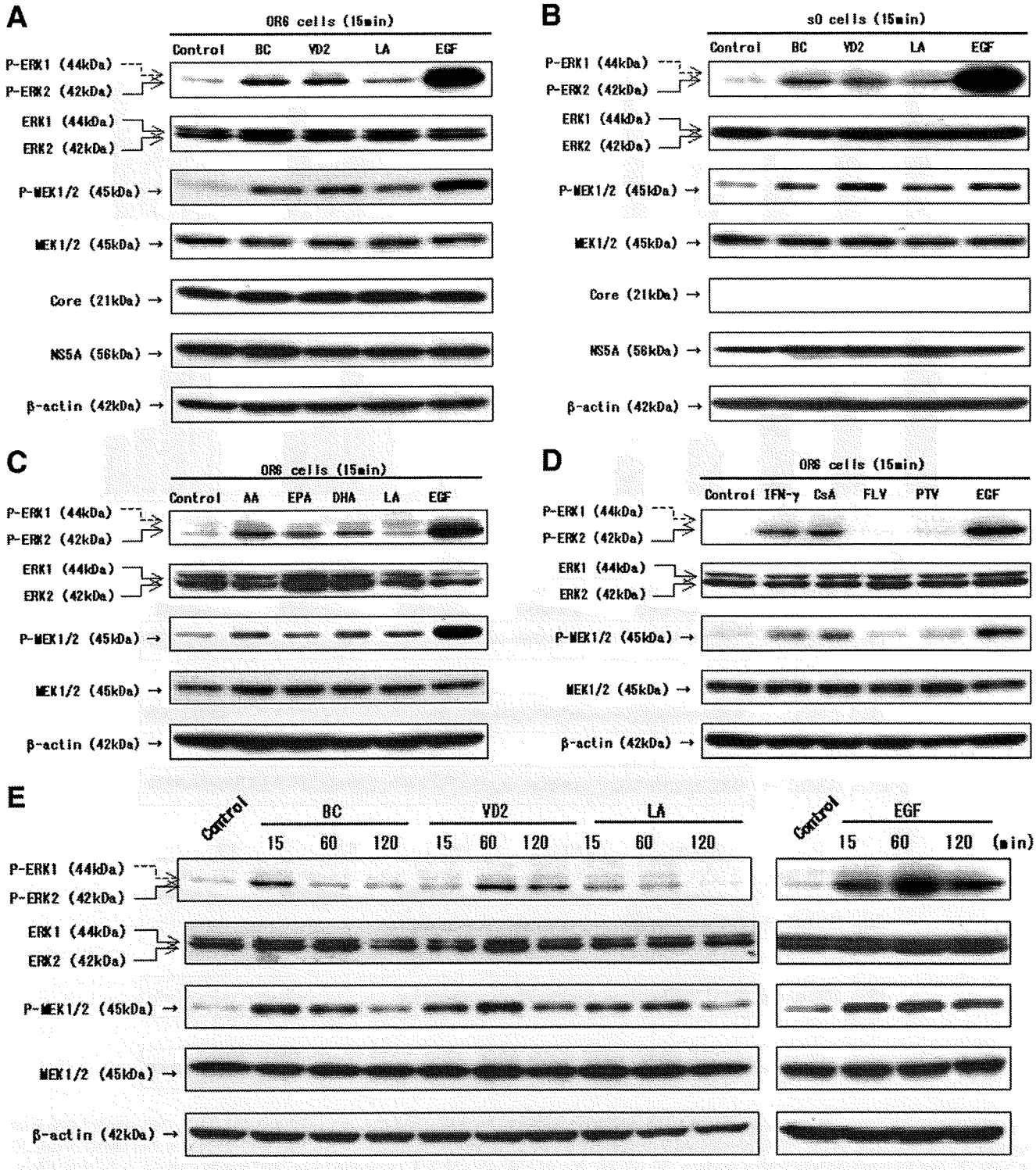

Fig. 4. UO126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients—BC, VD2, and LA—increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or sO cells (B) were maintained in FBS-free medium for 48 hours and then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF for 15 minutes. After treatment, cell lysates underwent western blot analysis using antibodies specific to phosphorylated ERK1/2, ERK1/2, phosphorylated MEK1/2, and MEK1/2. The appropriate expression of HCV core and NS5A was determined by way of immunoblotting with their respective antibodies. (C, D) IFN-γ, CsA, and the PUFAs, but not the statins, increased the phosphorylation of MEK-ERK1/2 in OR6 cells. OR6 cells were precultured as described in panels A and B, then treated with control medium, 100 μM AA, EPA, DHA, or LA, or 50 ng/mL EGF (C) and control medium, 2 IU/mL IFN-γ, 2 μg/mL CsA, 5 μM of FLV or PTV, or 50 ng/mL EGF (D), respectively, for 15 minutes. (E) Time-course western blot analysis of the increase of MEK-ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. Samples for analysis were harvested prior to treatment with the control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (0 time point) and at 15, 60, and 120 minutes posttreatment. After all of the treatments (C-E), cell lysates were subjected to western blot analysis of the activation of the MEK-ERK1/2 signaling pathway as described in panels A and B. β-actin was used as a control for the amount of protein loaded per lane in all analyses.

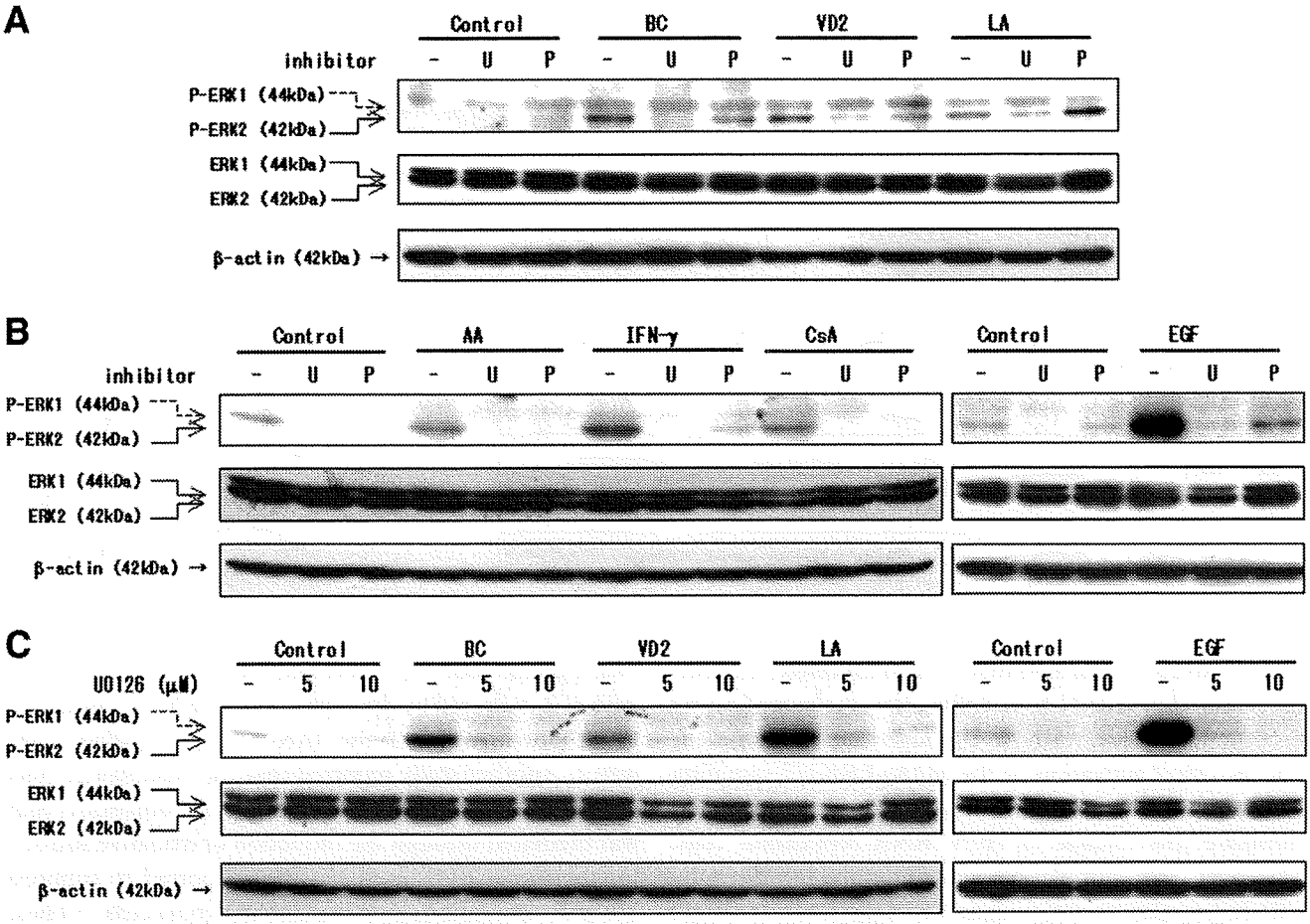

Fig. 5. U0126 strongly abolished ERK1/2 phosphorylation by the anti-HCV nutrients, anti-HCV reagents, and EGF. (A,B) Effects of the MEK1/2-specific inhibitors on ERK1/2 phosphorylation by anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with DMSO (−), 10 μM U0126: (U), or 20 μ M PD98059: (P) for 1 hour. Subsequently, the cells were treated with control medium, 20 μM BC, 10 μM VD2, or 100 μM LA (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, 2 μg/mL CsA, or 50 ng/mL EGF (B), respectively, in either the absence (DMSO) (−) or presence of U0126 (U) or PD98059 (P) for 15 minutes. (C) Dose effects of U0126 on ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. OR6 cells were precultured as described in Figs. 4A and 4B, then pretreated with DMSO (−) or 5 or 10 μM U0126 for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF in either the absence (−) or presence of U0126 for 15 minutes. After all treatments (A-C), cell lysates were subjected to western blot analysis using antibodies specific to phosphorylated ERK1/2 (top row) and ERK1/2 (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

7). Collectively, these results suggest that these nutrients and reagents induce ROS as an oxidant in HCV RNA replicating cells, leading to activation of the MEK–ERK1/2 signaling pathway and suppression of HCV RNA replication.

