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Full Length Research Paper

Atorvastatin modulates constitutive and lipopolysaccharide induced IL-6 secretion in precursors of human skeletal muscle

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It is well documented that, besides reducing blood LDL lipoproteins, HMG-CoA reductase inhibitors (statins) also suppress inflammatory markers and improve survival in sepsis. These beneficial effects can be at least partly explained by their capacity to inhibit the release of IL-6, which is generally regarded as a proinflammatory cytokine, although a variety of other actions including anti-inflammatory have been reported for this cytokine under various circumstances. In quantitative terms, IL-6 release is a major response of the skeletal muscle to various environmental stimuli and since muscle represents 40% of the body weight it can substantially contribute to the IL-6 blood level. The aim of our study was to provide more detailed insight into the effects of statins on the IL-6 release from the human skeletal muscle. Studying time and concentration dependency of the constitutive and lipopolysaccharide (LPS)-stimulated IL-6 release from the cultured human myotubes we found that 48 h pre-treatment with atorvastatin (AT) significantly inhibits constitutive IL-6 secretion at high (1 µM) and supra (10 µM and 100 µM) therapeutic concentrations. At these AT concentrations, LPS-stimulated IL-6 secretion was also significantly reduced by 48 h AT co- treatment or pre-treatment, but not by post-treatment; therapeutic (0.1 µM) AT concentration was efficient only in pre-treatment but not in co- treatment or post-treatment LPS protocols. This information is an important clue for the investigations of the molecular mechanisms underlying AT effects and its therapeutic applications.

Key words: Atorvastatin, interleukin (IL)-6, skeletal muscle, lipopolysaccharide (LPS), inflammatory response.

INTRODUCTION

Activation of the inflammatory system and increased cytokine production are the cornerstones of sepsis development and progression. This philogenetically old system, however, can be chronically (over) activated, which can lead to detrimental changes in several organs and systems. A substantial body of evidence has suggested that chronic inflammation is a key patho-physiological mechanism in various chronic diseases, such as atherosclerosis, diabetes mellitus type 2, the metabolic syndrome, cognitive decline (Taudorf et al., 2007; Yaffe et al., 2004), cachexia (Yeh et al., 2008; von Heahling et al., 2009) and chronic heart failure (Mann, 2002; Rauchhaus et al., 2000).

Events in inflammation are mediated and coordinated by cytokines. These proteins are active biological

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communication signals that influence activation of various cells participating in inflammation. The production of cytokines probably represents a biological mechanism that under appropriate conditions acts as a defence against changes in system homeostasis, while their overexpression can promote a vicious cycle that can eventually lead to system deterioration and disease progression. Among the most intensively investigated cytokines are tumour necrosis factor (TNF)-α and interleukin (IL)-6. Cytokine production can be induced through several mechanisms. Lipopolysaccharide (LPS), a major component of the outer leaflet of the outer membrane of Gram-negative bacteria, has been recognised as one of the most potent inducers of immune system activation and pro-inflammatory cytokine production (de Bont et al., 1998).

It has been established that cytokines are not produced only by the immune cells, where their release was first detected (Dinarello and Mier, 1986) but also from the other tissues and organs, including skeletal muscle (Febbraio and Pedersen, 2002; Pedersen and Febbraio, 2008; Brand and Pedersen, 2010; Pedersen 2011). The release of cytokines, including IL-6, IL-1, IL-8, IL-10, IL-15 and TNFα (Petersen and Pedersen, 2005; Peake et al., 2005; Nielsen and Pedersen, 2008), has already been demonstrated in the skeletal muscle making it an endocrine organ actively participating through this communication in the immunoregulatory, anti-inflammatory, regenerative and metabolic effects (Delaigle et al., 2004; Mann et al., 2011). Indeed, this local production of cytokines in skeletal muscle and myocardium in response to stressors (LPS, exercise and hypoxia) can be abundant, and can contribute substantially to the amounts of these cytokines in the systemic circulation, mimicking the responses generally observed in inflammatory disease (Febbraio and Pedersen, 2002; Prabhu, 2004; Prelovsek et al., 2006; Brandt and Pedersen, 2010; Pirkmajer et al., 2011). Recent studies have revealed a strong involvement of skeletal muscle in the pathophysiology of chronic disease and its role as an important target and generator of detrimental pathophysiological processes (Filippatos et al., 2005).

