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Chapter 3

Evidence for anti-inflammatory activity of statins, PPAR α -activators and an LXR-agonist in human C-reactive protein transgenic mice *in vivo* and in cultured human hepatocytes *in vitro*

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Abstract

Background: Inflammatory processes, aside from cholesterol, play a central role in atherogenesis. Human C-reactive protein (huCRP) signals systemic inflammation and independently predicts future cardiovascular risk. Cholesterol-lowering statins reduce atherosclerosis and plasma huCRP levels. Evidence is sought for a direct anti-inflammatory statin effect *in vivo*, independent of effects on plasma cholesterol and atherogenesis.

Results: The effect of atorvastatin and simvastatin on huCRP expression was studied in non-atherosclerotic huCRP transgenic mice and compared to other classes of lipid modulating compounds, PPAR α -activators, notably fenofibrate and Wy14643, and an LXR agonist, T0901317. Like statins, PPAR α -activators combine anti-atherosclerotic properties with huCRP-lowering effects. Dietary treatment with statins, PPAR α -activators or an LXR-agonist decreased basal and IL-1 β -induced plasma huCRP levels independently of cholesterol-lowering. These direct anti-inflammatory *in vivo* effects occurred at the transcriptional level and could be confirmed by statins and PPAR α -activators in cultured human liver slices and in human hepatoma cells transiently transfected with a huCRP promoter-driven luciferase reporter. A molecular rationale for the suppression of IL-1-induced huCRP transcription is provided by showing that statins and PPAR α -activators upregulate I κ B- α protein expression. This results in a reduced nuclear translocation of p50-NF κ B and thereby decreased amounts of nuclear p50-NF κ B~C/EBP β complexes, which determine the huCRP transcription rate.

Conclusions: Our results provide conclusive evidence for a direct suppressive effect of statins, PPAR α -activators and an LXR-agonist on huCRP expression independent of cholesterol-lowering and atherogenesis.

Introduction

It is now apparent that chronic inflammatory processes involving p50-NF κ B are among the dominant factors, aside from cholesterol, that modulate atherosclerosis.¹⁻⁴ Clinical studies demonstrate that human C-reactive protein (huCRP), a liver-derived, p50-NF κ B-regulated inflammation marker, independently predicts future cardiovascular events (summarized in^{1, 5}). Recent observations indicate that huCRP may also be directly involved in atherogenesis.⁶⁻¹⁰

Statins are cholesterol-lowering agents that reduce atherogenesis and concomitantly the inflammatory state as reflected by the decreased plasma levels of huCRP.^{1, 11, 12} The mechanism by which statins lower huCRP levels is still unknown. Since the atheromatous material itself also is a potent initiator of systemic inflammatory processes,^{2, 13} the question arises whether the huCRP-lowering effect of statins is a primary effect or a secondary effect, i.e. a consequence of the reduced atherogenesis. Because this issue is difficult to untangle in humans and CRP is not an acute phase protein in mice, huCRP transgenic (huCRPtg) mice have been used to address this question. HuCRPtg mice carry the entire huCRP gene, including the coding region and 17kb of the 5' flanking promoter region, and the expression of huCRP in these mice resembles its regulation in humans.^{14, 15} This enabled us to test whether statins, notably atorvastatin and simvastatin, exert a direct suppressive effect on huCRP expression under basal and inflammatory (IL-1-induced) conditions, independent of lipid-lowering and/or the atherogenic process. We have compared the effects of statins with another class of hypolipidemic drugs, notably fenofibrate and the specific PPAR α -activator Wy14643, which also combine anti-atherosclerotic properties with anti-inflammatory effects, including huCRP-lowering in hyperlipidemic patients.¹⁶⁻¹⁹ We previously reported that fenofibrate, Wy14643 and other activators of PPAR α directly suppress huCRP expression in primary human hepatocytes.²⁰ In addition to this, a second comparison was made with an additional putative anti-atherogenic compound with anti-inflammatory effects, the synthetic LXR-agonist, T0901317. In this study, we demonstrate that statins, PPAR α -activators and an LXR agonist reduce basal and IL-1 β -induced huCRP expression *in vivo* in huCRPtg mice independently of cholesterol lowering. A suppression of basal and IL-1 α -induced huCRP gene expression was also observed in *ex vivo* cultured human liver slices and in human HuH7 hepatoma cells using statins and PPAR α -activators. In previous studies we showed that huCRP gene transcription increases with increasing amounts of nuclear p50-NF κ B~C/EBP β complexes.²⁰ Here, evidence is provided that statins, similar to PPAR α -

activators, suppress IL-1-induced huCRP expression by reducing p50-NF κ B translocation into the nucleus via upregulation of I κ B α , resulting in a diminished amount of nuclear p50-NF κ B~C/EBP β complexes which are rate-limiting for huCRP transcription.

Materials and methods

Animals

Human CRP (huCRP) transgenic mice^{14, 15} were characterized by PCR and ELISA²¹ for huCRP expression. Experiments were approved by the Institutional Animal Care and Use Committee of TNO and were in compliance with European Community specifications regarding the use of laboratory animals. Mice had free access to water and food. Food intake and body weight were monitored throughout the study period.

Animal experiments

Male huCRP^{tg} mice were fed either standard mouse chow or one containing either 0.01%(w/w) or 0.1%(w/w) atorvastatin (Lipitor®, Pfizer, Capelle a/d IJssel, The Netherlands), 0.1%(w/w) simvastatin (Zocor®, MSD, Haarlem, The Netherlands), 0.03%(w/w) Wy14643 (Chemsyn, Lenexa, KS, USA), 0.1%(w/w) fenofibrate (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) or 0.01%(w.w) T0901317. Murine and human recombinant interleukin-1 α /-1 β (IL-1 α /-1 β) were purchased from Sanvertch (Heerhugowaard, The Netherlands) and used for *in vivo* and *in vitro* experiment, respectively. Comparable huCRP-inducing effects were obtained for both isoforms. For the *in vivo* experiments IL-1 β was injected intraperitoneally at a low (83,000 U/mouse) or a high (250,000 U/mouse) dose. HuCRP and total plasma cholesterol concentrations were determined in tail blood plasma samples by ELISA²¹ and kit No-1489437 (Roche Diagnostics, Almere, The Netherlands), respectively. Livers of animals sacrificed 10 hr after IL-1 β treatment were used to prepare mRNA²² and cDNA (kit #A3500, Promega, Leiden, The Netherlands). RT-PCR analysis was performed using RT-PCR mastermix (Eurogentec, Seraing, Belgium) and the ABI7700 RT-PCR system (PerkinElmer Biosystems, Nieuwekerk a/d IJssel, The Netherlands), following the guidelines of the manufacturer and using cyclophilin (PerkinElmer Biosystems) as a reference.