The Effects of EGF on HCV RNA Replication were Different than Those of the Anti-HCV Nutrients/Reagents. Because the study by Huang et al.[24] showed that EGF time-dependently suppressed the expressions of HCV nonstructural proteins in subgenomic replicon-harboring cells, we wondered whether EGF could suppress genome-length HCV RNA replication. EGF inhibited HCV RNA replication by approximately 25% at a concentration of 100 ng/mL. This anti-HCV activity was weaker than that of the anti-HCV nutrients and reagents tested in this study. However, as shown in the cell growth assay, EGF promoted OR6 cell proliferation in a dose-dependent manner (Supporting Fig. 6). These cell growth effects of EGF may have caused us to underestimate the actual anti-HCV activity of EGF. The other reagents and nutrients did not affect cell proliferation compared with EGF (Supporting Fig. 7).

Discussion

The previous studies using the MEK1/2-specific inhibitor and subgenomic replicon system showed that induction of the MEK–ERK1/2 signaling pathway might be required for the suppression of HCV RNA replication by some reagents.[24,25] In agreement with the study by Huang

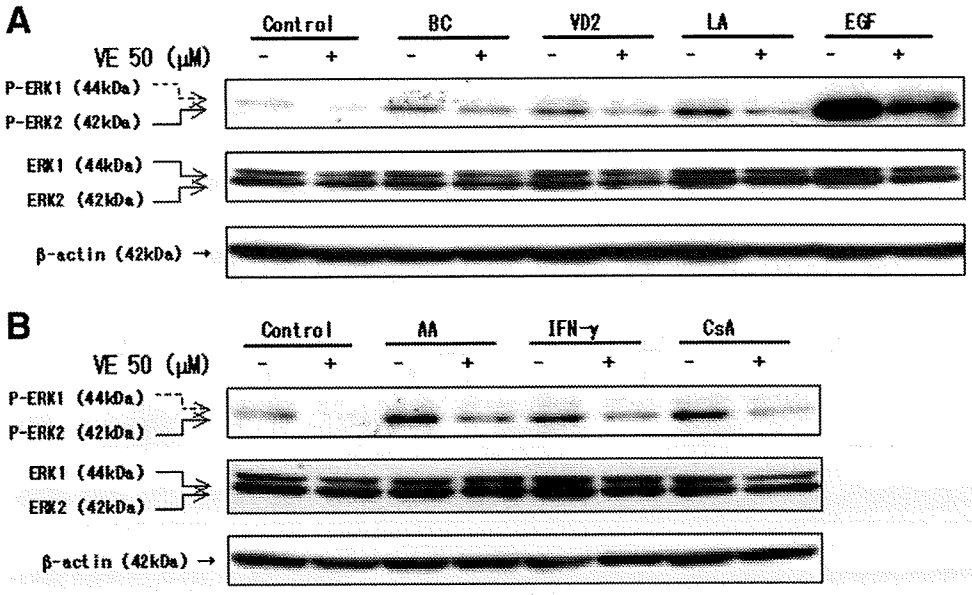

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, and 2 μg/mL CsA (B), respectively, in either the absence (ethanol) (−) or presence (+) of 50 μM VE for 15 minutes. After the treatment, cell lysates underwent western blot analysis as described in Fig. 5.

et al.,[24] we also confirmed that U0126 inhibited the anti-HCV activity of IFN-γ in OR6 cells stably replicating genome-length HCV RNA. Although they did not identify the direct activation of the MEK–ERK1/2 signaling pathway by IFN-γ, we demonstrated that IFN-γ could stimulate this cascade in HCV RNA replication cells. Moreover, this stimulation was not only inhibited by U0126 but also by antioxidant VE. This result indicates the involvement of oxidative stress in the anti-HCV activity of IFN-γ as well as the MEK–ERK1/2 signaling pathway. IFNs induce the transcription of IFN-stimulated genes through the JAK–STAT pathway, but the induction of IFN-stimulated genes by IFN-γ has been far more complex than that by IFN type I.[30] A study using a macrophage cell line revealed that IFN-γ activated ERK1/2, followed by the expression of IFN-γ–stimulated genes downstream of the JAK–STAT signaling pathway.[31] Another study reported that the defensive activity of IFN-γ against hepatitis B virus in hepatoblastoma cells was mediated through the induction of oxidative stress.[32] Furthermore, ROS itself has been reported to suppress HCV RNA replication in human hepatoma cells.[33] These reports support our proposal regarding anti-HCV activity of oxidative stress that the generation of intracellular ROS inhibits HCV RNA replication through activation of the MEK–ERK1/2 signaling pathway. Waris and Siddiqui[34] reported that calcium-dependent ROS generation induced cyclooxygenase-2 and prostaglandin E(2) via the activation of nuclear factor kappa B, leading to the suppression of HCV RNA replication. Choi et al.[35] also demonstrated that elevated calcium suppressed HCV RNA replication. The activation of nuclear factor kappa B by ROS was mediated through the MEK–ERK1/2 signaling pathway. Therefore, we suggest that the oxidative reagents and nutrients in this study also may induce anti-HCV status by calcium-dependent ROS generation.

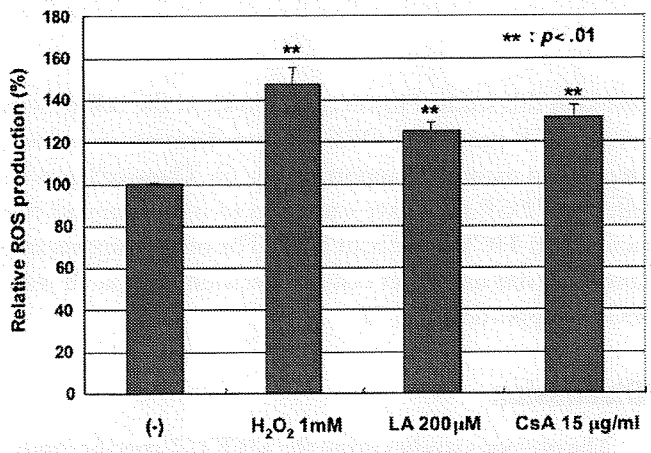

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. $^{**}P <$ 0.01 versus untreated cells.

In the course of our study of the anti-HCV activities of these three nutrients, we found that treatment with U0126 more strongly inhibited their anti-HCV activities than treatment with PD98059. U0126 has been shown to possess approximately 100-fold-higher MEK1/2-specific inhibitory activity than PD98059.[36] This different potential between the two inhibitors was considered to cause a gap in their effects on anti-HCV activities. We further found that, much like EGF, all three nutrients enhanced the phosphorylation of ERK1/2 and MEK1/2, which was reduced by treatment with U0126 or VE. In addition, the

present study was the first to observe that BC, which has been shown to produce ROS,[37] activates the MEK–ERK1/2 signaling pathway, an action that VD2[38] and LA[39] have already been shown to exhibit in leukemia cell and dendritic cell lines, respectively. Furthermore, we found the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of the three nutrients as well as various PUFAs, which were reported to be mediated through lipid peroxidation.[29] These results suggest that the anti-HCV nutrients BC, VD2, and PUFAs, including LA, as well as IFN-γ may suppress HCV RNA replication via activation of the MEK–ERK1/2 signaling pathway in response to ROS production.

We also investigated the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanism of anti-HCV reagents other than IFN-γ. In our previous study, the anti-HCV activity of CsA, but not FLV, was prevented by VE.[13] Consequently, these results implied that CsA, but not statins, could be potent activators of the MEK–ERK1/2 signaling pathway as oxidants, leading to down-regulation of HCV RNA replication. CsA has been demonstrated to bind to cyclophilins and suppress HCV RNA replication by abolishing their interaction with NS5B polymerase.[40] This CsA binding to cyclophilins, especially cyclophilin A (CyPA), has been shown to result in the generation of ROS through inhibition of the peptidylprolyl-cis-trans-isomerase–like activity of CyPA.[41] Moreover, CyPA was reported to be secreted in response to oxidative stress,[42] and to bind to a cell surface receptor, CD147, followed by ERK1/2 activation.[43] These reports and our results suggest that CsA, acting as an oxidant, may trigger activation of the MEK–ERK1/2 signaling pathway, both directly by producing ROS by way of interaction with CyPA in the early phase, and indirectly by secreting CyPA in the late phase. Both activations could lead to an inhibition of HCV RNA replication. Thus, CyPA may play a critical role as an intermediator in the oxidative anti-HCV activity of CsA. In the latest study, CyPA was identified as the most essential cellular cofactor of HCV RNA replication among cyclophilins.[44] Further studies will be needed to clarify whether CyPA is required for the oxidative suppressive mechanism of anti-HCV nutrients/reagents other than CsA.

Although we expected that strong activation of the MEK–ERK1/2 signaling pathway would suppress HCV RNA replication, EGF exhibited only slight anti-HCV activity in OR6 cells. The promotion of cell growth by EGF might prevent its primary inhibitory effect on HCV RNA replication. A portion of the ERK1/2 phosphorylation by EGF was also reduced by treatment with VE (Fig. 6A), suggesting that EGF might stimulate the MEK–ERK1/2 signaling pathway, in part, as an oxidant, and that this oxidative activity of EGF could exhibit its slight anti-HCV activity.

In this study, using MEK1/2 specific inhibitors, we revealed that the MEK–ERK1/2 signaling pathway is involved in the oxidative antiviral mechanism of the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Our results suggest that this oxidative induction of the MEK–ERK1/2 signaling pathway could be a novel therapeutic strategy for the eradication of HCV infection. Although oxidants themselves cause liver damage, they may work as anti-HCV factors during therapy in patients with chronic hepatitis C.

In conclusion, this study suggests that the anti-HCV activity of oxidative stress is closely linked to the activation of the MEK–ERK1/2 signaling pathway.

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Interleukin-27 Displays Interferon-γ–Like Functions in Human Hepatoma Cells and Hepatocytes

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Interleukin-27 (IL-27) is a cytokine belonging to the IL-6/IL-12 cytokine family. It is secreted by antigen-presenting cells, strongly acts on T cells, and also stimulates innate immune cells. In most studies, the effects of IL-27 on T cells were investigated; however, not much is known about possible effects of IL-27 on other cell types. IL-27 signals via the common IL-6–type cytokine receptor chain gp130 and the IL-27–specific chain WSX-1. Given the importance of gp130 in regulating liver responses such as the acute phase response or liver regeneration, we investigated whether IL-27 could also have a function in liver cells. We find that IL-27 stimulates hepatoma cells and hepatocytes by inducing a sustained signal transducer and activator of transcription (STAT)1 and STAT3 activation. Whereas the STAT3 mediated responses to IL-27 (γ-fibrinogen and hepcidin induction) are not detectable, we observe an interferon-gamma (IFN-γ)–like STAT1 response leading to the induction of interferon-regulated proteins such as STAT1, STAT2, interferon response factor (IRF)-1, IRF-9, myxovirus resistance A and guanylate binding protein 2. *Conclusion:* Our study provides evidence for a function of IL-27 in hepatoma cells and hepatocytes and shows that IL-27 responses are not restricted to the classical immune cells. Our results suggest that IL-27 exerts IFN-like functions in liver cells and that it can contribute to the antiviral response in these cells. (HEPATOLOGY 2009;50:585-591.)