Among the factors demonstrated to modulate the blood cytokine levels are also hydroxymethylglutaryl coenzyme A reductase inhibitors, known as statins. These substances have been introduced as a therapeutic approach for subjects with hypercholesterolemia and are currently still the most effective therapeutic of this condition. However, several studies have shown that statins also have other beneficial effects which are not directly connected with their anticholesterol actions. The recent JUPITER trial demonstrated a prognostic benefit of statin therapy in high-risk patients with elevated high-sensitivity C-reactive protein (hs-CRP) (Ridker et al., 2008). In patients with chronic heart failure, the statins can improve endothelial function by inducing constitutive nitric oxide synthase (cNOS) gene transcription (von Haehling et al., 2003) and reduce CRP levels (Kjekshus et al., 2007). McMurray et al. (2009) showed in the CORONA study that treatment with rosuvastatin led to better outcome in a subgroup of patients with elevated CRP. Clinical benefits of statin therapy have also been found in patients with chronic inflammation.

It has been suggested that many of the aforementioned effects are attributable to the ability of statins to induce modulation of cytokine production (Rauchhaus et al., 2000; Blaschke et al., 2009; Loppnow et al., 2011). Supporting this view are the studies demonstrating statin-mediated reduction of the release of pro-inflammatory cytokines in different statin-treated cell types, such as human vascular smooth muscle cells (Ito et al., 2002) and neonatal rat cardiomyocytes (Shang et al., 2006). High therapeutic concentrations of atorvastatin (AT) also significantly reduced inflammatory biomarkers and oxidative stress in low-grade inflammatory states, such as the metabolic syndrome (Fessler et al., 2007). However, a study that would systematically approach the effects of stains on the cytokine release from the human skeletal muscle, which is the largest human organ and can as such significantly contribute to the blood cytokine levels, is still missing. The aim of this study was to elucidate the effects of atorvastatin (AT), a widely used synthetic statin, on the IL-6 release from the human skeletal muscle. The release of IL-6 is in quantitative terms with the most important cytokine response of the skeletal muscle to the various environmental stimuli (Febbraio and Pedersen, 2002; Pedersen and Febbraio, 2008), including LPS. We studied the effects of AT on the constitutive and LPS-stimulated IL-6 release from the primary cultured human myotubes. Three AT concentrations (therapeutic, high therapeutic and supra-therapeutic) have been tested. In order to get better insight into the timing of AT effects, which is an essential clue for studying the molecular and cellular mechanisms of its actions, we compared the pre-treatment, co-treatment and post-treatment effects of AT on the LPS stimulated IL-6 release.

MATERIALS AND METHODS

Study design

Experiments were performed on primary cultures of human muscle at the myotube differentiation stage. The data were collected from experiments carried out on cultures prepared from different donors (subsequently), to avoid variations in cytokine production among donors. Myocytes were left to differentiate from myoblasts to myotubes. These human myotube cultures were arranged into five different sets. The first set served as the control and was used to determine the constitutive secretion of IL-6. The other sets of cultures were exposed to combinations of four different AT concentrations (0.1, 1.0, 10 or 100 µM) corresponding to different therapeutic doses, and LPS (100 ng/ml), to determinate the AT effects on the LPS-induced production of cytokines. Additionally, we exposed AT-pre-treated samples to LPS (100 ng/ml) in a time dependent manner. IL-6 concentrations in the culture supernatants were measured in all the samples using enzyme-linked immunosorbant assay (ELISA) kits (Thermo Scientific Pierce

