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Statins decrease TNF-α-induced osteoprotegerin production by endothelial cells and smooth muscle cells *in vitro*

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ABSTRACT

Recent reports have implicated osteoprotegerin (OPG) in cardiovascular disease processes. Endothelial and smooth muscle cells produce OPG and its expression in these cells is upregulated by inflammatory mediators. Statins, which besides their lipid lowering properties have various vasculoprotective effects, have been shown to regulate OPG expression in osteoblasts. We investigated whether statins affect the expression of OPG in human endothelial and smooth muscle cells. Using an ELISA we could demonstrate that statins reduce tumor necrosis factor-α (TNF-α)-induced OPG production in cultured human endothelial cells and smooth muscle cells. Atorvastatin also downregulated interleukin-1α (IL-1α)-induced OPG production in endothelial cells. A significant reduction of TNF-α-induced OPG was seen when statins were used in the nanomolar range. These results were confirmed at the level of specific mRNA expression by real-time-PCR. Using LDH leakage as a marker of cell damage we show that cell viability was not affected by statins at concentrations used in our study. The effect of statins on TNF-α-induced OPG production was reversed by mevalonate and geranyl–geranyl pyrophosphate at the level of protein production and at the level of mRNA expression, suggesting that it was brought about by inhibition of the mevalonic acid pathway and protein prenylation. Through our results we have added OPG to the list of molecules whose TNF-α-induced upregulation is counteracted by statins. If such an effect is also operative in the *in vivo* setting, one could postulate a role for statins in the modulation of cardiovascular disease processes possibly regulated by OPG.

1. Introduction

Osteoprotegerin (OPG) is a soluble receptor belonging to the tumor necrosis factor receptor superfamily [1] and serves as a decoy receptor by binding to receptor activator of NF-κB ligand (RANKL) and thus blocks the interaction of RANKL with its receptor [2]. RANKL induces osteoclast differentiation and activation thereby promoting bone absorbtion. Since OPG antagonizes this effect its expression protects from bone loss [3]. Besides its role in bone metabolism OPG has been implicated recently also in cardiovascular disease processes. Knockout mice lacking OPG showed severe osteoporosis but also profound calcification of the large arteries [4]. OPG was immunohistochemicaly localized in normal and atherosclerotic human vessels [5] and calcific aortic valve stenosis [6]. Furthermore OPG was described as an independent risk factor

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for the development of cardiovascular disease [7]. Increased levels of OPG are associated with severity of coronary artery disease [8] and OPG is a marker for vascular calcification [9]. Ueland et al. [10] identified OPG as a novel marker for cardiovascular mortality and clinical events in patients with acute myocardial infarction complicated with heart failure. In addition elevated levels of OPG have been found in chronic inflammatory diseases like rheumatoid arthritis [11] and atherosclerosis [12].

OPG is constitutively expressed by endothelial cells and smooth muscle cells and is upregulated in these cells by inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) and other modulators of vascular pathogenesis such as basic fibroblast growth factor (bFGF), angiotensin II and platelet derived growth factor (PDGF) and is downregulated by anti-inflammatory substances like transforming growth factor-β (TGF-β) and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands [13].

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or statins represent an established class of drugs for the treatment of hypercholesterolemia and large-scale clinical trials have emphasized their benefits in the primary and secondary prevention of atherosclerosis and its complications. The beneficial effects of statins are believed to result from their ability to reduce cholesterol biosynthesis and in addition from pleiotropic actions such as anti-inflammatory, anti-thrombotic and antioxidant effects [14].

Recently a stimulating effect of statins on OPG synthesis by human osteoblasts was described [15]. Possible effects of statins on OPG production in vascular cells, however, are currently unknown and investigations to study this subject have been suggested in a recent review [13]. Therefore, we here asked the question whether statins are involved in the regulation of OPG expression in endothelial cells under normal and inflammatory conditions.