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Interleukin-27 (IL-27) is a type I cytokine predominantly secreted by activated macrophages and dendritic cells. It can be allocated to the IL-6/IL-12 superfamily of cytokines. As a heterodimeric cytokine composed of the two subunits p28 and Epstein-Barr virus–induced gene 3,[1] IL-27 is a member of the IL-12 cytokine family, also encompassing IL-12 and IL-23. Like these cytokines, IL-27 has profound effects on T-cells and acts on innate immune cells.[2,3] Although IL-27 can have proinflammatory effects, most data point at the dominant role of IL-27 being immunosuppressive. Most studies have investigated the effects of IL-27 on CD4+ T-cells, and not much is known about possible effects of IL-27 on other cell types. IL-27 was shown to promote T helper 1 (TH1) responses through the induction of the transcription factors T-bet, up-regulation of IL-12Rβ2, and interferon-gamma (IFN-γ) production and suppression of the TH2 transcription factor GATA3.[1,4] However, IL-27 is also capable of suppressing both TH1 and TH2 responses during infection with a variety of pathogens.[5,6]

IL-27 signaling occurs via a receptor complex composed of the signal transducing receptor chains WSX-1 and glycoprotein (gp)130. Whereas WSX-1 is the IL-27–specific receptor chain,[7] gp130 is the common receptor subunit of IL-6–type cytokines.[8] Thus, IL-27 also belongs to this family. IL-6–type cytokines activate target genes involved in differentiation, survival, apoptosis, and proliferation. They can exert proinflammatory as well as anti-inflammatory properties and are major players in the acute phase response and the immune response of the organism. IL-6 is a major medi-

Abbreviations: FPV, fowl plague virus; GBP2, guanylate binding protein 2; IFN, interferon; IL, interleukin; IRF, interferon response factor; MxA, myxovirus resistance A; RIG-I, retinoic acid-inducible gene-I; STAT, signal transducer and activator of transcription; TH, T helper; OSM, oncostatin M.

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ator for the acute phase response of the liver as well as in liver regeneration.[9,10]

IL-6–type cytokines stimulate tyrosine phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, which can form STAT3 and STAT1 homodimers as well as STAT3/STAT1 heterodimers. However, the importance of the detected STAT1 phosphorylation by IL-6–type cytokines remains elusive. For example, IL-6 and oncostatinM (OSM) only seem to induce an IFN-γ–like response in STAT3 knockout cells.[11,12] There are multiple reasons for this inefficient STAT1 response.[13] Not only is STAT1 tyrosine phosphorylation after IL-6–type cytokine stimulation very transient,[11,14] but additionally, most of the phosphorylated STAT1 seems to be trapped in STAT1/STAT3 heterodimers.[13]

We describe a function of IL-27 in hepatoma cells and hepatocytes. We show that IL-27 elicits an efficient STAT1 response and leads to the expression of IFN-γ–regulated genes in these cells.

Materials and Methods

Cell Culture. The human hepatoma cell line HepG2 (DSMZ) was maintained in Dulbecco's modified Eagle medium/Nut. MixF-12 medium with Glutamax supplemented with 10% fetal bovine serum, 100 mg/L streptomycin, and 60 mg/L penicillin. The human hepatocyte cell line PH5CH8 was described previously.[15]

Isolation and Cultivation of Rat Hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats as described before.[16] Details are provided as Supporting Information.

Cell Lysis, Preparation of Nuclear Extracts for Electrophoretic Mobility Shift Assay, Western Blot Analysis, and Antibodies. All of these procedures were performed as previously described.[13] The antibodies used are listed as Supporting Information.

Viral Infections and Plaque Assay. Fowl plague virus (FPV) was propagated and used as described previously.[17] For infection, 7×10^5 HepG2 cells were left untreated or were pretreated with 50 ng/mL IL-27 for 18 hours. Cells were then washed with phosphate-buffered saline followed by incubation with FPV (0.001 multiplicity of infection) diluted in phosphate-buffered saline/BA (phosphate-buffered saline containing 0.2% bovine serum albumin, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) for 30 minutes at 37°C. The inoculum was aspirated, and cells were incubated for 24 hours with infection medium containing 0.2% bovine serum albumin and antibiotics supplemented either with or without 50 ng/mL IL-27. As a positive control for antiviral activity, infections were performed in the presence of 1000 U/mL interferon-alpha (IFN-α) for 24 hours. Plaque assays were performed as described previously.[18] Results are given as plaque-forming units per milliliter, and standard deviations are represented as error bars.

Statistical Analysis. The statistical analysis was performed using a Student t test. $P < 0.05$ was regarded as being statistically significant.

Results

IL-27 Acts on Human Hepatoma Cells and Cultured Human Hepatocytes. By screening different cell lines for their response to IL-27, we observed that human hepatoma cells are sensitive to IL-27. HepG2 cells were stimulated with increasing amounts of IL-27, and tyrosine phosphorylation of STAT3 (pY705) and STAT1 (pY701) was assessed by western blot analysis. Stimulation of HepG2 cells for 15 minutes with IL-27 leads to a phosphorylation of both STAT3 and STAT1 in a dose-dependent manner (Fig. 1A). As a control, HepG2 cells were also stimulated with IL-6. STAT1 as well as STAT3 tyrosine phosphorylation occurred on treatment of these cells with IL-6 for 15 minutes. The levels of phosphorylated STAT1 and STAT3 were higher in IL-6–stimulated HepG2 cells than in those treated with IL-27. We next investigated the kinetics of STAT phosphorylation on IL-27 stimulation in HepG2 cells and in the human hepatocyte cell line PH5CH8. Both of these cell lines express the IL-27 receptor WSX-1 on their surface (Supporting Fig. 1). As a control, the cells were also stimulated with IL-6, IFN-γ, and IFN-α. IL-27 induces a sustained phosphorylation of STAT1 and STAT3 (Fig. 1B,C; lanes 2-5) in both HepG2 and PH5CH8 cells, whereas IL-6 only leads to a sustained, albeit more pronounced, STAT3 phosphorylation (lanes 10-13). STAT1 activation after IL-6 stimulation was very transient (Supporting Fig. 2). However, a prominent STAT1 phosphorylation was observed on stimulation of the cells with interferons (lanes 6-9 and lanes 14-17), and STAT2 phosphorylation was only observed on treatment with IFN-α. Of note, up-regulation of both STAT1 and STAT2 was observed when the cells were stimulated with IL-27, IFN-γ, or IFN-α, an indication for an efficient STAT1 activation.

IL-27 Leads to a Prolonged STAT1 and STAT3 Activation in Liver Cells. As we previously reported, the STAT1 phosphorylation observed on treatment of hepatoma cells and primary human macrophages with IL-6–type cytokines such as IL-6 and OSM does not necessarily lead to the formation of active STAT1 ho-

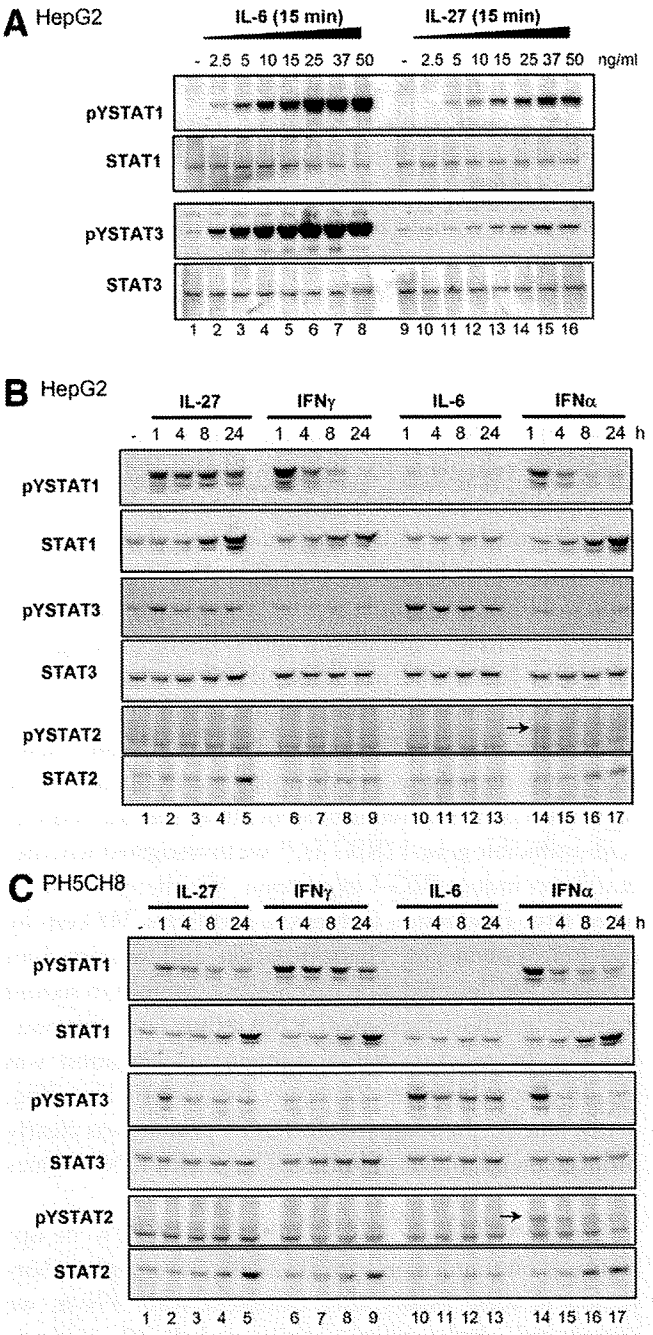

Fig. 1. IL-27 phosphorylates STAT1 and STAT3 in hepatoma cells and hepatocytes. (A) HepG2 cells were stimulated with the indicated amounts of IL-6 and IL-27. After 15 minutes, the cells were lysed, proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and tyrosine phosphorylation of STAT1, and STAT3 was detected by western blot analysis using phospho-specific antibodies for pY701-STAT1 and pY705-STAT3. Equal loading of the samples was assessed by stripping and reprobing the blot with antibodies recognizing STAT1 and STAT3. (B, C) Western blot analysis showing STAT1, STAT2, and STAT3 phosphorylation on stimulation of HepG2 hepatoma cells (B) or the cultured hepatocyte cell line PH5CH8 (C) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Western blot analysis was performed as described above.