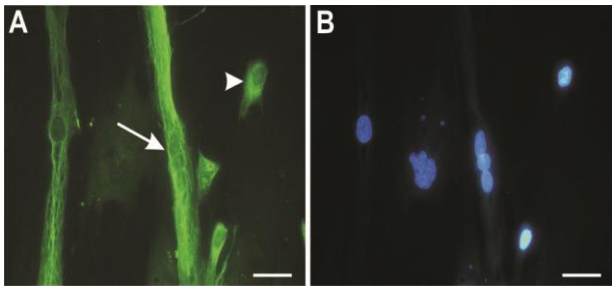

Figure 1. Identification of myocytes by desmine staining. Representative micrograph (400× magnification) of cultured primary human myotubes, after staining for desmine (A) and their nuclei with Hoechst 33342 (B). The vast majority of the cells in culture show multinucleated desmine-positive myotubes (arrow), with a few desmine-positive mononucleated myoblasts seen (arrowhead). Bars = 20 µm.

Endogen, Rockford, USA). The study was performed in accordance with the Declaration of Helsinki and was approved by the National Ethical Commission at the Ministry of Health of the Republic of Slovenia (No: 63/01/99).

Preparation of human muscle cultures

The myotube cultures used were derived from primary myoblast cultures as described previously (Prelovsek et al., 2006). In brief, human muscle cells were derived from the satellite cells released from the muscle tissue, cleaned of adhering connective tissue, cut into small pieces and trypsinised. The resulting cell cultures were grown at clonal densities in advanced minimum essential medium (aMEM; Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK) under standard *in-vitro* conditions. Myoblast colonies were identified, trypsinised and further expanded. To induce myogenic differentiation, subconfluent cultures were shifted from growth medium to differentiation medium (aMEM supplemented with 2% foetal bovine serum). After fusion of the cells to form myotubes, they were maintained in culture in six-well dishes for up to 15 days, with renewal of the medium every 3 days. Myotube cultures used in experiments were further evaluated for fusion index (number of nuclei per cell), only myotube cultures with sufficient mean fusion index (4 and more) were used for further experiments.

Treatment with atorvastatin and lipopolysaccharide

The myotube cultures were exposed under different experimental conditions to AT and LPS treatments, as follows: 48 h AT exposure; 48 h co-exposure to AT and LPS; 48 h pre-exposure to AT followed by 12 h co-exposure to LPS and AT; 24 h pre-exposure to LPS after which the medium was removed to be followed by 12 h exposure to AT. Under all these experimental conditions, we used four different AT (Krka Pharmaceutical Company, Novo mesto, Slovenia) concentrations (0.1, 1.0, 10 and 100 µM) for each set of cultures. An AT concentration of 0.1 µM is regarded as therapeutic; a concentration of 1.0 µM is regarded as high therapeutic and the concentrations of 10 and 100 µM are regarded as supra-therapeutic (Singh et al., 2008). These final concentrations of AT were prepared from a stock solution of AT prepared by dissolving the lyophilised hemi-calcium AT salt in aMEM at 37°C. In the controls, the cells were incubated for the same times under the same medium conditions both without addition of AT or LPS (control for constitutive IL-6 secretion) and without addition of AT in combination with LPS (control for LPS effects). The culture supernatants were collected after completion of each treatment and were frozen at -80°C for further analysis.

Determination of IL-6 concentrations

The concentrations of the secreted IL-6 in the myotube supernatants collected from the cultures after these treatments were measured using ELISA kits (Endogen, Rockford, USA), according to the manufacturer instructions and as previously described (Prelovsek et al., 2006).

Identification of myotube cultures and cytotoxicity

Myotubes were identified by desmin staining. The cells were grown on glass coverslips coated with gelatine, fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline for 15 min and permeabilised by addition of 0.5% Triton X-100 (Sigma Aldrich) in phosphate-buffered saline at room temperature. Desmin was detected using a mouse monoclonal anti-desmin antibody (DAKO, Glostrup, Denmark) followed by fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, USA.). Total cell numbers were evaluated by Hoechst 33342 nucleus staining. The cells were visualised using an Olympus IX81F inverted microscope (Tokyo, Japan).