Experiments with human hepatocytes

Experiments with human liver were approved by the Institutional Committees of the Leiden University Medical Center and TNO-Prevention and Health. Human liver slices and primary human hepatocytes were prepared and cultured as reported.²⁰ Briefly, liver slices and primary cells were pre-treated for 16 hours with 3 μ M simvastatin (Merck, Amsterdam, The Netherlands), 250 μ M Wy14643 (Chemsyn, Lenexa, KS, USA) or vehicle (DMSO), and subsequently stimulated with 25 ng/ml IL-1 α for an additional 24 hours in the continuous presence of simvastatin, Wy14643 or vehicle. *In vitro* experiments evaluating PPAR α -activation have been performed with Wy14643 since fenofibrate is not applicable in tissue culture because it requires *in vivo* conversion into fenofibric acid. HuCRP was determined in the supernatant of the treated cells by ELISA.²¹

Transfection experiments

Human HuH7 hepatoma cells were cultured as specified.²⁰ Reporter gene activity was determined using the dual-luciferase reporter assay system (Promega, Leiden, The Netherlands) essentially as described.²⁰ A renilla luciferase construct was co-transfected to correct for differences in transfection efficiency. Briefly, cells were pre-incubated with simvastatin (1, 3, 10 μ M), atorvastatin (1, 3, 10 μ M) or vehicle for 24 hours as established previously²³ or with Wy 14643 (10, 30, 100 μ M) for 5 hours.²⁰ Then, 100 ng of a luciferase reporter plasmid carrying a 300bp fragment of the huCRP promoter (phuCRP-luc)²⁰ or 3 consecutive NF- κ B binding sites (p3xNF κ B-luc)²³ were transiently transfected into 1.2×10^5 HuH7 cells using the FUGENE6 transfection reagent (Roche Diagnostics). Subsequently, cells were stimulated with IL-1 α (5 ng/ml) and harvested after an additional 18 hours in the presence of the above indicated concentrations of simvastatin, atorvastatin, Wy14643 or vehicle.

Western Blotting and co-immunoprecipitation

Cellular and nuclear extracts were prepared in the presence of proteinase inhibitors (PI) (Roche Diagnostics) as reported.²⁰ Western blotting and co-immunoprecipitation experiments were performed following detailed protocols²⁰ using the antibodies anti-p50-NF κ B(sc-1190), anti-I κ B α (sc-371), anti-C/EBP- β (sc-150) and the control antibodies anti-MMP8(sc-8848), anti- β -actin(sc-1615), and anti-histone H1(sc-8616). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands). Immunoblots were visualized using the Super Signal West Dura Extended Duration Substrate (Pierce, St Augustin, Germany) and the luminescent image workstation (Roche Diagnostics).

Statistical methods

Significant differences were established by an ANOVA test (SPSS version 11.5 for Windows) followed by a Least Significant Difference (LSD) post hoc analysis. The level of statistical significance was set at $P < 0.05$. Data are presented as means \pm SEM unless stated otherwise.

Results

Effect of statins, PPAR α -activators and LXR-agonist on plasma cholesterol and basal huCRP levels

HuCRP tg mice were fed a chow diet containing either vehicle (control) or a low dose of atorvastatin (0.05%(w/w); LD) for 4 weeks. Plasma was collected weekly for cholesterol and huCRP measurements. As shown in Figure 1A, LD atorvastatin treatment resulted in a significant 21% ($P < 0.05$) decrease in cholesterol levels after 2 weeks. Plasma cholesterol levels did not further decrease after prolonged treatment. After 4 weeks, cholesterol levels of the LD group were reduced by 16% ($P < 0.05$) as compared to controls. Plasma huCRP levels in the LD group and the control group remained constant and did not significantly change over the 4 weeks treatment period (Figure 1C; left panel).

In a second experiment, the mice were treated with a higher dose of atorvastatin (0.1%(w/w); HD). As compared to LD, HD atorvastatin treatment more rapidly lowered

plasma cholesterol levels, reaching maximal reduction (27%; $P < 0.05$) already after one week (Figure 1B). The reduction in cholesterol levels in the HD group at the end of the treatment period (22%; $P < 0.05$) was very comparable to that in the LD group.

In contrast to LD, however, HD atorvastatin treatment very strongly reduced huCRP levels, resulting in a 93% ($P < 0.05$) decrease after 4 weeks (Figure 1C; right panel). These results indicate that the anti-inflammatory potential of atorvastatin is demonstrable at concentrations that are higher than required for cholesterol-lowering. For comparison, mice were also treated with another statin, simvastatin. Treatment with 0.1%(w/w) simvastatin resulted in a comparable cholesterol reduction (on average 21%; $P < 0.05$) as observed with atorvastatin, but a less pronounced reduction (75%; $P < 0.05$) of plasma huCRP (Figures 1D and 1E). This suggests that atorvastatin has a stronger anti-inflammatory potential than simvastatin when compared at the same dose.

In another series of experiments, huCRP^{tg} mice received a chow diet containing either 0.1%(w/w) fenofibrate, 0.03%(w/w) Wy14643 or vehicle. Four weeks of fenofibrate treatment resulted in a 66% ($P < 0.05$) reduction of plasma huCRP levels in the absence of an effect on plasma cholesterol (Figures 1F and 1G). An even stronger huCRP-lowering effect (99%; $P < 0.05$) was observed with the specific PPAR α -activator Wy14643; Wy14643 did not affect plasma cholesterol levels (Figures 1F and 1G). In another set of experiments, huCRP^{tg} mice received a chow diet containing 0.01% (w/w) of the LXR agonist, T0901317. Four weeks of treatment resulted in a 56% ($P < 0.05$) reduction of plasma huCRP levels. These effects were observed in spite of an increase in total plasma cholesterol (Figure 1H). In all, the data demonstrate that statins, PPAR α -activators and an LXR agonist reduce basal huCRP expression *in vivo* independently of lowering plasma cholesterol.

Effect of statins and PPAR α -activators on IL-1 β -induced huCRP expression

To test whether statins and PPAR α -activators can also suppress the *in vivo* induction of huCRP expression by IL-1, i.e. an acute inflammatory stimulation, we first determined the time profile of the effect of a single intraperitoneal injection of IL-1 β . As shown in Figure 2, an intraperitoneal injection of $2.5 \cdot 10^5$ U IL-1 β /mouse resulted in a time-dependent increase in plasma huCRP levels. A maximally 15-fold ($P < 0.05$) induction was reached between 18h and 24h. Plasma huCRP levels then gradually declined reaching near control values after 120h.