modimers. Most of the phosphorylated STAT1 is rather trapped in STAT1/STAT3 heterodimeric complexes.[13] Thus, we performed electrophoretic mobility shift assays to examine whether phosphorylated STAT1 is forming homodimers on treatment of liver cells with IL-27 (Fig. 2). As controls, we used cells stimulated with IL-6, IFN-γ, or IFN-α. On stimulation of HepG2 or PH5CH8 cells with IL-27, the sustained formation of STAT1/STAT1 (lanes 2-5) complexes shows that IL-27 induces a persistent STAT1 activation. Although STAT3 homodimers also can be detected, STAT3 activation is weak if compared with IL-6. Of note, STAT1/STAT1 dimers are hardly observed on stimulation of the cells with IL-6 at these time points, indicating that the prominent but transient STAT1 phosphorylation observed 15 minutes after stimulation (Fig. 1A) is not translated into a STAT1 response. IL-27, however, should be capable of inducing STAT3 as well as STAT1 responses, because both factors are activated in a sustained manner and bind DNA in their homodimeric form. As expected, IFN-γ and IFN-α mainly induced the formation of STAT1 homodimers. Overall, both interferons show a more sustained STAT1 activation in PH5CH8 cells than in HepG2 cells (Figs. 1B,C, 2).

Compared with IL-6, IL-27 Does Not Induce the STAT3-Dependent Genes γ-Fibrinogen and Hepcidin. To assess whether IL-27 induces STAT3-dependent genes, we investigated the induction of the acute-phase protein genes γ-fibrinogen and hepcidin in both HepG2− and PH5CH8 cells (Supporting Fig. 3). In contrast to IL-6, neither IL-27, IFN-γ, nor IFN-α seem to induce significant levels of these genes.

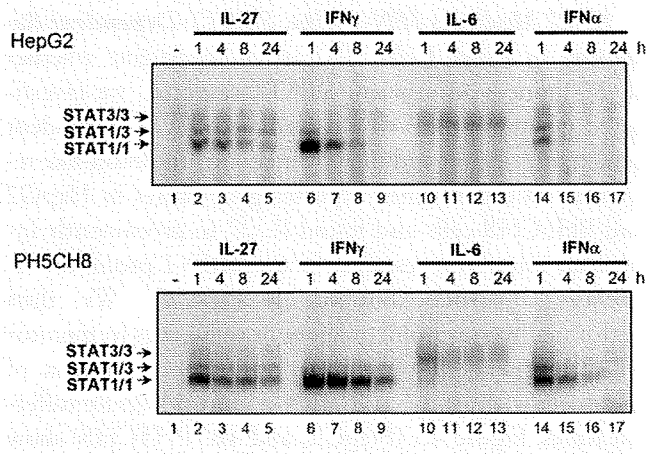

Fig. 2. IL-27 leads to a sustained STAT1 and STAT3 activation. HepG2 cells and PH5CH8 cells were stimulated with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for the times indicated, and nuclear extracts were prepared. These were analyzed by electrophoretic mobility shift assays, and STAT3/3, STAT3/1 and STAT1/1 dimer species were visualized by autoradiography.

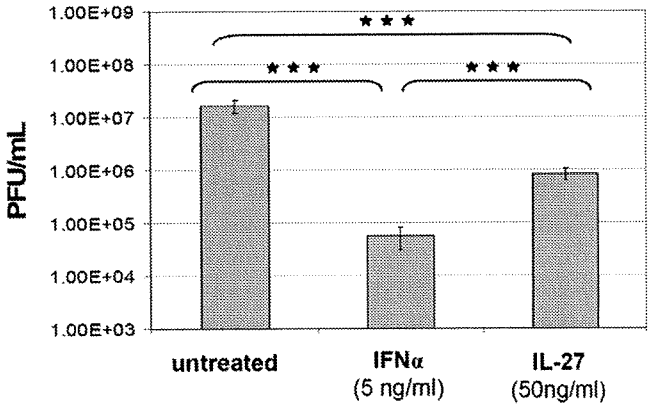

Fig. 3. IL-27 displays antiviral activity. HepG2 cells were pretreated with 50 ng/mL IL-27 for 18 hours before infection with FPV for 24 hours. The infection medium also contained 50 ng/mL IL-27. Antiviral activity was monitored by plaque assay. As a positive control, cells were infected in the presence of 5 ng/mL (1000 U/mL) IFN-α for 24 hours. Results are given in plaque-forming units per milliliter, and standard deviations are represented as error bars (n = 4; ***P < 0.001).

IL-27 Displays Antiviral Activity. Because IL-27 leads to a sustained STAT1 activation, we investigated possible antiviral activities of IL-27 by performing infection assays with HepG2 cells using the fowl plague virus FPV. HepG2 cells were pretreated with IL-27 for 18 hours before infection. Cells were then infected with FPV for 24 hours in the presence or absence of IL-27. As a positive control, infection assays were also performed in the presence of IFN-α, a cytokine that is well known to suppress virus replication. Figure 3 shows that treatment of HepG2 cells with IL-27 reduces the amount of progeny viruses after 24 hours of infection. Similar albeit less pronounced effects could be observed with a human influenza virus A7Puerto-Rico/8/34 isolate (data not shown).

IL-27 Mediates IFN-γ–like, STAT1-Dependent Responses in Liver Cells. To further investigate whether IL-27 induces an efficient STAT1 response, we investigated whether IL-27 would regulate STAT1-dependent gene transcription and thereby mediate interferon-like responses. We performed reporter gene assays in HepG2 and PH5CH8 cells and found IL-27 to prominently induce an interferon response factor (IRF)-1 promotor luciferase construct (Supporting Fig. 4). We then performed western blot analyses in these cells to monitor STAT1-dependent protein expression on treatment of these cells with IL-27, IL-6, IFN-γ, and IFN-α for different times. Figure 4A (HepG2) and 4B (PH5CH8) show that IL-27 up-regulates the STAT1-dependent genes STAT1 (lanes 4, 5), STAT2 (lanes 4, 5), and IRF-1 (lanes 2-5). Up-regulation of these genes also can be observed on treatment of the cells with IFN-γ or IFN-α, although the STAT2 up-regulation is barely detectable in HepG2 cells stimulated with IFN-γ (lane 9). In contrast, IL-6 fails to up-regulate any of the investigated STAT1-dependent genes. We further checked whether IL-27 would induce other interferon-regulated genes such as guanylate binding protein 2 (GBP2) and myxovirus resistance A (MxA), which are regulated by IFN-γ and IFN-α, respectively. Both genes are implicated in the antiviral response after interferon treatment of cells.[19] IL-27 up-regulates GBP2 in a similar manner to IFN-γ (lanes 5 and 9), whereas IFN-α does not induce GPB2. In contrast, IFN-α leads to a prominent up-regulation of MxA protein expression (lanes 15-17), whereas IL-27 only shows a weak induction (lane 5). IFN-γ only induces MxA expression in PH5CH8 cells (lane 9). Because MxA is known to be regulated by type I interferons, we investigated whether its IL-27–mediated induction could be attributable to the up-regulation of IFN-α or IFN-β by IL-27 (Supporting Fig. 5). However, stimulation of HepG2 cells with IL-27 in the presence of neutralizing antibodies directed against IFN-α or IFN-β did not affect IL-27–mediated induction of MxA, suggesting a direct induction by IL-27.

Because the IL-27–regulated transcription factor IRF-1 was recently reported to play a central role in the regulation of the antiviral protein RNA helicase retinoic acid–inducible gene-I (RIG-I),[20] we investigated whether IL-27 can induce RIG-I expression. We therefore monitored RIG-I induction in HepG2 and PH5CH8 cells by real-time polymerase chain reaction and western blot analysis. We detected a relatively weak twofold to sixfold increase in RIG-I messenger RNA expression (Supporting Fig. 6), whereas RIG-I protein up-regulation was hardly detectable after 24 hours by western blot (Fig. 4A,B; lane 5). In comparison, RIG-I protein was clearly induced on treatment of both cell lines with IFN-α (lanes 15-17).

Because we found both STAT1 and STAT2 to be up-regulated on stimulation with IL-27 (Fig. 4) and phosphorylation of these factors after treatment with IFN-α, we investigated whether prestimulation with IL-27 could enhance subsequent IFN-α–mediated signaling in PH5CH8 cells. We found that pretreatment with IL-27 enhances subsequent STAT1 and STAT2 phosphorylation on IFN-α treatment (Supporting Fig. 7A) and also induces the expression of IRF-9 (Supporting Fig. 7B), which forms the transcription factor complex interferon-stimulated gene factor 3 together with pYSTAT1 and pYSTAT2. However, we did not detect increased expression of the IFN-α–regulated genes RIG-I and MxA (Supporting Fig. 7C).