2. Materials and methods

2.1. Materials

Atorvastatin (kindly provided by Pfizer, Sandwich, U.K.), fluvastatin (kindly provided by Novartis, Basel, Switzerland) and simvastatin (kindly provided by Merck Sharpe and Dome, Ballydine, Ireland) were handled as described previously [16]. Mevalonate and geranyl–geranyl pyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO, USA). Human recombinant TNF-α was purchased from Roche (Basel, Switzerland). Human recombinant IL-1α and human recombinant PDGF-AB were obtained from R&D (Minneapolis, MN, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords by mild collagenase treatment and cultured and characterized as described [17]. Human coronary artery smooth muscle cells (HCASMC) were isolated from pieces of coronary artery by the explant technique and cultured as recently described [16]. All cells used in this study were between passages 3 and 8. For experiments outlined below the cells were seeded into 24 well culture dishes and grown to confluence (30,000 cells/well for HUVEC; 10,000 cells/well for HCASMC). All human material was obtained and processed according to the recommendations of the hospital's Ethics Committee and Security Board.

2.3. Quantification of osteoprotegerin

Endothelial cells and smooth muscle cells were incubated in Medium 199 (M199; Sigma) containing 1.25% supplemented calf serum (Biochrom, Berlin, Germany) with different concentrations of TNF-α or the respective statin for the indicated time periods. In some experiments cells were treated with TNF-α or the respective statin in combination with mevalonate or GGPP, respectively. After incubation, the culture supernatants were collected following removal of cell debris by centrifugation and stored at −80 °C until used. In some experiments after collection of supernatants the remaining cell-monolayers were washed with phosphate-buffered saline (PBS), pH 7.4 and lysed by addition of 0.5 ml PBS containing 0.1% Triton X 100 per well. After centrifugation the lysates were stored at −80 °C until used. OPG antigen in conditioned media and lysates was determined by a specific enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (Biomedica, Vienna, Austria) according to the manufacturer's instruction.

2.4. Determination of cell viability

In order to determine possible cytotoxic effects of TNF-α, IL-1α, PDGF-AB or the statins used, lactate dehydrogenase (LDH) leakage was measured in cultures treated with TNF-α, IL-1α,

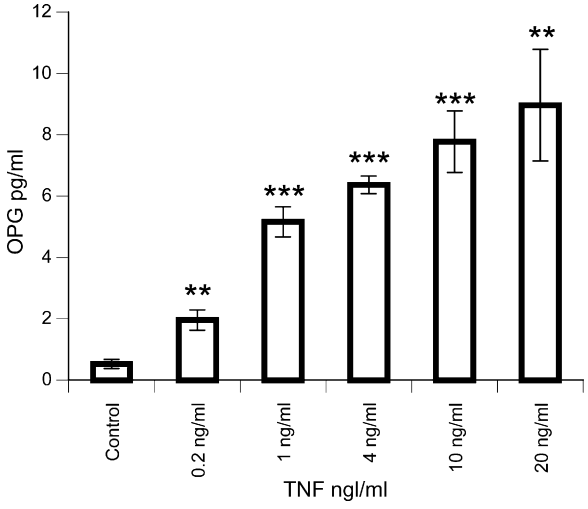

Fig. 1 – Dose-dependent effect of TNF-α on OPG protein expression. Confluent monolayers of HUVEC were incubated with or without TNF-α at the indicated concentration for 24 h. Conditioned media of such treated cells were collected and OPG was determined as described under Section 2. Values represent mean values ± S.D. of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. Data were compared by ANOVA. Values of $p < 0.05$ were considered significant. $^{***}p < 0.001$, $^{**}p < 0.01$ compared to control cells.

PDGF-AB or statins using a commercially available assay for photometric determination of LDH activity (Sigma).