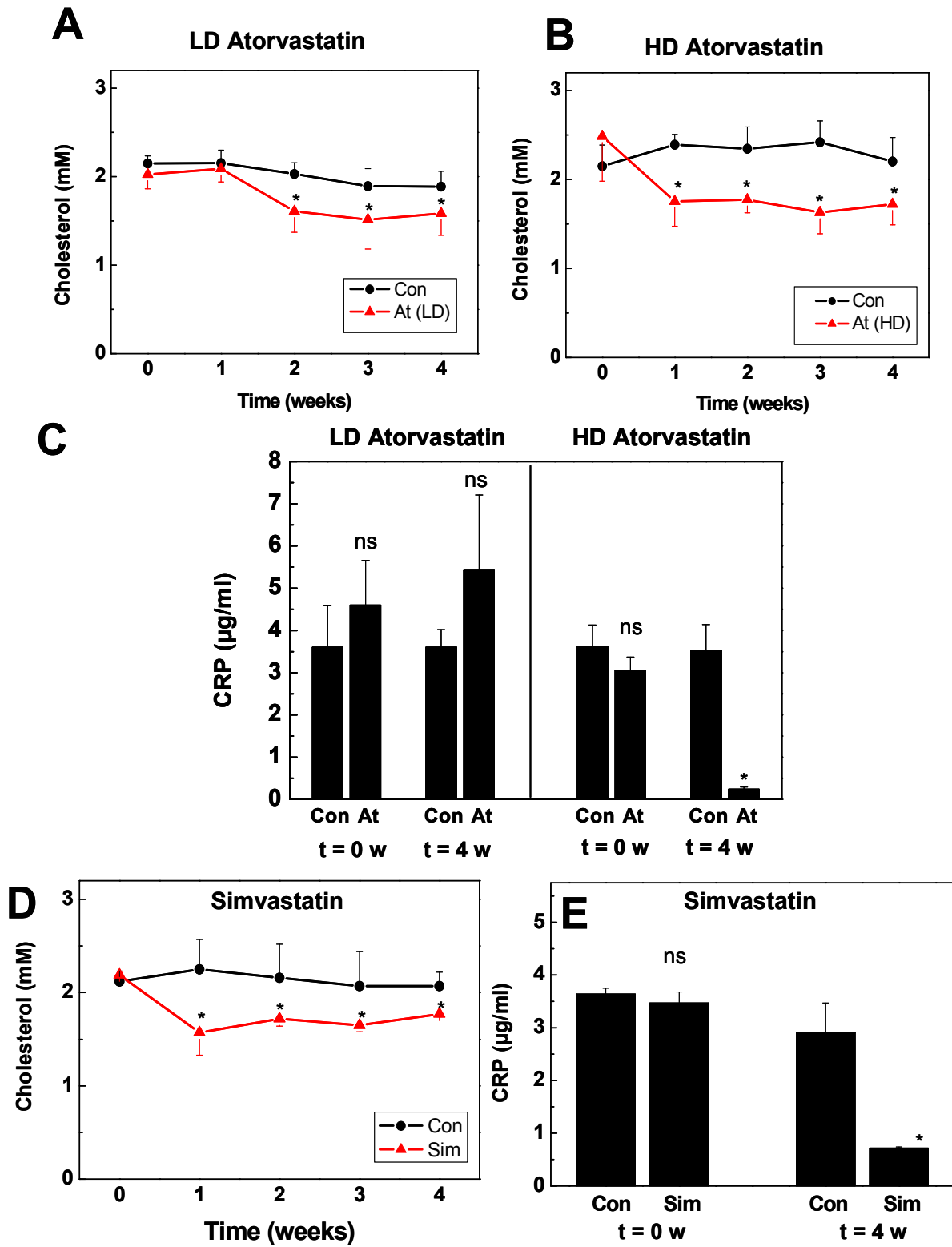


Figure 1. (see legend on next page)