IL-27 Acts on Primary Rat Hepatocytes. To verify whether IL-27 also acts on hepatocytes in primary culture, we isolated primary rat hepatocytes and stimulated these cells with IL-27, IFN-γ, or IL-6 for different times. Treatment of

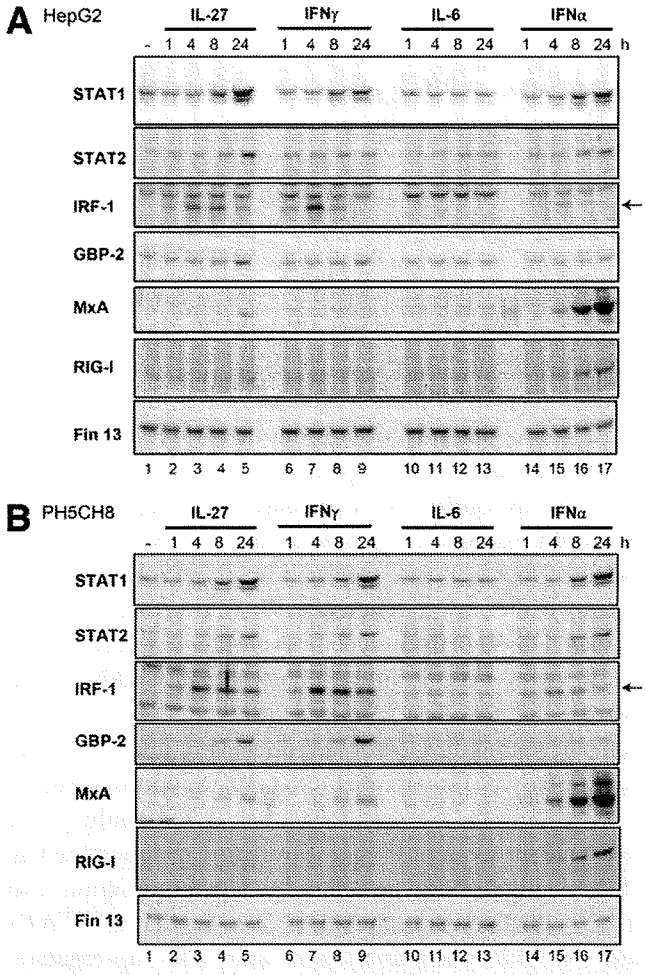

Fig. 4. IL-27 mediates STAT1 responses in hepatoma cells and cultured hepatocytes. (A, B) Western blot analysis monitoring up-regulation of STAT1, STAT2, IRF-1, GBP2, MxA, and RIG-I protein expression on stimulation of HepG2 cells (A) or PH5CH8 cells (B) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Expression levels of Fin13 are provided to compare the protein amount in the samples.

these cells with IL-27 induces a sustained phosphorylation of both STAT1 and STAT3 (Fig. 5), showing that primary hepatocytes respond to IL-27. Whereas the IL-27–mediated STAT1 phosphorylation is comparable to the one obtained after treatment with IFN-γ, the STAT3 response is much weaker than the one initiated by IL-6, confirming the results we obtained in the cell lines.

Discussion

Within the gastrointestinal system, a role for IL-27 was reported in the context of concanavalin-induced hepatitis,[21] Crohn disease,[22] as well as colon carcinoma.[23] However, all of these studies highlight IL-27 functions in infiltrating immune cells such as T cells and natural killer cells. IL-27 also acts on liver cells, namely, human hepatoma cells, cultured human hepatocytes, and primary rat hepatocytes. We find IL-27 to induce a sustained activation of STAT1 and STAT3 in these cells (Figs. 1 and 5).

IL-6–type cytokine signaling is characterized by a sustained STAT3 activation mediated via the different contributing receptor chains gp130, leukemia inhibitory factor receptor, and oncostatinM receptor. This STAT3 activation is of primordial importance for various functions in the liver such as the acute-phase response and liver regeneration. We therefore investigated the potency of the STAT3 activation mediated by IL-27, the new member of the IL-6–type cytokine family. For this, we selected γ-fibrinogen and hepcidin, two type II acute-phase proteins that are synthesized by hepatocytes in response to IL-6. In our experiments, IL-27 (as well as IFN-γ) was not able to lead to a significant induction of these genes (Supporting Fig. 3). These results suggest that IL-27 at most very weakly contributes to the acute-phase response of the liver and that it is in general a quite weak initiator of STAT3 responses in parenchymal liver cells. Because of the weak but sustained STAT3 activation that we observe after IL-27 stimulation (Figs. 1 and 2), it may be of interest to investigate a larger panel of acute-phase proteins to further dissect the potential contribution of IL-27 to the expression of type II acute-phase response genes.

However, the observed activation of STAT1 on stimulation of hepatoma cells and hepatocytes with IL-27 was of special interest because IL-6–type cytokines such as IL-6 and OSM fail to induce an efficient STAT1 response despite the fact that STAT1 phosphorylation is observed.[11-13] Thus, the detection of mere STAT1 phosphorylation (as detected after a 15-minute stimulation in Fig. 1A) does not allow drawing conclusions about STAT1 activity. Together with the very

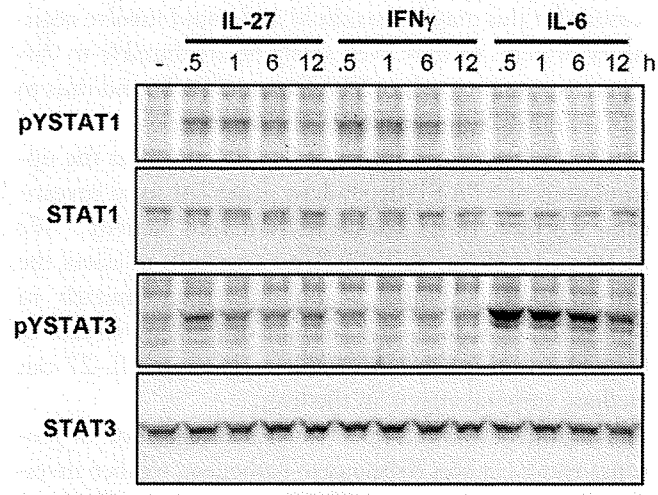

Fig. 5. IL-27 induces STAT1 and STAT3 tyrosine phosphorylation in primary hepatocytes. Primary rat hepatocytes were treated with 20 ng/mL IL-27, IL-6, or IFN-γ for the times indicated. Phosphorylation of STAT1 and STAT3 was monitored by western blot analysis.

transient phosphorylation of STAT1 after stimulation of hepatoma cells with IL-6, the fact that most of the phosphorylated STAT1 is found in STAT1/STAT3 heterodimers contributes to the lack of STAT1-dependent gene induction after treatment of cells with IL-6.[13] It also provides an explanation for the fact that IL-6 and OSM induce an interferon-like response in STAT3 knockout cells,[11,12] because the lack of STAT3 prevents the formation of heterodimers and thereby favors STAT1 homodimer formation. Here we show that IL-27 leads to a sustained STAT1 activation characterized by the formation of STAT1 homodimers (Fig. 2). Investigating whether the observed STAT1 activation translates to the induction of STAT1 target genes, we show that IL-27 up-regulates STAT1, STAT2, IRF-1, and IRF-9 protein expression (Fig. 4; Supporting Fig. 7B). This induction is comparable to the up-regulation after stimulation of these cells with IFN-γ or IFN-α, with the exception of an impaired STAT2 up-regulation by IFN-γ in HepG2 cells (Fig. 4A). This shows that IL-27 mounts an efficient STAT1 response and can mediate interferon-like responses in liver cells. This result corroborates previous data obtained in CD4+T cells and macrophages that highlight the importance of STAT1 for distinct biological activities mediated by IL-27.[4,24,25]

It is an interesting thought that the extent of STAT1 and STAT3 activation may be differently regulated as STAT3 responses are mediated via the gp130 receptor chain, whereas STAT1 responses will most likely only efficiently be mediated via the IL-27–specific WSX-1 receptor chain. This may lead to case-sensitive STAT1 or STAT3 responses. For example, it was recently reported that IL-27 activates both STAT1 and STAT3 in early activated T cells, whereas it displays a preferential activation of STAT3 in fully activated CD4+T cells.[26] The reasons for this may be manifold and could involve regulatory proteins or may even solely be attributable to different expression levels of STAT1 and STAT3 and thus to different distributions of STAT-dimer species.

The prolonged activation of STAT1 as well as the up-regulation of STAT1-dependent genes led us to investigate whether IL-27 possesses antiviral activity. We performed a plaque assay in hepatoma cells to assess the antiviral potency of IL-27 and show that, similarly to IFN-α, IL-27 reduces the amount of progeny viruses in HepG2 cells (Fig. 3). This result suggests that IL-27 can mediate antiviral effects in the liver.

To further assess the IL-27–mediated regulation of proteins involved in host resistance to pathogens, we then investigated the regulation of RIG-I, MxA, and GBP2 on stimulation of HepG2 and PH5CH8 cells with IL-27. The RNA helicase RIG-I is induced by retinoic acid as well as interferons and constitutes the first line of defense against viral infections by sensing viral double-stranded RNA.[27] Because it was recently shown that IRF-1 plays a central role in the regulation of RIG-I expression, we investigated whether IL-27 would induce this sensor for viral double-stranded RNA. Although we detected an increase in RIG-I messenger RNA levels on IL-27 stimulation in both human hepatoma cells and cultured human hepatocytes (Supporting Fig. 6), RIG-I protein was barely detectable. This shows that although IRF-1 expression may be necessary for RIG-I induction,[20] its expression alone is not sufficient. One may speculate that additional cellular signals may lead to an up-regulation of RIG-I protein expression by IL-27 and IFN-γ. Investigating the induction of the IFN-γ–induced protein GBP2 and the IFN-α–regulated MxA protein, we found IL-27 to up-regulate both proteins. IL-27 regulated these genes in a manner comparable to that of IFN-γ treatment. Because MxA is a gene regulated by type I interferons, we tested whether its induction after IL-27 treatment could be mediated through the induction of type I interferons. Experiments with neutralizing antibodies against IFN-α and IFN-β1 did not affect MxA nor GPB2 and STAT1 induction, suggesting that the observed regulation by IL-27 does not involve type I interferon production (Supporting Fig. 5).

Furthermore, we tested whether IL-27 could prime cells for a subsequent IFN-α stimulation (Supporting Fig. 7). Most interestingly, we found that prestimulation with IL-27 enhances subsequent IFN-α–mediated STAT1 and STAT2 phosphorylation and also up-regulates IRF-9. However, we did not detect increased expression of the IFN-α–regulated proteins RIG-I and MxA at the different doses of IFN-α tested. Nevertheless, it would be of interest to investigate other genes induced by the transcription factor complex interferon-stimulated gene factor 3 composed of pYSTAT1, pYSTAT2, and IRF-9.

Recent reports suggest that IL-27 can have antiviral activities in peripheral blood mononuclear cells, CD4+T cells, and macrophages and can inhibit human immunodeficiency virus 1 replication.[25,28] It was shown that IL-27 significantly induces interferon-inducible antiviral genes such as myxovirus protein 1, 2′-5′-oligoadenylate synthetase 2 and RNA-dependent protein kinase in macrophages, suggesting that IL-27 inhibits human immunodeficiency virus replication by eliciting an interferon-like response.[25] Together with our data, this suggests that IL-27 can elicit a multifaceted antiviral response.

The results presented in this study suggest that IL-27 may be a potential candidate for studies on combination therapies against hepatitis C. The standard care for a chronic hepatitis C infection is a combination therapy of IFN-α plus ribavirin. For a standard treatment, the response rate is approximately 50% for patients with hepatitis C virus genotype 1 and abut 80% for genotypes 2 and 3.[29] For the development of future

therapies, interests are focusing on combination therapies with different classes of anti–hepatitis C virus drugs such as protease or polymerase inhibitors. Furthermore, novel IFN-based products are being developed.[29] Our results that IL-27 acts on hepatocytes and hepatoma cells and displays IFN-like signaling in these cells as well as the antiviral effects of IL-27 observed in immune cells[25,28] indicate that treatment with IL-27 could be used in the therapy of hepatitis C virus infection.