To assess the myotubes for the integrity of their cell membranes after treatment with AT, which might cause cytotoxic effects (Campos-Lara and Mendoza-Espinoza, 2011), we determined the concentrations and activities of lactate dehydrogenase (LDH; EC 1.1.1.27) in the supernatant using cytotoxicity detection kits (LDH) (Roche Applied Science).

Statistics

All experimental data were expressed as means ± SD of four samples, which represented at least three separate experiments. Significance was determined by Student's *t*-tests. A P value less than 0.05 was considered statistically significant. Univariate two-way ANOVA was used to test for differences in IL-6 secretion under different experimental conditions. The data were compared using SPSS 13.0 (SPSS, Chicago, IL, USA) for Windows, and Microsoft Excel (Microsoft Excel 2003).

RESULTS

For the modulatory effects of AT on IL-6 signalling, an *in-vitro* model of primary human myotube cultures was used. Desmin staining was used to identify and evaluate the purity of the myotube cultures. These multinucleated cells in cultures were desmin positive (Figure 1), and were seen as 81 ± 2% of all of the cells in the tested cultures, with the rest of the cells in the cultures as mononucleated cells, with 72 ± 5% of these seen to be desmin positive. Neither AT nor LPS caused significant increases in LDH concentrations under the experimental conditions used. No differences in LDH release were seen between the control versus AT-treated cultures or in cultures treated with AT in combination with LPS; the LDH activities were all under the level of significance (data not shown).

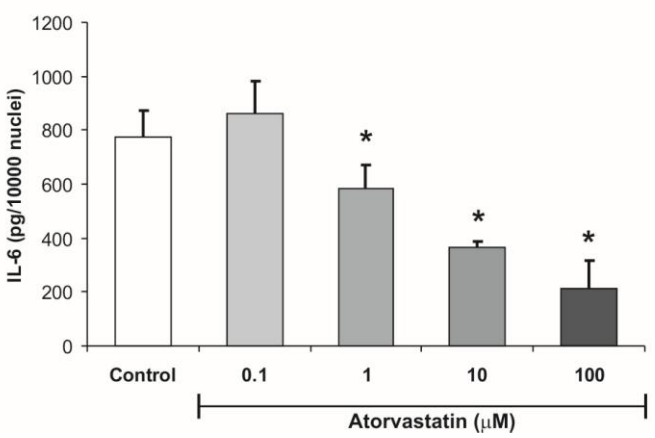

Figure 2. Effects of atorvastatin on constitutive IL-6 secretion in cultured primary human myotubes. The cell cultures were treated for 48 h with different AT concentrations (0.1, 1.0, 10 and 100 µM). Control: cell culture without AT for 48 h. The inhibitory effects of AT were statistically significant at concentrations of 1.0 µM and higher. *P < 0.05 Student's T-test, compared to control.

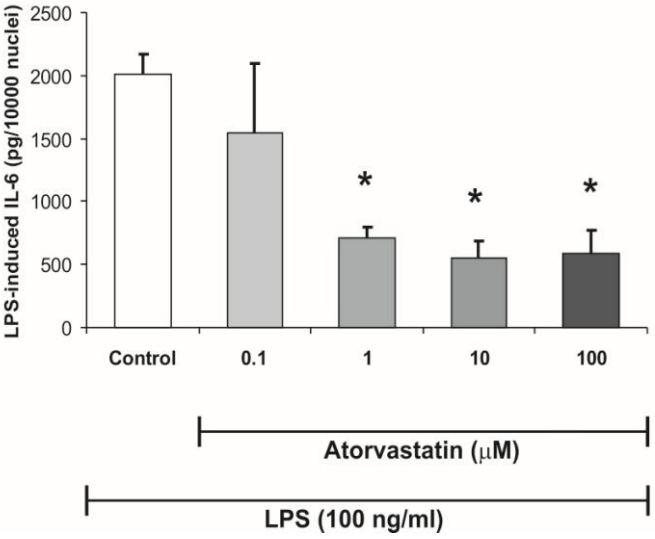

Figure 3. Effects of atorvastatin and lipopolysaccharide co-treatment on IL-6 secretion in cultured primary human myotubes. Human myotube cultures were concomitantly treated for 48 h with different AT concentrations (0.1, 1.0, 10 and 100 µM) and LPS (100 ng/ml). Control: cell cultures treated with LPS for 48 h. The inhibitory effects of AT were statistically significant at AT concentrations of 1.0 µM and higher. *P < 0.05 Student's T-test compared to control.

