cell lines compared with PBMC.

Kyn/trp in unstimulated PBMC was reduced by atorvastatin; no such difference was observed for neopterin concentrations. It also appears that baseline levels of kyn/trp and neopterin concentrations are due to stimulation of cells, albeit at a low level, and atorvastatin is able to down-regulate this activity. However, neopterin concentrations measured in control cells were close to the limit of detection of the ELISA used, and the assay may not be sensitive enough to detect a further reduction of neopterin concentrations.

Human MdM produce increased amounts of neopterin upon stimulation with IFN-γ [9,15]. Therefore, measurement of neopterin concentration allows monitoring of the extent of cellular immune activation [7,16,17]. Increased neopterin formation coincides with increased production of reactive oxygen species formed by activated MdM and with tryptophan degradation by IDO, both the latter also representing part of the microbicidal armature of MdM [10,18–20]. By decreasing the activation status, statins may counteract oxidative power of activated cells. Reactive oxygen species released by MdM stimulated with IFN-γ potently oxidize nitric oxide (NO) to form peroxynitrite ($ONOO^-$) [21]. Thereby the cytotoxic activity is increased at the expense of vasodilatory NO. Thus, if statins reduce the amounts of oxidizing compounds formed from activated cells, they might reduce the conversion of NO to $ONOO^-$, increasing residual NO. Our data may support the notion that benefits of statins in cardiovascular disorders do not only result from their influence on HMG-CoA reductase and cholesterol biosynthesis; in parallel, vasodilatory effects could be achieved which contribute to further amelioration of atherogenesis [22]. Statins have been found previously to up-regulate NO production in conscious dogs [23].

It is unclear, at which biochemical pathway atorvastatin interferes with immune activation cascades and whether its effects still involve changes related to cholesterol biosynthesis. Statins lead to a decreased synthesis of cholesterol as well as its precursors, including farnesyl- and geranylgeranylpyrophosphate (FPP and GGPP). FPP is essential for membrane attachment and biological activity of small G-proteins from the Ras-family, and GGPP is required for RhoA translocation to cell membranes [23,24]. When adding FPP and GGPP to our test system there was no effect on

the inhibitory capacity of atorvastatin observed (data not shown), suggesting no association between inhibited FPP and GGPP production and decreased neopterin formation and tryptophan degradation.

IFN-γ, which is released during cellular immune response, induces IDO to convert tryptophan to kynurenine [8–10]. Kyn/trp is usually high during cellular immune activation because of increased IDO activity and correlates closely with neopterin concentrations in various diseases [11,12]. Interferon-induced IDO activation has been linked to microbicidal effects due to tryptophan deprivation [20]. In mice, tryptophan depletion as a result of IDO activation was found to be required for tolerance induction during pregnancy [25]. Similarly, tryptophan degradation induced by IFN-γ in monocytes/macrophages is capable of inducing T cell unresponsiveness *in vitro* [26,27]. These observations may explain why enhanced production of IFN-γ and tryptophan degradation by IDO often coincides with acquired immunodeficiency during disease [11,28]. From the data it could be concluded that statins might also be able to improve T cell responsiveness by reducing cytokine-induced tryptophan degradation, and thus statins may represent useful immunmodulators in chronic inflammatory diseases besides atherosclerosis, e.g. autoimmune disorders or neurodegenerative diseases such as Alzheimer's dementia [5].

In conclusion, atorvastatin is able to suppress biochemical effects in cytokine-treated monocytes. As well as an influence of statin on the interaction between T cells and monocytes, direct effects on monocytes have also been found.

ACKNOWLEDGEMENTS

This work was supported by the by the Austrian Funds 'Zur Förderung der wissenschaftlichen Forschung', project 14154Med and by the Austrian Federal Ministry of Social Affairs and Generations.

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