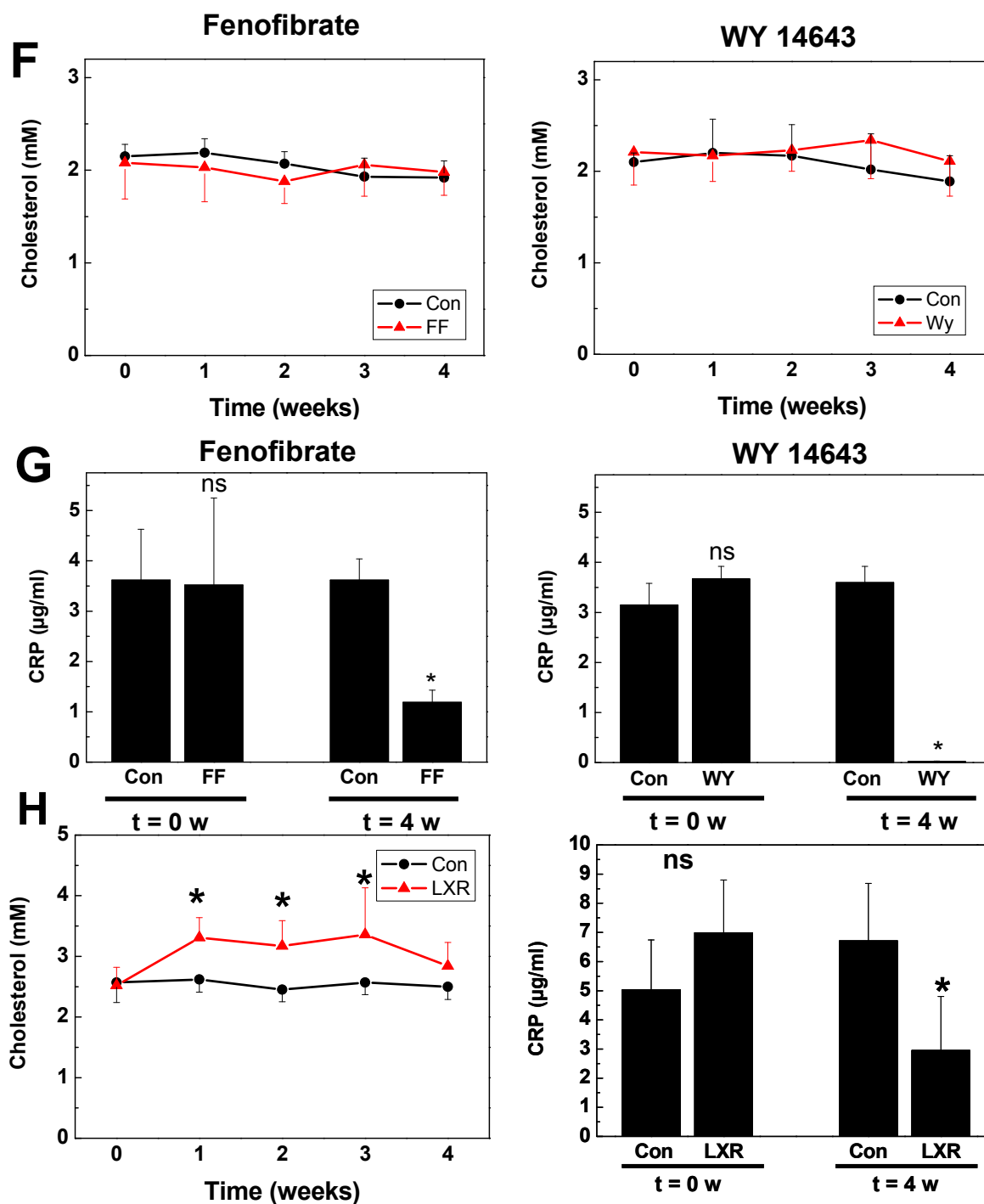


Figure 1: Effect of atorvastatin, simvastatin, fenofibrate, Wy14643 and T0901317 (LXR) on plasma cholesterol and basal huCRP expression. **(A)** Groups of huCRP^{tg} mice ($n \geq 5$) received a chow diet containing a low dose (LD; 0.01%(w/w)) of atorvastatin (At) or vehicle control (Con) for 4 weeks. Data are expressed as means \pm sd. **(B)** As in (A) but mice received a high dose (HD; 0.1%(w/w)) of atorvastatin. **(C)** Plasma huCRP levels of mice are expressed as means \pm SEM. **(D)** As in (A) but mice received a dose of 0.1%(w/w) simvastatin. **(E)** As in (C). **(F)** As in (A) but mice received 0.1%(w/w) fenofibrate (FF) or 0.03%(w/w) Wy14643. **(G)** As in (C). **(H)** As in (A) and (C). Statistical significant difference compared to control animals is indicated by * $P < 0.05$, ns=not significant.

Subsequently, the effect of atorvastatin and simvastatin on IL-1 β -induced huCRP expression was investigated. HuCRP^{tg} mice were treated for three weeks with LD or HD atorvastatin or vehicle. Then mice received an intraperitoneal injection of either a low dose ($8.3 \cdot 10^4$ U/mouse) or a high dose ($2.5 \cdot 10^5$ U/mouse) of IL-1 β .

Plasma huCRP levels were determined 18h after stimulation with IL-1 β . In control animals, injection of a low or a high dose of IL-1 β resulted in a 5.4-fold ($P < 0.05$) and a 25.0-fold ($P < 0.05$) increase in huCRP, respectively (Figure 3A).

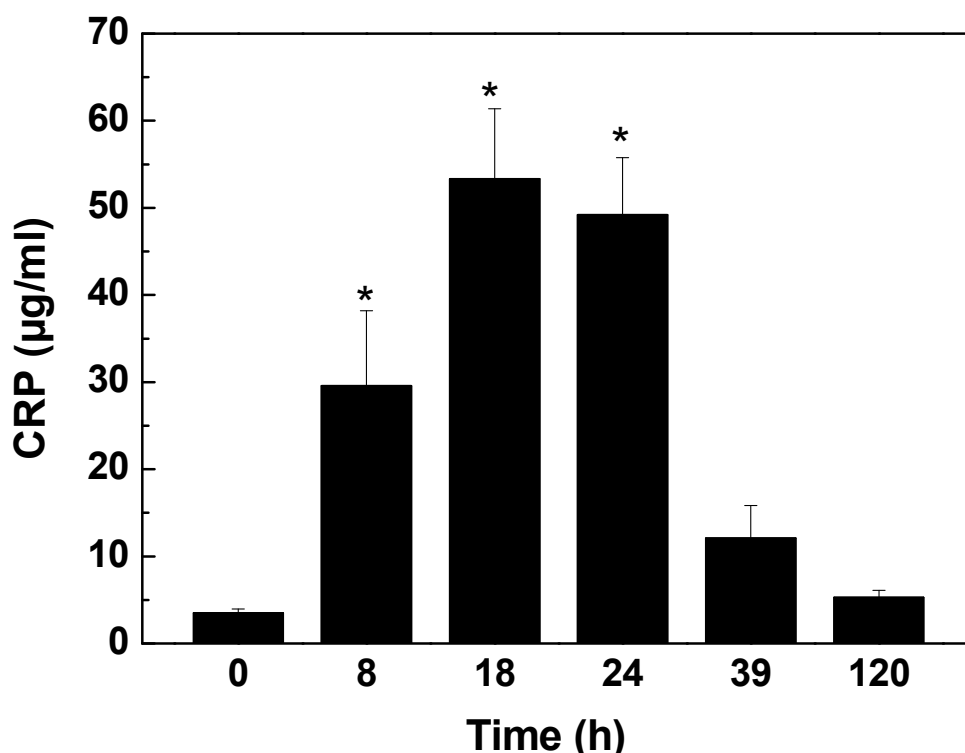


Figure 2: Induction of huCRP expression in huCRP^{tg} mice. HuCRP^{tg} mice (n=17) received an intraperitoneal injection of $2.5 \cdot 10^5$ U IL-1 β /mouse. Plasma huCRP were determined in tail blood samples collected at the indicated time points. Data represent means \pm SEM. * $P < 0.05$ compared to t=0.

LD atorvastatin treatment did neither significantly affect basal huCRP levels nor low dose or high dose IL-1 β -induced huCRP expression (Figure 3A). However, HD atorvastatin treatment significantly reduced basal huCRP expression levels by 93% ($P < 0.05$), and suppressed low dose and high dose IL-1 β -induced huCRP expression by 66% ($P < 0.05$) and 86% ($P < 0.05$), respectively (Figure 3A). These data indicate that atorvastatin can also suppress IL-1 β -induced huCRP levels and reiterate that these suppressive effects are only observed at atorvastatin concentrations that are beyond the doses required for cholesterol-lowering.