2.5. mRNA purification

Cells were stimulated as described above, supernatant was removed and RNA was isolated using High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

2.6. Quantification of OPG mRNA by real-time-polymerase chain reaction

Real-time-PCR was performed using LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturer's instructions. Primers were designed using the Primer3 Software (http://frodo.wi.mit.edu/), GAPDH forward primer: 5′-ACA GTC CAT GCC ATC ACT GCC-3′, GAPDH reverse primer: 5′-GCC TGC TTC ACC ACC TTC TTG-3′, OPG forward primer: 5′-TCT ATA CTG CAG CCC CGT GT-3′, OPG reverse primer: 5′-AGG AGG GCA GCT CCT ATG TT-3′. The reverse transcriptase reaction consisted of an initial incubation at 61 °C for 20 min, incubation at 95 °C for 30 s, followed by 50 amplification cycles of 95 °C for 1 s, the respective annealing temperature (67° for OPG, 65° for GAPDH) for 10 s and 72 °C for 10 s. A melting step was performed from 45 to 95 °C increasing 0.1 °C per second. Data was analysed using LightCycler Software Version 3.5 (Roche). RNA was isolated using High Pure RNA Isolation Kit (Roche).

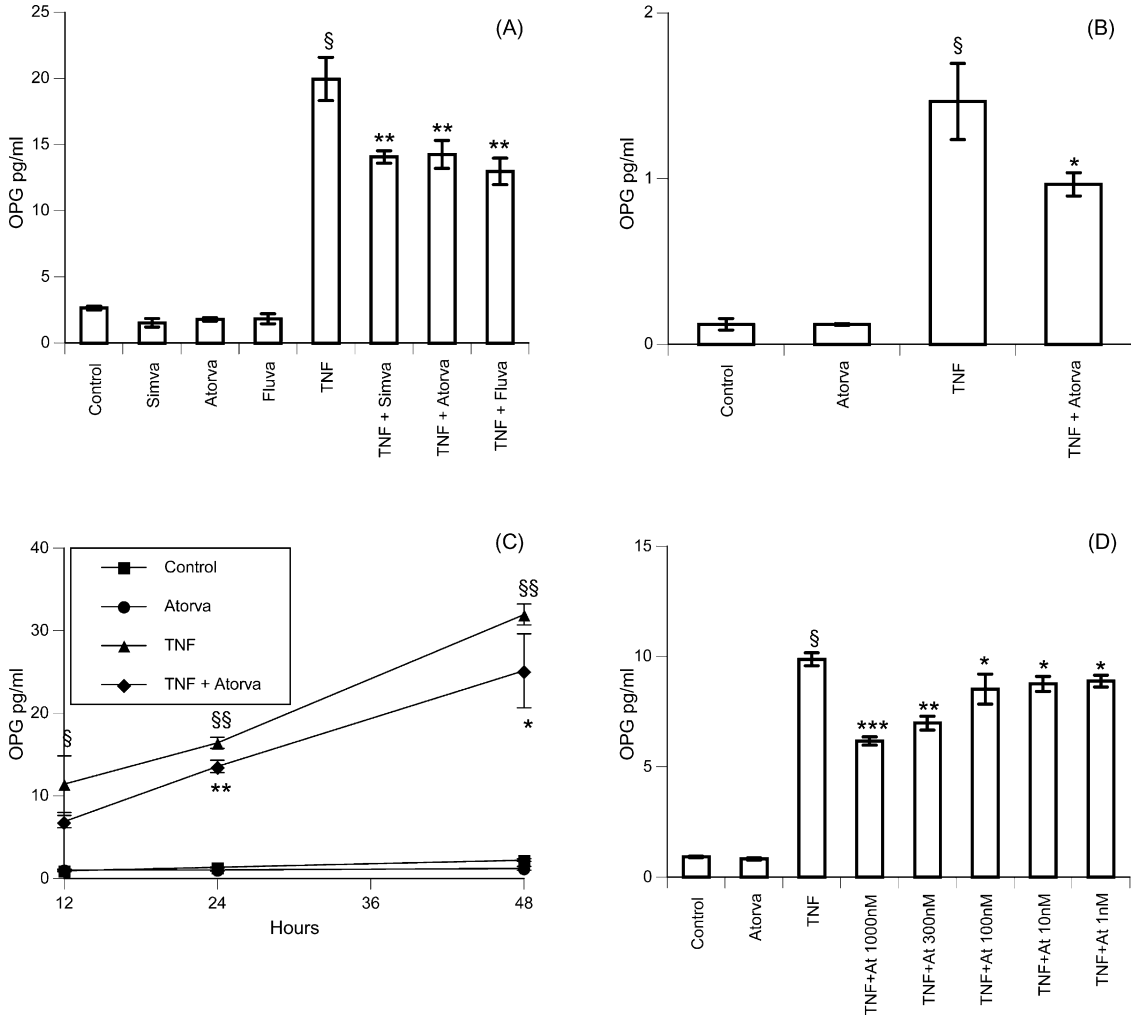

Fig. 2 – Effect of statins on TNF-α-induced OPG protein production in HUVEC. (Panels A and B) Confluent monolayers of HUVEC were incubated with the indicated statins, TNF-α (20 ng/ml) or a combination of one of the statins and TNF-α, at the respective concentration, for 24 h. (Panel C) Confluent monolayers of HUVEC were incubated for 12, 24 and 48 h with or without atorvastatin (0.5 μM) or TNF-α (20 ng/ml) alone or a combination of the statin and TNF-α, at the respective concentration. (Panel D) Confluent monolayers of HUVEC were incubated for 24 h with or without TNF-α (20 ng/ml) alone or with TNF-α (20 ng/ml) in combination with the indicated concentration of atorvastatin. Conditioned media (panels A, C and D) and lysates (panel B) of such treated cells were collected and OPG was determined as described under Section 2. Values shown in panels A–D represent mean values ± S.D. of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. Data were compared by ANOVA: $^{\S}p < 0.005$, $^{\S\S}p < 0.01$ compared to control cells, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to TNF-α-treated cells.

2.7. Statistical analysis

Data were compared statistically by ANOVA. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of TNF-α, IL-1α, PDGF-AB or statins on cell viability