Taken together, we present data showing that IL-27 acts on hepatocytes and hepatoma cells, elicits IFN-γ–like STAT1-mediated responses in these cells, and is able to regulate genes involved in host resistance to pathogens.

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Hypoxia-Inducible Factor 1α Is Up-Regulated by Oncostatin M and Participates in Oncostatin M Signaling

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The interleukin-6–type cytokine oncostatin M (OSM) acts via the Janus kinase/signal transducer and activator of transcription pathway as well as via activation of mitogen-activated protein kinases and is known to critically regulate processes such as liver development and regeneration, hematopoiesis, and angiogenesis, which are also determined by hypoxia with the hypoxia-inducible factor 1α (HIF1α) as a key component. Here we show that treatment of hepatocytes and hepatoma cells with OSM leads to an increased protein level of HIF1α under normoxic and hypoxic conditions. Furthermore, the OSM-dependent HIF1α increase is mediated via Janus kinase/signal transducer and activator of transcription 3 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 pathways. OSM-mediated HIF1α up-regulation did not result from an increase in HIF1α protein stability but from increased transcription from the *HIF1α* gene. In addition, we show that the OSM-induced *HIF1α* gene transcription and the resulting enhanced HIF1α protein levels are important for the OSM-dependent vascular endothelial growth factor and plasminogen activator inhibitor 1 gene induction associated with several diseases. *Conclusion:* HIF1α levels increase significantly after treatment of hepatocytes and hepatoma cells with OSM, and HIF1α contributes to OSM downstream signaling events, pointing to a cross-talk between cytokine and hypoxia signaling in processes such as liver development and regeneration. (HEPATOLOGY 2009;50:253-260.)

Oncostatin M (OSM) is an interleukin (IL)-6–type cytokine produced by monocytes and macrophages, T cells, and several other cell types. OSM receptors are widely expressed and are composed of the common signal transducer gp130 in complex with the LIFR or the OSMR. OSM has pleiotropic effects that in part overlap with those of other IL-6–type cytokines; examples include inflammation, neurogenesis, regulation of cell proliferation, and fibrosis.[1-3] In addition, OSM plays a crucial role in the orchestration of hematopoiesis and liver development.[3]

Upon OSM-induced receptor clustering, Janus kinases—mainly Janus kinase 1—are activated, phosphorylate tyrosines within the receptor that recruit other signaling proteins with matching SH2 domains such as signal transducers and activators of transcription (STATs) or adapter proteins for the mitogen-activated protein kinases to the receptor. The major signaling cascades activated by OSM include STAT3 as well as the extracellular signal-regulated kinase (Erk) 1/2 and p38 pathways.[2-4]

Several processes such as hematopoiesis, angiogenesis, liver development, metabolism, inflammation, and tu-

Abbreviations: Erk, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HIF1α, hypoxia-inducible factor 1α; HRE, hypoxia response element; IL, interleukin; mRNA, messenger RNA; OSM, oncostatin M; PAI1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

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morigenesis are also crucially influenced by the ambient oxygen tension of the tissue. Hypoxia-inducible factors (HIFs) act as master regulators for the expression of genes essential in a hypoxic microenvironment. The best characterized factor is HIF1, which regulates more than 100 genes. HIF1 is formed by dimerization of the oxygen-sensitive hypoxia-inducible factor 1α (HIF1α) subunit with the constitutively expressed beta-subunit, which is also known as ARNT. Under normoxic conditions, the HIF1α subunit is hydroxylated and quickly degraded via the proteasome. Under hypoxic conditions, the activity of the hydroxylases is reduced, and HIF1α protein is stabilized and can bind to hypoxia-response elements (HREs) within the regulatory areas of HIF target genes and efficiently recruit cofactors.[5]

In addition to hypoxia, HIF1α has also been shown to be up-regulated under normoxia in response to growth factors, thrombin, lipopolysaccharide, angiotensin II, insulin, or the cytokines IL-1 and tumor necrosis factor α (TNF-α).[6] Although several details have been unraveled regarding the ability of hypoxia to stabilize HIF1α, the mechanisms by which those factors (especially cytokines) induce HIF1α have not been fully elucidated.

Interestingly, OSM has been described to orchestrate the hypoxia-influenced processes of hematopoiesis, angiogenesis, liver development, and regeneration.[3,7] This suggests a possible cross-talk of the OSM and hypoxia signaling pathways. The expression of the HIF1 target genes vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor 1 (PAI1)—which are crucial for angiogenesis and tissue remodeling, respectively—can also be up-regulated by the cytokine OSM.[8-12] OSM supports *in vitro* differentiation of fetal hepatic cells into liver-like structures, which is paralleled by enhanced VEGF expression.[11] Moreover, OSM mediates differentiation of oval cells into hepatocytes.[13] OSM plays a crucial, nonredundant role in liver regeneration, as shown for OSM receptor knock-out mice after partial hepatectomy or CCl_4 treatment,[14] and OSM gene therapy attenuates liver damage induced by dimethylnitrosamine.[15] Interestingly, HIF1α is also expressed during liver regeneration,[16] and HIF1α supports the growth of hepatoma cells *in vivo* and *in vitro*.[17,18]

Because both OSM and HIF1α play a pivotal role in liver-related processes (development, regeneration, carcinogenesis), the aim of the present study was to investigate whether the OSM signaling pathway has an impact on the HIF1 system in hepatoma cells and hepatocytes.

Materials and Methods

Cell Culture and Reagents. HepG2 hepatoma cells were maintained in DMEM/NUT-MIX-F12 medium (Lonza) supplemented with 10% fetal bovine serum (PAA), 100 mg/L streptomycin, and 60 mg/L penicillin (Cytogen). The human hepatocyte cell line PH5CH8 has been described.[19] Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. Hypoxia treatment was performed at 37°C in a water-saturated atmosphere at 5% CO_2 and 6% oxygen. HepG2 cells were transfected using the Fugene reagent (Roche) according to the manufacturer's recommendations. Cotransfections of small interfering RNA (siRNA) and reporter gene constructs are described in the Supporting Information. Human recombinant OSM was obtained from Peprotech. Actinomycin D and cycloheximide were obtained from Calbiochem. Stattic[20] was from Sigma.

Western Blot Analysis and Antibodies. All steps of cell lysis and immunoprecipitation were performed at 4°C using ice cold buffers. Cells were lysed on a dish with lysis buffer containing 30 mM Tris/HCl (pH 6.7), 5% glycerol, 2.5% mercaptoethanol, and 1% sodium dodecyl sulfate. The lysates were further analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies against HIF1α, STAT3, STAT1, and Fin13 were obtained from BD Transduction Laboratories. Antibodies against phospho-STAT3, phospho-STAT1, Erk1/2, phospho-Erk1/2, p38, and phospho-p38 were obtained from Cell Signaling. ECL signals were detected as described.[21] Before reprobing, blots were stripped as described.[4]

Reporter Gene Assays. HepG2 cells were transfected with 1 μg of the β-galactosidase control plasmid (pCH110, Amersham Biosciences) and 1.5 μg of the respective reporter gene construct. Twenty-four hours after transfection, the cells were treated with the different stimuli as described in the figure legends. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI) (see Supporting Information for further details). All experiments were performed at least in triplicate, and biological triplicates were also performed within one experiment. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. For some experiments the luciferase activity values after OSM stimulation were additionally normalized to values from control unstimulated cells.

Quantitative Real-Time Polymerase Chain Reaction. The exact protocol is described in the Supporting Information. Total RNA was extracted using the RNeasy Mini Kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. One microgram of total RNA was reverse-transcribed with a ThermoScript RT-PCR System (Invitrogen). Quantita-

tive real-time polymerase chain reaction (PCR) was performed on an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories). Standard curves using four 10-fold dilutions (1×, 0.1×, 0.01×, 0.001×) were produced to ensure that the amplification efficiencies were similar and in the range of 95% to 105%. The messenger RNA (mRNA) level of each target gene was normalized to the relative amount of the housekeeping gene TBP. The comparative threshold cycles (C_T) method, $2^{-\Delta CT}$, was used to calculate the changes in gene expression for each target gene.

Statistical Analysis. Each experiment was performed at least three times. Representative data are shown and are expressed as the mean ± standard deviation. Depending on datasets, statistical analysis was performed using a *t* test, Mann-Whitney test, or analysis of variance. *P* values of <0.05 were considered significant.

Results

OSM Increases Expression of Functional HIF1α Under Normoxic Conditions. Exposure of HepG2 hepatoma cells to OSM for different times led to a profound and transient increase in HIF1α protein levels that lasted up to 24 hours. The up-regulation reached a maximum after about 6 hours of stimulation. As expected, OSM induced STAT3, Erk1/2, and p38 phosphorylation (Fig. 1A). HIF1α was also induced upon stimulation of the human hepatocyte cell line PH5CH8 with OSM (Fig. 1B). Quantitation of western blots showed that upon OSM stimulation, the HIF1α protein is up-regulated by factors of 2.66 ± 0.3 in PH5CH8 cells and 2.7 ± 0.9 in HepG2 cells.

To investigate whether OSM-induced HIF1α is functional, we tested the effect of OSM on HepG2 cells transfected with a HIF1-responsive luciferase reporter gene construct. OSM treatment increased luciferase activity three-fold (Fig. 1C). Thus, OSM-induced HIF1α is transcriptionally active even under normoxic conditions.