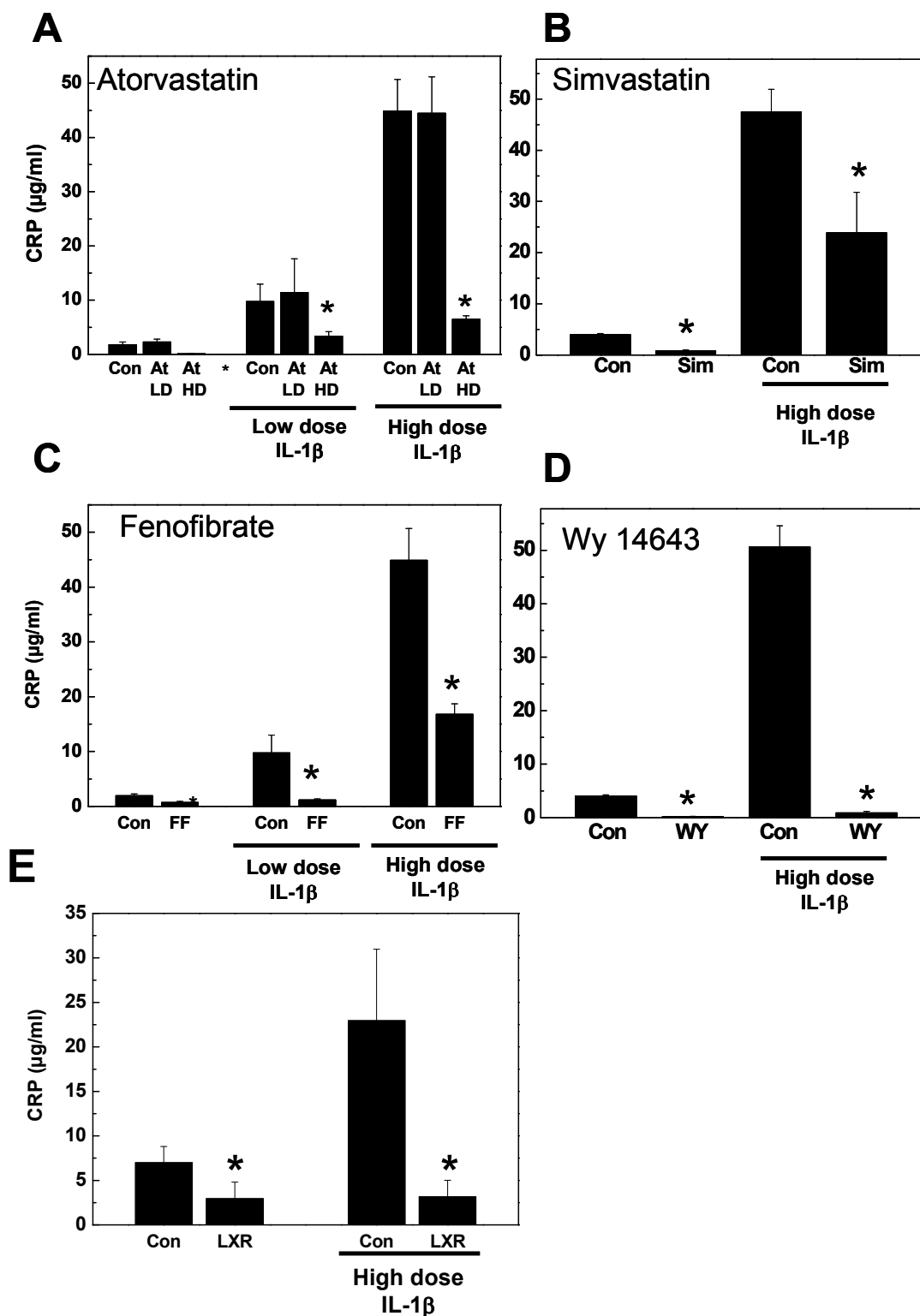


Figure 3: Effect of atorvastatin, simvastatin, fenofibrate, Wy14643 and T0901317 (LXR) on IL-1 β -induced huCRP expression. **(A)** Groups of huCRPtg mice ($n \geq 5$) received a chow diet containing atorvastatin (At) at a low dose (LD; 0.01%(w/w)) or at a high dose (HD; 0.1%(w/w)), or vehicle (Con) for three weeks. **(B)** As in (A) but mice were fed 0.1%(w/w) simvastatin (Sim). **(C)** As in (A) but mice were fed 0.1%(w/w) fenofibrate (FF). **(D)** As in (A) but mice were fed 0.03%(w/w) Wy 14643 (WY). **(E)** As in (A) but mice were fed 0.01%(w/w) T0901317 (LXR). Data represent means \pm SEM. * $P < 0.05$ compared to control.

When compared to HD atorvastatin, treatment with the same dose of simvastatin suppressed high dose IL-1 β -induced huCRP expression by only 50% ($P < 0.05$), thus underscoring the more potent anti-inflammatory potential of atorvastatin (Figure 3B). HuCRP tg mice were also treated with 0.1%(w/w) fenofibrate, 0.03%(w/w) Wy14643 or vehicle for 3 weeks, and subsequently stimulated with the above two doses of IL-1 β to induce huCRP expression. Fenofibrate-treatment reduced basal huCRP expression by 63%($P < 0.05$) and strongly suppressed induction of huCRP expression with the two doses of IL-1 β by 88%($P < 0.05$) and 63%($P < 0.05$), respectively (Figure 3C). For comparison, mice treated for 3 weeks with 0.03%(w/w) Wy14643 suppressed the high dose IL-1 β -induced huCRP expression by 98%($P < 0.05$) demonstrating that of the compounds tested, Wy14643 is the most potent anti-inflammatory drug (Figure 3D). In addition, mice treated for 4 weeks with 0.01%(w/w) T0901317 suppressed the high dose IL-1 β -induced huCRP expression by 86% ($P < 0.05$; Figure 3E). Together these data indicate that PPAR α -activators, like statins, also can reduce IL-1 β -induced huCRP expression *in vivo*.

Next, the effect of atorvastatin and fenofibrate on huCRP gene expression was analyzed by RT-PCR in livers of mice sacrificed 10 hours after IL-1 β injection. HD atorvastatin and 0.1%(w/w) fenofibrate suppressed IL-1 β -induced huCRP gene expression by 67% ($P < 0.05$) and 91% ($P < 0.05$), respectively, indicating that the suppressive effect is at the level of transcription (data not shown). The effectiveness of the atorvastatin treatment was confirmed by a 13-fold ($P < 0.05$) upregulated hepatic expression of HMG-CoA reductase in HD atorvastatin treated animals (data not shown). In these animals, the mRNA expression level of the specific PPAR α target gene acyl-CoA oxidase (ACO) was unchanged indicating that atorvastatin did not exert its action via activation of PPAR α (cf. fenofibrate-treated animals which displayed a 27-fold ($P < 0.05$) upregulation of ACO gene expression (data not shown)).

A molecular mechanism for the down-regulation of IL-1 α -induced huCRP expression by statins in human hepatocytes

To elucidate the molecular mechanism by which statins reduce huCRP expression in human liver cells, freshly prepared human liver slices were incubated with simvastatin and, for comparison, the specific PPAR α -activator Wy 14643. After 24 h of treatment, huCRP levels were determined in the culture medium by ELISA. Simvastatin reduced basal huCRP expression by 38% ($P < 0.05$), and a 48% ($P < 0.05$) reduction was observed with

Wy 14643 (Figure 4A). Incubation of control liver slices with IL-1 α significantly increased huCRP expression 1.5-fold ($P < 0.05$). Simvastatin and Wy 14643 suppressed this induction by 40% ($P < 0.05$) and 30% ($P < 0.05$), respectively (Figure 4A). Luciferase reporter gene experiments using a 300bp fragment of the huCRP promoter, which harbors known IL-1-sensitive responsive elements for C/EBP β and p50-NF κ B²⁰, demonstrated that statins suppressed the effect of IL-1 α at the transcriptional level. IL-1 α induced huCRP promoter activity 5.9-fold in control cells; treatment with simvastatin dose-dependently suppressed this induction by maximally 60% ($P < 0.05$) which is less pronounced than the suppression obtained with atorvastatin at the same dose (76%; $P < 0.05$) or Wy 14643 (65%; $P < 0.05$) (Figure 4B). Because IL-1 α -induced huCRP gene expression is regulated by the expression and nuclear translocation of the transcription factors p50-NF κ B and C/EBP β ²⁰, the effect of simvastatin on nuclear accumulation of p50-NF κ B and C/EBP β under basal and IL-1 α -stimulated conditions was analyzed next. Western blot experiments using total extracts and nuclear extracts of vehicle-treated and simvastatin-treated human HuH7 hepatoma cells stimulated or not with IL-1 α showed that simvastatin did not inhibit the IL-1 α -induced intracellular generation of p50-NF κ B and C/EBP β but reduced the translocation of p50-NF κ B into the nucleus (Figure 5A).