When HUVEC or HCASMC were incubated with fluvastatin, simvastatin or atorvastatin for 24 h at a concentration of 1 μM, cell viability was not significantly affected by this treatment as LDH leakage did not exceed 105% of control. A cytotoxic effect was only observed at concentrations >5 μM of the respective statins (data not shown). No cytotoxic effect of TNF-α, IL-1α or PDGF-AB was observed at the concentrations used in this study (data not shown).

3.2. Statins affect TNF-α-induced OPG secretion in HUVEC and HCASMC

As can be seen from Fig. 1 TNF-α (0.2–20 ng/ml) dose-dependently induced OPG protein secretion in HUVEC in agreement with previous studies [18]. Incubation of HUVEC with a combination of the respective statin (0.5 μM) together with TNF-α (20 ng/ml) resulted in a significant reduction of the secretion of OPG in these cells as compared to HUVEC treated with TNF-α alone at 24 h (Fig. 2A). A similar effect was seen when OPG was measured in the lysates of such treated HUVEC (Fig. 2B). The effect on OPG secretion was also evident at 12 and 48 h of incubation as can be seen from Fig. 2C. Similar to the results obtained with TNF-α OPG secretion was also increased in HUVEC by IL-1α (50 ng/ml) in agreement with previous observations [18]. Incubation of HUVEC with a combination of atorvastatin (0.5 μM) together with IL-1α (50 ng/ml) resulted in a significant reduction of the secretion of OPG in these cells as compared to HUVEC treated with IL-1α alone at 24 h (control: 0.5 ± 0.1 pg/ml, IL-1α: 18.7 ± 1.7 pg/ml, atorvastatin: 0.4 ± 0.1 pg/ml, IL-1α + atorvastatin: 10.5 ± 1.2 pg/ml). In contrast to these results PDGF-AB (50 ng/ml) had no effect on OPG secretion in HUVEC (data not shown).

When HUVEC were treated with different concentrations of atorvastatin (1 nM to 1 μM) together with TNF-α (20 ng/ml), atorvastatin significantly downregulated TNF-α-induced OPG production up to a concentration of 1 nM (Fig. 2D).

For RNA analysis mRNA was collected from HUVEC treated for 2, 6 and 24 h with a combination of TNF-α (20 ng/ml) and atorvastatin (0.5 μM). The mRNA data suggests a sustained inhibitory effect of atorvastatin on TNF-α-induced OPG mRNA upregulation, as OPG mRNA levels of cells treated with a combination of TNF-α and atorvastatin were lower than mRNA levels of HUVEC treated with TNF-α alone at all time points tested (Fig. 3).