The myotubes constitutively secreted IL-6. These constitutive IL-6 levels were 777 ± 95 pg/10,000 cell nuclei, as in agreement with previous reports (Prelovsek et al., 2006; Pirkmajer et al., 2010). After exposing the myotube cultures to different AT concentrations for 48 h,a concentration-dependant inhibitory effect on constitutive

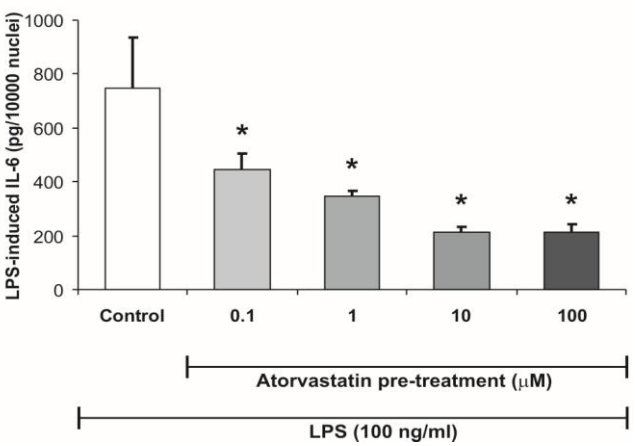

Figure 4. Effects of atorvastatin pre-exposure on lipopolysaccharide induced IL-6 secretion in cultured primary human myotubes. Human myotube cultures were pre-treated for 48 h with the different AT concentrations (0.1, 1.0, 10 and 100 µM), and then LPS (100 ng/ml) was added afterwards for 12 h. Control: cells cultured 48 h without special treatments followed by LPS exposure for 12 h. Inhibitory effects of AT were detected at concentrations 0.1 µM and higher. *P < 0.05 Student's T-test, compared to control.

IL-6 secretion was observed. The inhibitory effects of AT were statistically significant at AT concentrations of 1.0 µM and higher ($P < 0.05$) (Figure 2).

In order to be able to compare results with those performed in previous studies we performed a control set of experiments, where 24 h constitutive versus LPS-induced IL-6 secretion was compared. 24 h exposure to LPS (100 ng/ml) significantly stimulated IL-6 secretion from these human myotube cultures. A statistically significant 1.8-fold level over the control constitutive IL-6 secretion (563 versus 1006 pg/10,000 nuclei; $P < 0.01$) was observed, which is comparable with that observation in previous studies.

After co-exposure of these myotube cultures to LPS (100 ng/ml) and with AT at increasing concentrations for 48 h, a statistically significant inhibition of LPS-induced IL-6 production was observed at AT concentrations of 1.0 µM and higher (Figure 3).

When myotube cultures were pre-exposed to AT for 48 h before addition of LPS, this again showed a dose-dependant inhibition of LPS-induced IL-6 secretion (Figure 4). However, with myotubes stimulated with LPS for 24 h before a 12 h treatment with AT, no significant effects of the AT were seen on the LPS-induced IL-6 secretion, although there was a trend to an inhibitory effect at the highest concentration of AT tested (100 µM) (Figure 5).