This can be explained by an increased expression of the intracellular inhibitor of NF κ B, I κ B- α , by simvastatin (Figure 5B). The induction of I κ B- α by simvastatin was comparable to the effect of Wy14643, an established inducer of I κ B- α ²⁰, and suggests a more general suppression of NF κ B activity. Indeed, simvastatin, atorvastatin and Wy 14643 also dose-dependently inhibited IL-1 α -induced NF κ B-driven reporter gene activity by maximally 42% ($P < 0.05$), 75% ($P < 0.05$) and 57% ($P < 0.05$), respectively (Figure 5C).

We next questioned whether simvastatin, as a consequence of its inhibitory effect on p50-NF κ B translocation, would reduce the amount of nuclear p50-NF κ B~C/EBP β complexes that drive huCRP gene transcription. Co-immunoprecipitation experiments demonstrated that simvastatin reduced the amount of p50-NF κ B~C/EBP β complexes induced by IL-1 α in nuclei of HuH7 cells and human primary hepatocytes (Figure 6).

Together these data indicate that simvastatin suppresses IL-1 α -induced huCRP gene expression by upregulating I κ B- α and reducing p50-NF κ B translocation, resulting in a diminished amount of nuclear p50-NF κ B~C/EBP β complexes and thereby reduced huCRP gene transcription.

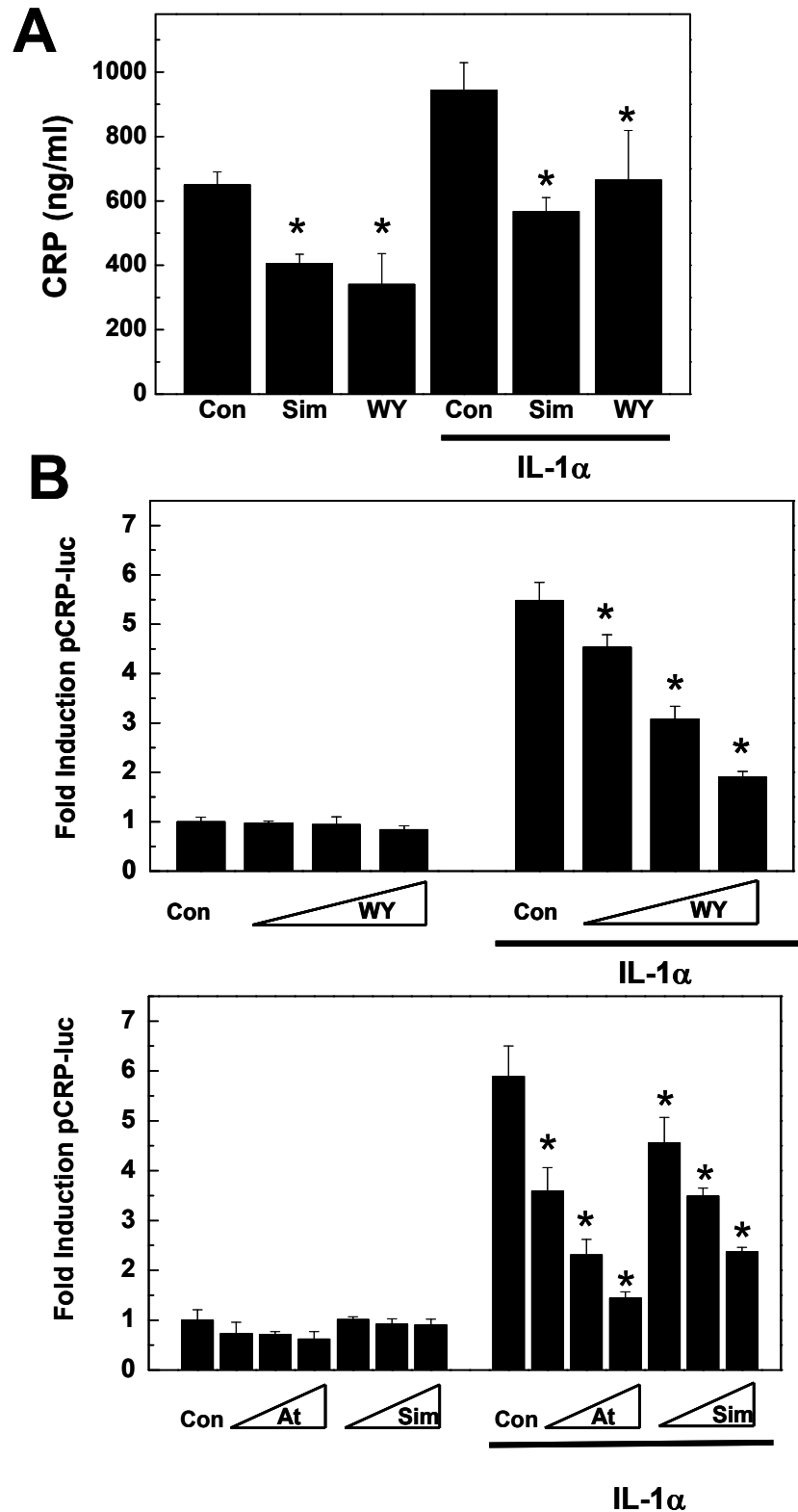


Figure 4: Statin treatment suppresses IL-1-induced huCRP gene expression in human liver cells. (A) Human liver slices were incubated with 5 μ M simvastatin (simva), 250 μ M Wy14643 (WY) or vehicle (DMSO; Con) for 24 hours and then stimulated with 25 ng/ml IL-1 α for an additional 24 hours. Data represent means \pm sd of three experiments. (B) HuH7 cells were treated with Wy 14643 (10, 30, 100 μ M), atorvastatin (1, 3, 10 μ M) or simvastatin (1, 3, 10 μ M), transiently transfected with pCRP-luc, and subsequently were stimulated with 10 ng/ml IL-1 α for 18 hours. Shown data represent means \pm sd. *P<0.05 compared to control.

Discussion

It is now well-established that atherosclerosis is not solely a lipid disorder but also an inflammatory disease.^{2, 3} Statins and fibrates are hypolipidemic drugs that reduce atherosclerosis development and concomitantly lower plasma levels of huCRP, a sensitive inflammation marker and independent predictor of future cardiovascular events.^{1, 17, 18, 24} An important question is whether the huCRP levels are lowered as a result of a direct anti-inflammatory action of these drugs, or whether they are lowered as a consequence of reduced atherogenesis. We here provide evidence that statins, i.e. atorvastatin and simvastatin, PPAR α -activators, i.e. fenofibrate and Wy14643, and a synthetic LXR agonist, T0901317 reduce basal and IL-1 β -induced huCRP gene expression in non-atherosclerotic huCRP^{tg} mice.