TNF-α (20 ng/ml) also increased the secretion of OPG in HCASMC significantly. When these cells were in incubated with a combination of atorvastatin (0.5 μM) together with TNF-α (20 ng/ml) a significant reduction of the secretion of OPG in

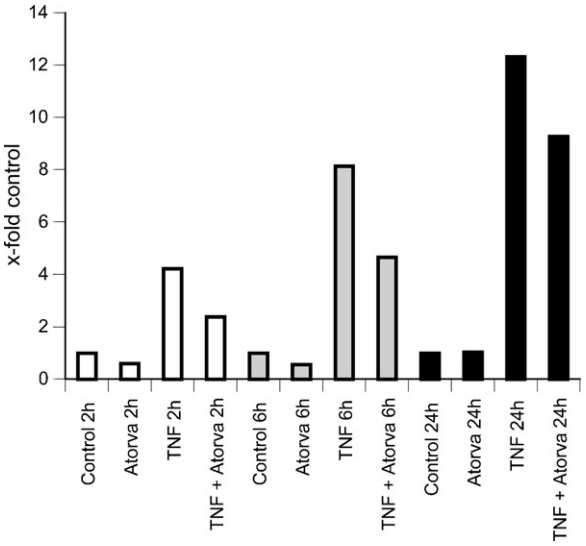

Fig. 3 – Effect of statins on TNF-α-induced OPG mRNA expression in HUVEC. Confluent monolayers of HUVEC were incubated for 2, 6 and 24 h with or without atorvastatin (0.5 μM) or TNF-α (20 ng/ml) alone or a combination of the statin and TNF-α, at the respective concentration. RNA was prepared and real-time-PCR was performed as described under Section 2. Values were normalized to the housekeeping gene GAPDH and are given as x-fold control. Experiments were performed two times. A representative experiment is shown.

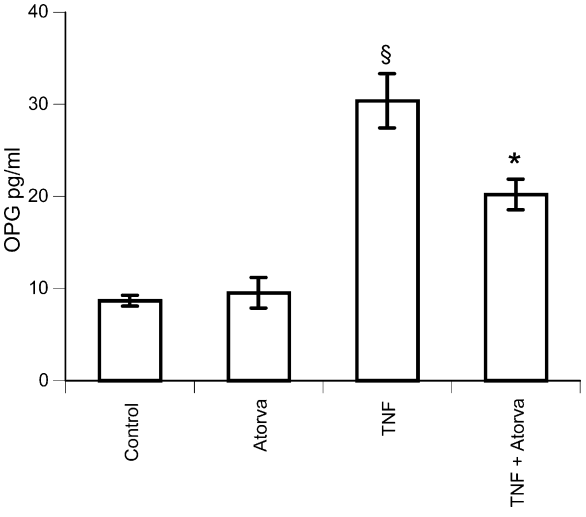

Fig. 4 – Effect of atorvastatin on TNF-α-induced OPG protein production in HCASMC. Confluent monolayers of HCASMC were incubated with atorvastatin (0.5 μM), TNF-α (20 ng/ml) or a combination of atorvastatin and TNF-α, at the respective concentration, for 24 h. Conditioned media of such treated cells were collected and OPG was determined as described under Section 2. Values represent mean values ± S.D. of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. Data were compared by ANOVA: $^{\S}p < 0.005$ compared to control cells, $^{*}p < 0.05$ compared to TNF-α-treated cells.