OSM Augments Hypoxia-Dependent HIF1α Induction and Hypoxia-Mediated Target Gene Expression. Next, we compared the induction of HIF1α by OSM under normoxia with that induced by hypoxia. In addition, we examined whether OSM may affect the hypoxia-dependent induction of HIF1α. We found that HIF1α levels induced by OSM under normoxia were slightly lower than those induced by hypoxia after 4 and 6 hours of induction, respectively. Interestingly, when cells were treated with OSM under hypoxia, the increase in HIF1α protein levels was higher than under each treatment alone (Fig. 2A). Quantitation of western blots showed that the HIF1α protein is up-regulated by factors of 3.6 ± 0.19

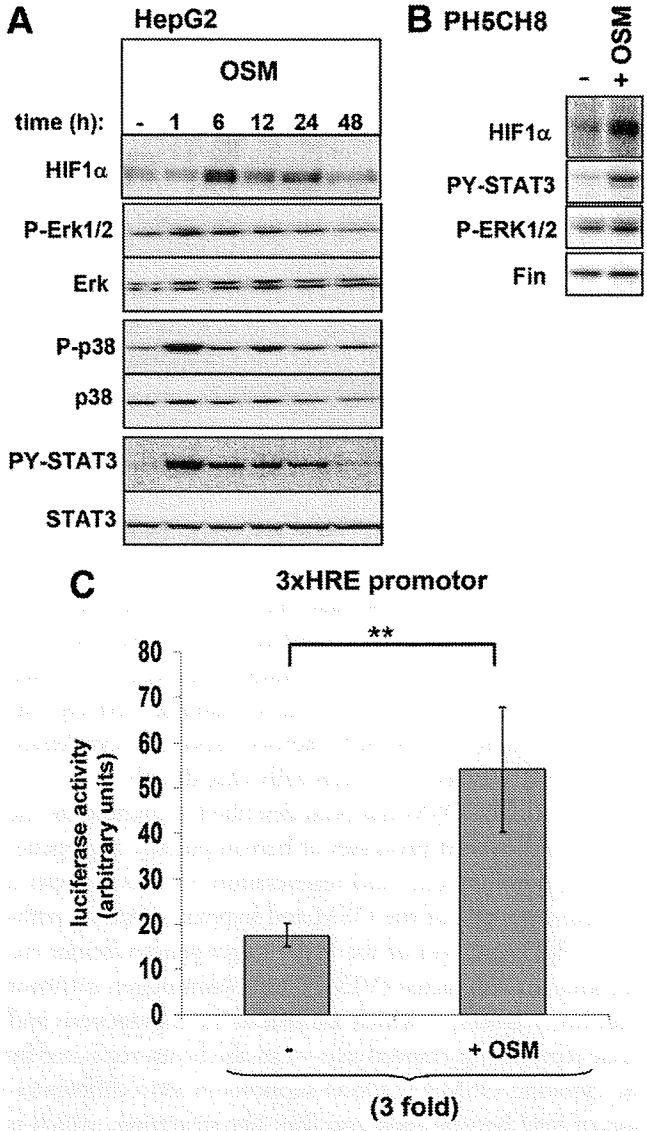

Fig. 1. OSM induces HIF1α protein levels over an extended period, and the induced HIF1α is transcriptionally active. (A) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, phospho-Erk1/2, phospho-p38, STAT3, Erk1/2, and p38 antibodies. (B) PH5CH8 cells were stimulated for 4 hours with OSM (10 ng/mL) or left untreated. Western blots of the membranes were detected as described in (A). (C) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the medium was exchanged and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared, and the reporter gene activity was measured as described in Experimental Procedures. $^{**}P < 0.01$.

upon hypoxia, 2.7 ± 0.9 upon OSM, and 6.4 ± 1.8 upon combined treatment with hypoxia and OSM.

We then investigated HIF1α mRNA expression under the same conditions and found that OSM induces HIF1α mRNA levels stronger (3.4-fold) than hypoxia (2.3-fold). Combined treatment with hypoxia and OSM led to an

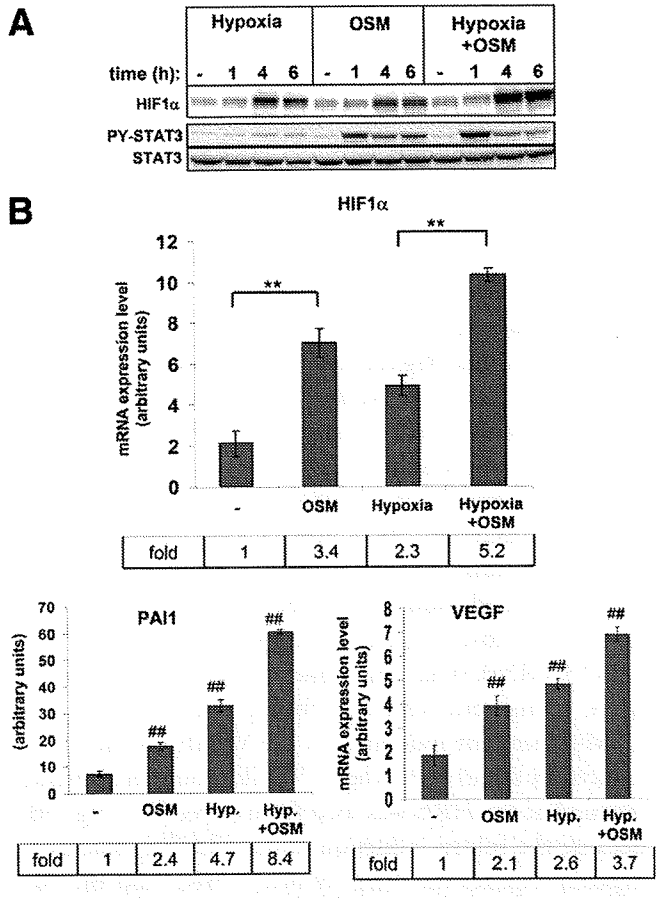

Fig. 2. OSM enhances hypoxia-induced HIF1α, PAI1, and VEGF expression. (A) HepG2 cells were treated for the indicated periods with hypoxic conditions and/or OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with antibodies directed against HIF1α, phospho-STAT3, and STAT3. (B) HepG2 cells were stimulated for 4 hours with hypoxic conditions and/or OSM (10 ng/mL). RNA was prepared, and HIF1α mRNA levels were analyzed via quantitative PCR. $^{**}P < 0.01$. (C) HepG2 cells were treated as described in (B) and VEGF and PAI1 mRNA levels were analyzed via quantitative PCR. $^{\#\#}P < 0.01$ versus untreated controls.

even higher induction of HIF1α mRNA (5.2-fold) (Fig. 2B). Thus OSM significantly increases HIF1α mRNA under normoxia and hypoxia, which matches with the protein up-regulation seen in the western blots (Fig. 2A). Two important target genes of the hypoxic response, PAI1 and VEGF, were also significantly up-regulated by OSM, and a combined treatment with hypoxia and OSM led to an even stronger induction of PAI1 and VEGF mRNA (Fig. 2C).

OSM-Mediated Up-Regulation of HIF1α Protein Levels Is Due to De Novo Transcription but not Regulation of Protein Stability. To find out whether OSM influences HIF1α protein stability, we aimed to measure HIF1α protein half-life. Therefore, we stimulated HepG2 cells with OSM or $CoCl_2$ (a hypoxia mimetic) for 6 hours to induce a robust HIF1α expression before the translation inhibitor cycloheximide was added for different periods. We found that the OSM-induced HIF1α protein disappeared completely after a 10-minute treatment with cycloheximide (Fig. 3A, right panel). In contrast, $CoCl_2$-induced HIF1α was still well detectable after 1 hour; it disappeared after 3 hours of cycloheximide treatment (Fig. 3A, left panel). There was no difference in HIF1α protein stability between $CoCl_2$ and the combined treatment with OSM and $CoCl_2$. These data show that the OSM-mediated HIF1α up-regulation is not due to an enhanced stability of the protein.

In contrast, experiments with the transcription inhibitor actinomycin D showed that less HIF1α protein was detectable when cells were treated with OSM in the presence of actinomycin D relative to cells treated with OSM alone (Fig. 3B). This was even more remarkable because actinomycin D treatment prevents the STAT3-induced up-regulation of the feedback inhibitor SOCS3, which suppresses STAT3 activation upon OSM. Despite the higher STAT3 activation observed in actinomycin D–treated cells, no HIF1α expression could be observed. Actinomycin D did not affect HIF1α expression in cells exposed to hypoxia, which was expected because hypoxia essentially increases protein stability. Here, the HIF1α

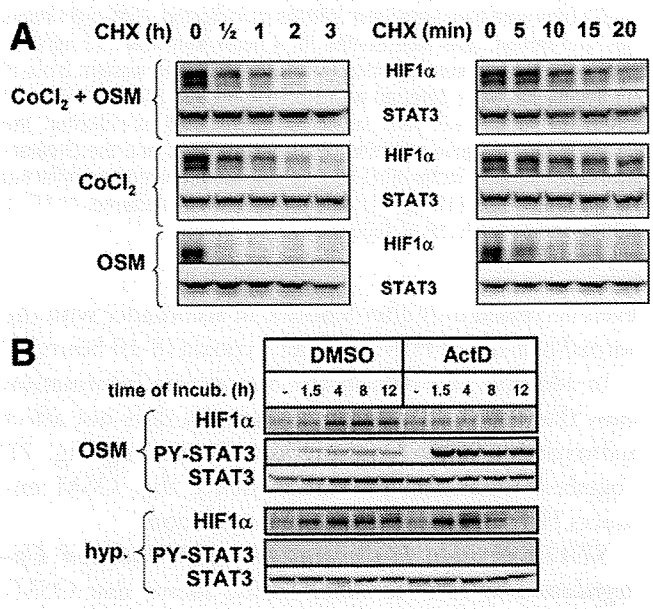

Fig. 3. OSM increases transcription of HIF1α but does not affect HIF1α protein stability. (A) HepG2 cells were stimulated for 6 hours with $CoCl_2$ (50 μM) and/or OSM (10 ng/mL). Cycloheximide (10 μg/mL) was then added for the indicated periods before lysates were prepared. Western blots were detected with HIF1α and STAT3 antibodies. For HIF1α detection, the western blots were exposed so that the band intensity of the untreated lane for all treatments was comparable. (B) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL) or hypoxia (hyp.) in the presence of dimethyl sulfoxide alone or actinomycin D (5 μg/mL). Western blots of lysates separated via SDS-PAGE were detected with HIF1α, phospho-STAT3, and STAT3 antibodies.