These anti-inflammatory effects are demonstrable at statin concentrations that are higher than required for cholesterol-lowering *per se* or occur without altering plasma cholesterol levels in the case of fenofibrate and Wy14643. The LXR-agonist even displays its anti-inflammatory effects in hyperlipidemic environment. The data indicate that statins, PPAR α -activators and an LXR-agonist exert direct anti-inflammatory effects unrelated to atherosclerosis and lowering plasma cholesterol levels. This notion is strengthened by experiments with cultured human hepatocytes in which simvastatin and Wy14,643 also reduce huCRP gene expression, and by providing a mechanistic explanation for this action. The anti-inflammatory action of statins and PPAR α -activators involves upregulation of an inhibitor of NF- κ B, I κ B α , which traps p50-NF κ B in the cytosol. As a consequence, the nuclear translocation of p50-NF κ B is reduced and the amount of nuclear p50-NF κ B~C/EBP β complexes, which are crucial for huCRP gene transcription,^{20, 25} is decreased. The results provide the first conclusive evidence for a direct down-regulating effect of statins and PPAR α -activators on huCRP expression *in vivo*.

Although it is widely accepted that the clinical benefit obtained with statins is a direct result of their lipid-lowering properties, there is still debate as to whether the additional, so-called pleiotropic effects²⁶ of statins contribute to the clinical outcome in vascular disease, or whether all beneficial effects of statins are due to lowering plasma lipids. Recent studies in mice and cynomolgus monkeys demonstrated that statins reduce the development of atherosclerosis beyond the effect achieved by their plasma cholesterol-lowering effect alone.²⁷⁻²⁹ It was suggested that these cholesterol-independent effects of statins were the result of their anti-inflammatory action as reflected by the reduced expression of TNF α ,

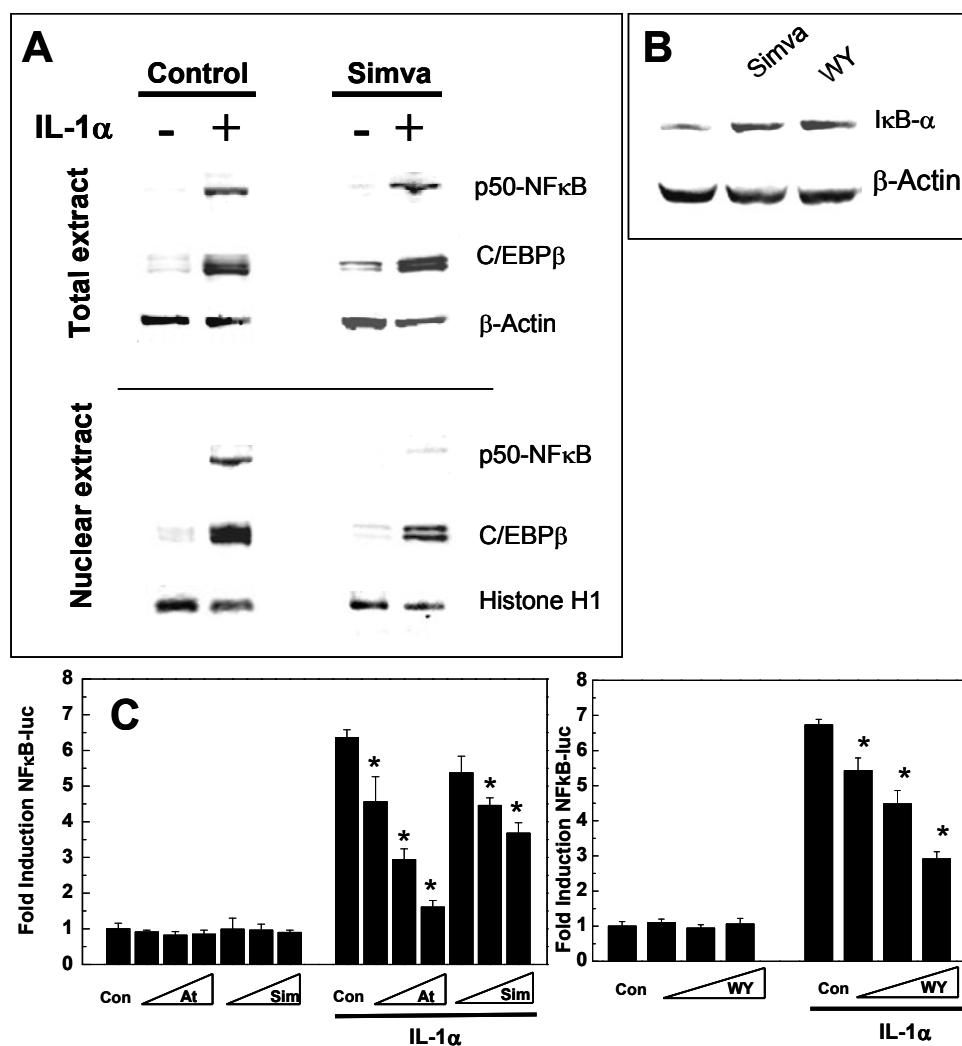


Figure 5: Statin treatment inhibits NFκB activity. (A) HuH7 cells were preincubated with 3 μM simvastatin (Simva) for 24 h and subsequently were stimulated with 10 ng/ml IL-1α for 18 hours. Western blot analysis of total cellular and nuclear extracts for p50-NFκB and C/EBPβ expression. (B) HuH7 cells were incubated with simvastatin or Wy 14643 (WY) for 24 hours and analyzed for IκBα expression in total cell extracts. Control β-actin and histone H1 expression levels confirm equal loading. (C) HuH7 cells were incubated with Wy 14643 (10, 30, 100 μM), atorvastatin (1, 3, 10 μM), simvastatin (1, 3, 10 μM), transiently transfected with p3xNFκB-luc, and subsequently were stimulated with 10 ng/ml IL-1α for 18 h. Data represent means±sd. *P<0.05 compared to control.