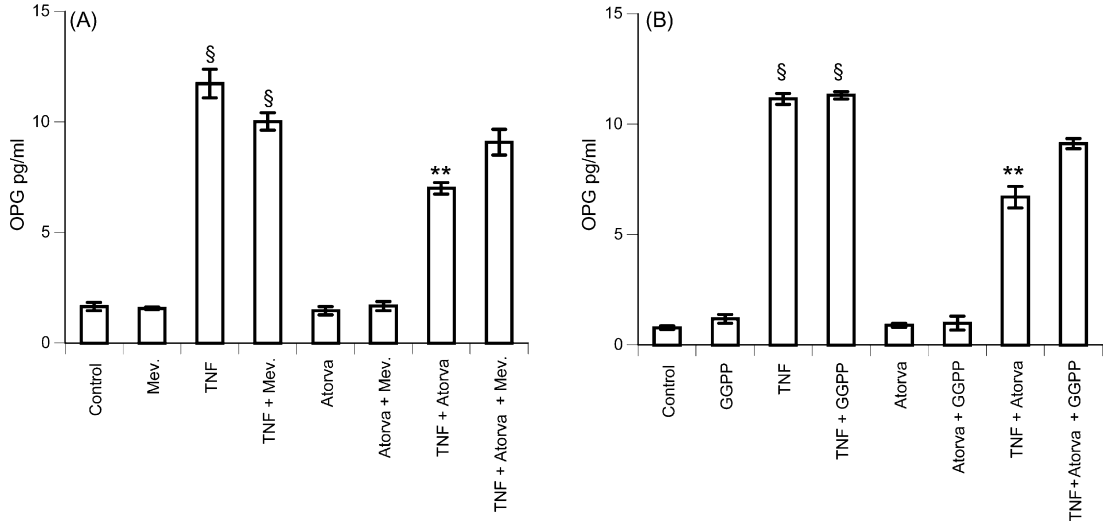

Fig. 5 – Effect of atorvastatin on TNF-α-induced OPG expression is reversed by mevalonate and geranyl–geranyl pyrophosphate. (Panel A) Confluent monolayers of HUVEC were incubated for 24 h with or without TNF-α (20 ng/ml), mevalonate (100 μM) or atorvastatin (0.5 μM) alone, or with the indicated combination at the respective concentration. (Panel B) Confluent monolayers of HUVEC were incubated for 24 h with or without TNF-α (20 ng/ml), GGPP (10 μM) or atorvastatin (0.5 μM) alone, or with the indicated combination at the respective concentration. Conditioned media of such treated cells were collected and OPG was determined as described under Section 2. Values represent mean values ± S.D. of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. Data were compared by ANOVA. $^{\S}p < 0.001$ compared to control cells, $^{**}p < 0.001$ compared to TNF-α-treated cells.

these cells as compared to HCASMC treated with TNF-α alone at 24 h was observed (Fig. 4).

3.3. *Mevalonate and geranyl–geranyl pyrophosphate reverse the effect of statins on OPG secretion*

To determine whether the effects of the statins on TNF-α-induced OPG secretion depend on their capacity to inhibit mevalonate synthesis by HMG-CoA reductase, HUVEC were treated with atorvastatin (0.5 μM) together with TNF-α (20 ng/ml) in the absence or presence of 100 μM mevalonate or 10 μM GGPP. When mevalonate or GGPP were present during the incubation period for 24 h the downregulating effect of atorvastatin on TNF-α-induced OPG was reversed (Fig. 5A). The addition of GGPP to HUVEC treated with atorvastatin and TNF-α, also reduced the downregulating effect of atorvastatin on OPG secretion as shown in Fig. 5B. Similar to the results obtained at the level of protein secretion mevalonate and GGPP reversed the downregulating effect of atorvastatin on TNF-α-induced OPG expression also at the level of specific mRNA in HUVEC treated for 6 h with atorvastatin (0.5 μM) together with TNF-α (20 ng/ml) in the absence or presence of 100 μM mevalonate or 10 μM GGPP (Table 1).

Table 1 – Effect of atorvastatin on TNF-α-induced OPG mRNA expression is reversed by mevalonate and geranyl–geranyl pyrophosphate

	OPG mRNA
Control	1.0
TNF-α	8.3
TNF-α + atorvastatin	4.5
TNF-α + atorvastatin + mevalonate	7.2
TNF-α + atorvastatin + GGPP	7.5

Confluent monolayers of HUVEC were incubated for 6 h with or without TNF-α (20 ng/ml) in the presence or absence of mevalonate (100 μM) or GGPP (10 μM). RNA was prepared and real-time-PCR was performed as described under Section 2. Values were normalized to the housekeeping gene GAPDH and are given as x-fold control. Mevalonate or GGPP alone had no effect on TNF-α-induced upregulation of OPG mRNA (data not shown). Experiments were performed two times. A representative experiment is shown.