DISCUSSION

The main findings of our study are as follows: 1) AT

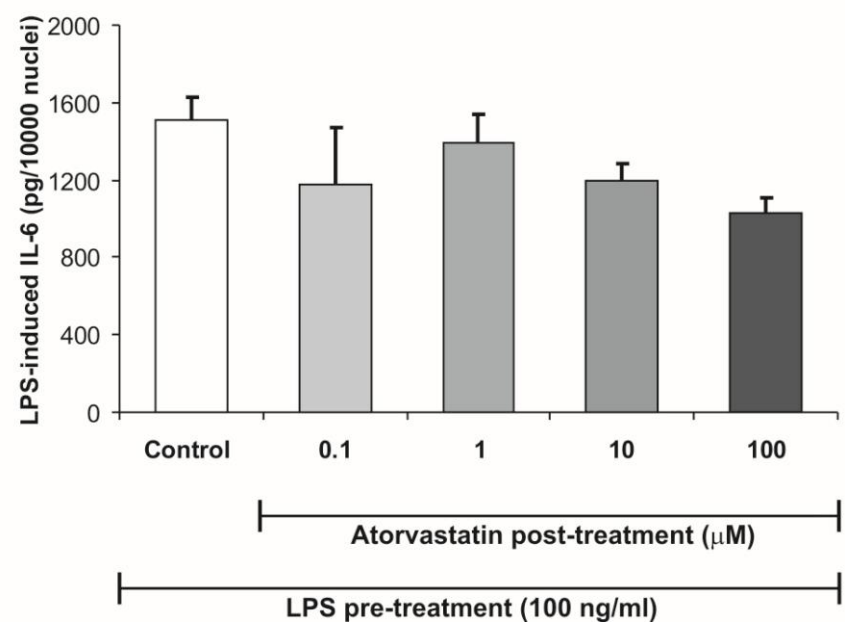

Figure 5. Effects of lipopolysaccharide pre-exposure on atorvastatin efficiency to inhibit IL-6 secretion in cultured primary human myotubes. Human myotube cultures were pre-treated for 24 h with LPS (100 ng/ml) followed by exposure to AT at different concentrations (0.1, 1.0, 10 and 100 μM) for 12 h. Control: cell cultures were exposed to LPS for 24 h. The inhibitory effects of AT at the highest dose tested did not reach statistical significance.

decrease the constitutive and LPS-induced IL-6 release from the cultured human myotubes; 2) these AT effects become statistically significant at the high therapeutic and supra-therapeutic concentrations, while the therapeutic concentration appears rather inefficient with this regard, and 3) only AT pre-treatment significantly reduces the LPS-induced IL-6 release, while AT post-treatment is apparently without any effect. These findings will be essential for the investigations of the molecular and cellular mechanisms underlying the AT effects on the IL-6 release. To the best of our knowledge, this is the first study where AT effects on constitutive and LPS-induced IL-6 production in human skeletal muscle cells were examined.

The detailed molecular mechanisms of AT actions are not yet known, however there are several proposals based on the studies carried out in the experimental models other than skeletal muscle. Our findings are in accordance with the reports of Rauchhaus et al. (2000), Blaschke et al, (2009) and Loppnow et al. (2011) who reported statin induced decrease of IL-6 release in monocytes, smooth muscle cells and fibroblasts. These findings and the results of our study are especially important in the light of growing evidenced that IL-6 acts not only as a pro-inflammatory, but also as an anti-inflammatory cytokine, variety of IL-6 action is tissue-dependent and dose-dependent and differ with regard to the circumstances and the presence of other factors co-activated (Pedersen, 2011).

More recently, myocytes have raised a lot of interest as 'endocrine' cells, as it has been demonstrated that they can produce several signalling molecules, including, in particular, the cytokines with IL-6 constitutively secreted, and also produced in response to various stress stimuli (Pedersen et al., 2001; Filippatos et al., 2005; Prelovsek et a., 2006; Shang et al., 2006). Primary cultures of human myotubes were used here as an *in-vitro* model for human skeletal muscle. In this study, constitutive IL-6 secretion by this human skeletal muscle cell model, as well as LPS-induced increases in the production of IL-6, were demonstrated. Primary cultures of human myotubes (Miranda, 1994) are beside *in-vitro* innervated muscle cells, the only suitable *in-vitro* experimental model for studying processes in adult skeletal muscle, although results can not be completely extrapolated to the *in-vivo* conditions.