MCP-1 and VCAM-1 in atherosclerotic lesions and the down-regulation of liver-derived plasma inflammation markers; however, decisive evidence for this conclusion was lacking. In the present study we unequivocally demonstrate that statins possess anti-inflammatory potential by themselves and directly, i.e. unrelated to cholesterol lowering and in absence of atherosclerosis, suppress basal and IL-1β-induced huCRP gene expression. This additional effect may also contribute to the reduced risk of cardiovascular events in normocholesterolemic patients treated with statins, an observation that cannot fully be explained by the cholesterol-lowering properties of the drugs.^{27, 30, 31} In that respect it may

also be significant that other anti-inflammatory, plasma lipid-modulating drugs such as activators of PPAR α and liver X receptors (LXRs) show anti-atherogenic activity unrelated to their plasma cholesterol-altering activities.^{16,32} For example, fenofibrate was recently found to reduce atherosclerosis in ApoE-deficient mice.¹⁷ Similarly, the LXR agonist GW3965 was shown to inhibit atherogenesis in ApoE-deficient mice and LDLR-deficient mice despite of unfavorable effects on plasma lipids.³³

Recent observations indicate that huCRP may also be directly involved in the process of atherosclerosis.⁶⁻⁹ A study using huCRP transgenic or nontransgenic apoE-deficient mice demonstrates that elevated huCRP plasma levels lead to accelerated atherosclerosis documenting a proatherogenic role for huCRP *in vivo*.¹⁰ Our data show that treatment with statins and PPAR α -activators reduces huCRP plasma levels, suggesting that these drugs may retard the process of atherosclerosis beyond the effect achieved by their lipid-lowering properties alone. The anti-inflammatory action of statins and fibrates on huCRP expression in hepatocytes is based on upregulation of the cytosolic inhibitor of NF κ B, I κ B- α , resulting in a reduced NF κ B-activity. This effect is not limited to hepatocytes since several statins have recently been shown to upregulate the expression of I κ B- α in cultured human aortic endothelial cells.³⁴ Furthermore, statins have also been reported to inhibit NF κ B-activation in human mesothelial cells and human aortic smooth muscle cells (haSMC).^{23, 34} Similarly, fibrates have been found to induce I κ B- α expression in huSMC and hepatocytes.^{20, 35} This more general effect of statins and fibrates is in accordance with the finding that atorvastatin reduces NF κ B-activation in atherosclerotic lesions of rabbits³⁶ and may explain the decreased expression of the NF κ B-regulated genes MCP-1 and VCAM-1 in atherosclerotic lesions.^{28, 29} Since NF κ B coordinates the expression of a wide variety of genes that control immune responses and is involved in many inflammatory diseases,^{4, 37, 38} statins and fibrates may also have beneficial effects in diseases with an inflammatory component other than atherosclerosis.³⁹ Together, our results provide convincing evidence that statins, PPAR α -activators and a synthetic LXR agonist reduce huCRP gene expression by direct anti-inflammatory activities unrelated to cholesterol-lowering and atherosclerosis. It remains to be investigated whether these activities also suppress other mediators of inflammatory processes, which may lead to extended therapeutic indications based on the anti-inflammatory properties of statins and fibrates.

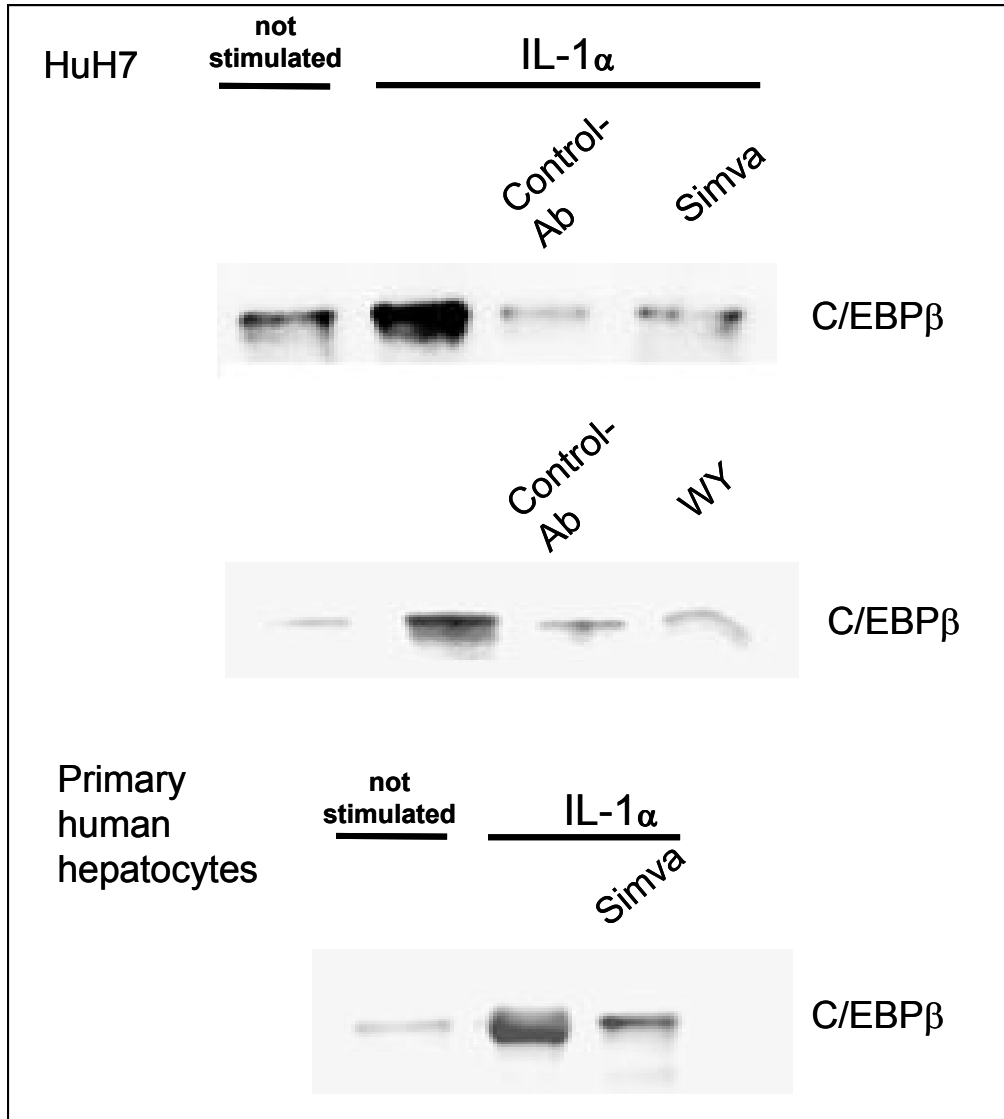
p50-NF κ B pull-down / anti-C/EBP β Western

Figure 6: Statin treatment reduces the nuclear amount of p50-NF κ B~C/EBP β complexes. Co-immunoprecipitation was performed on nuclear extracts prepared from HuH7 cells and freshly isolated primary human hepatocytes preincubated for 17 hours with 3 μ M (HuH7 cells) and 10 μ M (primary hepatocytes) simvastatin (Simva), 50 μ M Wy 14643 (WY) or vehicle and stimulated with 10 ng/ml IL-1 α (HuH7 cells) or 25 ng/ml IL-1 α (primary hepatocytes) for 18 hours. Anti-p50-NF κ B or anti-MMP-8 control antibody (Control-Ab) were used for immunoprecipitation and C/EBP β bound to precipitated p50-NF κ B was detected by Western blot.

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Chapter 3

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