4. Discussion

Recently evidence has been accumulated implicating OPG in the pathophysiology of cardiovascular disease. Increased OPG serum levels are associated with the presence and severity of coronary artery disease and vascular calcification and were identified as an independent risk factor of cardiovascular disease [8]. A recent report showed that men with OPG polymorphism mutations both in the promoter and the signal peptide region had an increased risk of coronary artery disease and exhibited significantly higher circulating OPG serum levels [19]. However, it is not clear at this point of time if circulating levels of OPG are directly involved in vascular calcification or reflect an attempt of the organism to correct overmineralization and thus are vasculoprotective or are indicators of vascular disease. In that respect it should also be mentioned that OPG might contribute to or facilitate monocyte chemotaxis in inflammatory lesions via syndecan-1

activation independent of the RANKL pathway and thereby could be involved in modulating inflammatory injury in the vascular system [20].

Vascular smooth muscle cells and endothelial cells express OPG constitutively and its expression is upregulated in these cells by inflammatory cytokines such as TNF-α and IL-1 [13]. In agreement with these studies we report here that human endothelial cells and human vascular smooth muscle cells *in vitro* produce OPG and that this production is induced by TNF-α. In further agreement with a recent report we also show in this paper that human smooth muscle cells isolated from coronary arteries produce more OPG than human umbilical vein endothelial cells, which suggests a significant contribution of smooth muscle cells to pathophysiological processes regulated by OPG [21]. Furthermore we report here for the first time that statins significantly reduce TNF-α-induced OPG in human vascular smooth muscle cells and TNF-α- and IL-1α-induced OPG in human endothelial cells and that the effect on TNF-α-induced OPG was time and dose-dependent in the latter cell type. It should be mentioned that PDGF-AB had no effect on OPG production in our endothelial cells. A significant reduction of TNF-α-induced OPG was seen even when atorvastatin was used in the nanomolar range in endothelial cells. Following clinical administration of atorvastatin maximum plasma levels in patients were between 6 and 200 nM [22]. It should be emphasized that in our experiments statins also decreased the basal production of OPG by endothelial cells slightly, but not significantly. As indicated by the results of a cell viability assay using LDH leakage as a marker of cell damage the effect of the HMG CoA reductase inhibitors on TNF-α-induced OPG was not due to cytotoxicity. LDH leakage was not affected in endothelial cells by statins at concentrations used here. Our results obtained on the protein level were also reflected on the level of mRNA expression because TNF-α-stimulated mRNA levels specific for OPG were also reduced when cells were treated with atorvastatin as determined by real-time-PCR. In our study the effect of statins on the TNF-α-induced upregulation of OPG production by endothelial cells was reversed by mevalonate and GGPP, suggesting that this effect was brought about by inhibition of the mevalonic acid pathway and protein prenylation. It should be mentioned, that, in contrast to our data presented here, statins increase the expression of OPG in osteoblasts [15]. This might be related to the difference in cell type.

In contrast to statins, whose protective and anti-inflammatory effects on the cardiovascular system are well defined, the exact role of OPG in the inflammation process in general and in cardiovascular disease in particular remains to be fully understood [23]. As discussed above OPG is robustly upregulated by cytokines such as TNF-α and IL-1 in vascular cells and is present in normal vessels and in atherosclerotic plaques [5,13]. We provide evidence that statins counteract the OPG production in endothelial cells after inflammatory activation through TNF-α. This is in line with other studies showing that statins inhibit the effects of this inflammatory cytokine on the expression of modulators of cardiovascular injury such as adhesion molecules and plasminogen activator inhibitor 1 [16,24].

In conclusion we have added OPG to the list of molecules whose TNF-α-induced upregulation is counteracted by statins. If such an effect is also operative in the *in vivo* setting, one could postulate a role for statins in the modulation of cardiovascular disease processes possibly regulated by OPG.

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