Consistent with previous studies (Ito et al., 2002; Prelovsek et al., 2006), LPS emerged as a strong stimulus for IL-6 secretion in our study, which therefore confirms that skeletal muscle represents an important source of cytokine production in inflammatory states. The exact mechanisms of this LPS-induced IL-6 production in myocytes remain to be defined, although there are several possible pathways that have already been proposed. These include activation of signalling pathways through activation of the Toll-like receptors (Gallaci et al.,

1998) and production of NF-κB transcriptional factor (Wright et al., 2002) and reactive oxygen species (Shang et al., 2006), all of which can increase the production and secretion of IL-6 and other cytokines like TNF-α.

Recent studies have revealed that as well as having a lipid-lowering ability, the statins can have important modulatory effects on immune response activation. In the presented study, AT clearly inhibited IL-6 secretion when administered in combination with LPS, thereby demonstrating its anti-inflammatory effects. These AT inhibitory effects were also concentration dependent. A wide range of AT concentrations was tested in our study, with selection of therapeutic and high- therapeutic and supra-therapeutic target, which means that well known pleiotrophic physiological effects of AT (Jacobson 2004; Zhou and Liao, 2010) have been tested. These data are consistent with the findings of previous studies (Ito et al., 2002) which have suggested a mechanism of mevalonate starvation. Mevalonate is a product of HMG-CoA conversion by HMG-CoA reductase, and it is a precursor to many isoprenoid compounds. These compounds serve as lipid-binding sites for intracellular signalling molecules, such as the small Rho guanosine triphosphate-binding proteins, the membrane localisation and function of which are dependent on isoprenylation (Acimovic et al., 2008; Adam and Laufs, 2008). Proteins like RhoA represent an important step in IL-6 and TNF-α production (Ito et al., 2002; Shang et al., 2006), and their inhibition can limit IL-6 and TNF-α secretion. Although, far from being completely understood, this suggested mechanism does sound appealing, especially with the correlation with results from other studies, where mevalonate or geranylgeranyl-pyrophosphate (an isoprenoid product of mevalonate metabolism) completely reversed the effects of statins on IL-6 synthesis in vascular smooth muscle cells (Ito et al., 2002).

The findings in this study relating to this IL-6 production are consistent with previous reports in similar models (Ito et al., 2002). In human vascular smooth muscle, IL-6 secretion was also inhibited by AT under basal conditions and in a concentration-dependent manner that was independent of exposure timing and the combination with LPS (Rauchhaus et al., 2000). It is most likely that the mechanism involved is isoprenylation of this GTPase (e.g. RhoA), and an interplay with the IL-6 production cascade. As in this study, where AT efficiently blocked both constitutive as well as LPS-induced IL-6 production, it can be speculated that in skeletal muscle cells, LPS-induced production of IL-6 works as an enhancement of an already existent IL-6 production signalling pathway.

Mechanistic insights are beyond the scope of the current experiments, and there are only a few reports in this field, so, further investigations are needed. Of course, having studied what we assume are normal skeletal muscle cells (in culture), our results might not be directly applicable to patients with chronic diseases, although they do provide further clear insight into the immunomodulatory effects of the statins. At this stage, our results are not directly applicable to clinical medicine, although they do support the findings from the CORONA and JUPITER trials, where positive benefit of rosuvastatin therapy were demonstrated in patients with elevated CRP levels (Ridker et al., 2008; von Haehling et al., 2003). Future studies are needed to investigate and define the precise mechanisms involved here, and it would be interesting to test statins with different pharmacokinetic profiles, along with other hypolipidemic drugs (e.g. ursodeoxcholic acid, fibrates).

Our study has demonstrated constitutive and LPS-induced IL-6 production *in-vitro* model of human skeletal muscle cells. Skeletal muscle might therefore represent an important source of cytokine secretion, and their expression might be involved in processes such as muscle wasting. AT inhibits constitutive and LPS-induced IL-6 secretion in skeletal muscle. This inhibition is concentration and time-of-exposure dependent. Such concentration-dependency and time-dependency appears to be of great importance when considering statin-induced modulation of cytokine production. However, the exact molecular mechanisms of IL-6 production and the role of the statins in these pathways remain defined. Our results thus provide further evidence of the immunomodulatory abilities of the statins, which should, over time, provide new approaches into these disease-treatment strategies.

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