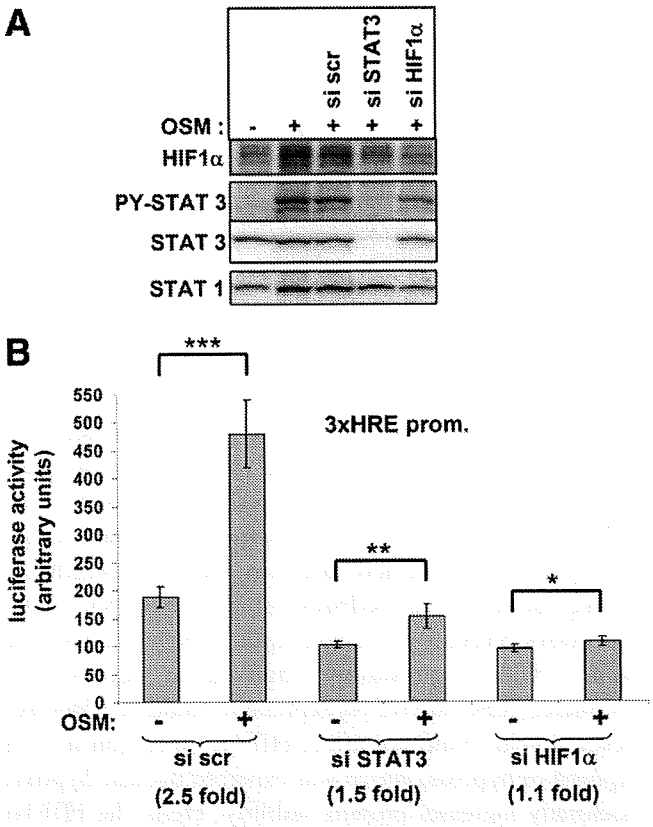

Fig. 4. Knockdown of STAT3 by siRNA decreases HIF1α protein levels and HIF1α-responsive reporter gene activity upon OSM treatment. (A) HepG2 cells were transfected with siRNAs as indicated. Forty-eight hours after transfection, cells were treated for 4 hours with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, STAT3 and STAT1. (B) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. The cells were treated for additional 16 hours with OSM (10 ng/mL) before lysates were prepared. $***P < 0.001$. $**P < 0.01$. $*P < 0.05$.

levels decreased only after 8 hours, in accordance with the normal half-life of HIF1α under hypoxia (8-10 hours).[22]

In addition, our data in the Supporting Information show that OSM, in contrast to hypoxia, does not affect hydroxylation and ubiquitination (Supporting Fig. 2). Together, these data provide evidence that OSM enhances HIF1α via transcriptional regulation.

STAT3 Protein Is Crucial for OSM-Induced Up-Regulation of HIF1α. After having shown that OSM-mediated up-regulation involves transcriptional regulation, we addressed the role of STAT3, playing a pivotal role in signaling of IL-6–type cytokines. Suppression of STAT3 by siRNA led to a loss of HIF1α expression upon OSM treatment, similar to the HIF1α siRNA used as a positive control (Fig. 4A). The unspecific control siRNA did not show these pronounced effects. Upon transfection of STAT3 or HIF1α siRNAs together with the 3xHRE reporter gene construct, OSM only weakly induced the reporter gene activity, whereas OSM induced reporter gene activity 2.5-fold when the unspecific control siRNA was transfected (Fig. 4B). In addition, the STAT3 inhibitor Stattic or dominant negative STAT3 also inhibited OSM-dependent effects (increase of HIF1α protein and mRNA levels, HRE promotor activity, or target gene induction) (Supporting Figs. 3-5). Furthermore, we provide evidence for the relevance of Erk signaling in OSM-mediated induction of HIF1α protein and activity (Supporting Figs. 3-5).

OSM-Induced HIF1α Is Crucially Involved in the Transcriptional Regulation of the Genes for VEGF and PAI1. To investigate the relevance of HIF1α expression in OSM signal transduction, we examined the effects of HIF1α suppression on the target genes PAI1 and VEGF. We found that the OSM-dependent induction of both the VEGF and PAI1 mRNA were decreased upon HIF1α suppression (Supporting Fig. 6). In addition, the OSM-mediated induction of the VEGF and PAI1 promotor was down-regulated by HIF1α siRNA and by STAT3 siRNA (Fig. 5A), while a control siRNA had no effect. Consistent with this finding, we found that the OSM-dependent induction of the VEGF promoter was reduced by about 50% when a VEGF promoter construct mutated at the HRE was used for transfection (Fig. 5B). Because the robust induction of the VEGF promoter by hypoxia requires the integrity of the HRE and the AP1 site, we also used a construct where the AP1 site was mutated; the OSM-dependent induction of this promoter was reduced by about 25% (Fig. 5B). Importantly, mutation of the previously described STAT3 binding site within the VEGF promoter had no effect on the OSM-dependent induction of reporter gene activity (Fig. 5B). Thus, OSM-induced VEGF transcription seems to be mediated via HIF1 and AP1, rather than by STAT3.

In addition, HIF1 seems to play a crucial role in OSM-mediated activation of the PAI1 promotor, for which a reduction of about 40% was observed when the HRE was mutated (Fig. 5C).

Together, these data indicate that *de novo*–transcribed HIF1α importantly contributes to the OSM-induced VEGF and PAI1 transcription.

Discussion

The first major finding of the present study is that the cytokine OSM, which activates the STAT3, Erk1/2, and p38 signaling pathways, can induce a robust up-regulation of HIF1α protein levels in hepatocytes and hepatoma cells under normoxic conditions and leads to the formation of transcriptionally active HIF1 complexes. OSM-induced HIF1α protein up-regulation was stronger compared with other cytokines (IL-6, IL-1β, TNF-α, interferon-γ), some of which have been implicated in

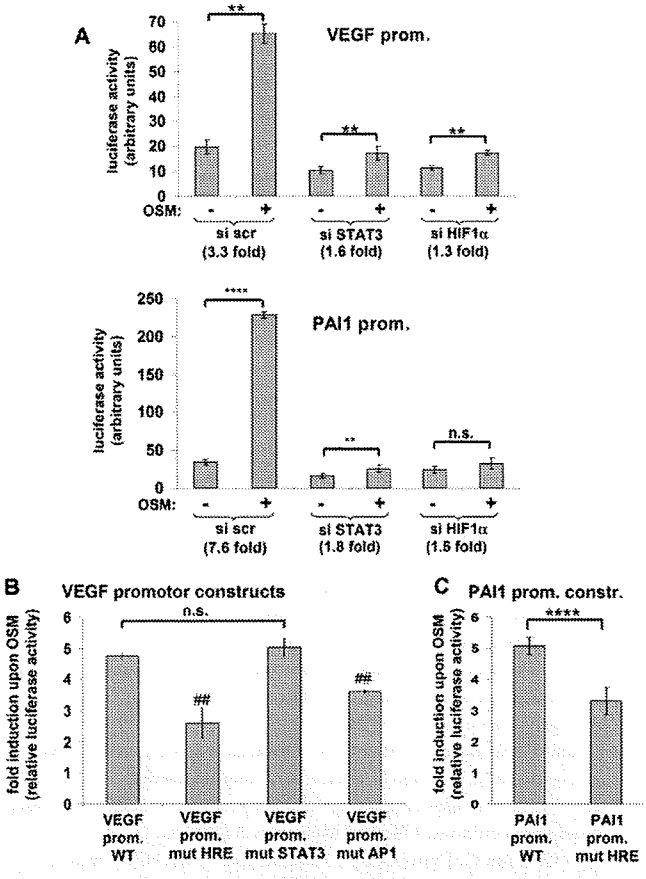

Fig. 5. Regulation of the VEGF and PAI1 gene promotors by OSM. (A) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-VEGF-Luc or pGL3-hPAI1-796 and the β-galactosidase expression vector pCH110, and processed as described in Fig. 4B. $^{****}P < 0.0001$. $^{**}P < 0.01$. n. s., not significant. (B) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-VEGF-Luc or the following mutants thereof: pGL3-VEGFmutHRE-Luc (containing a mutated HIF1α binding site), pGL3-VEGFmutSTAT3-Luc (containing a mutated STAT3 binding site), or pGL3-VEGFmutAP1-Luc (containing a mutated AP1 binding site). The β-galactosidase expression vector pCH110 was cotransfected. Twenty-four hours after transfection, the medium was exchanged, and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared. Values for luciferase activity (relative to β-galactosidase activity) are shown as fold induction compared with untreated samples. $^{\#\#}P < 0.01$ versus wild-type construct. (C) HepG2 cells were transfected with pGL3-hPAI1-796 or with pGL3-hPAI1-796-M2 containing a mutation in the HRE and processed as described in (B). $^{****}P < 0.0001$.

HIF1α up-regulation before (IL-6, IL-1β, TNF-α) (Supporting Fig. 1). Moreover, the OSM-increased HIF1α protein was shown to be involved in the enhanced expression of the HIF1 target genes PAI1 and VEGF.

Our study also shows that OSM and hypoxia differ in their mechanism of HIF1α up-regulation. Regulation of HIF1α activity is complex and under normoxic conditions, HIF1α has an extremely short half-life because it is continuously degraded due to the initial hydroxylation at two proline residues and transcriptional activity is reduced due to hydroxylation of asparagine 803. Hypoxia reduces the activity of the oxygen-utilizing hydroxylases, thereby stabilizing the protein and increasing its transactivity.[5] However, our data provide evidence that OSM does not contribute to an increased stability or increased transactivity as shown in the experiments with the Gal-HIF1α-TADN or TADC gene constructs (Supporting Fig. 2C). Furthermore, we could show that OSM-induced HIF1α was clearly ubiquitinated, which was less the case for hypoxia-stabilized HIF1α (Supporting Fig. 2A,B). We propose that transcriptional mechanisms are responsible for the OSM-mediated HIF1α protein up-regulation because it was inhibitable by the transcriptional inhibitor actinomycin D, whereas this was not the case for hypoxic treatment. The importance of HIF1α regulation at the mRNA level is further supported by findings showing that hepatocyte growth factor, angiotensin-II, lipopolysaccharide, IL-1, thrombin, or hypoxia also enhance HIF1α mRNA levels in different cell types (see Bonello et al.[23] and references therein).

Moreover, our tests with inhibitors for Janus kinases, STAT3, and mitogen-activated protein kinase kinase indicate that these pathways play an important role in OSM-dependent induction of HIF1α mRNA and protein expression (Supporting Fig. 3), HIF1α-dependent reporter gene activity (3x HRE promotor) (Supporting Fig. 4A), as well as in the regulation of the target genes VEGF and PAI1 (Supporting Fig. 5). In contrast, inhibitor tests suggested that p38 mitogen-activated protein kinases or the PI3K/Akt pathway are not involved in OSM-induced HIF1α expression (data not shown).

Experiments with siRNA further revealed that STAT3 plays a crucial role in OSM-regulation of HIF1α protein levels and HIF1α-dependent transcriptional activity. Moreover, dominant negative STAT3 (STAT3-DN) also led to a down-regulation of HIF1α-dependent transcriptional activity (Supporting Fig. 4B). It has been shown that HIF1α transcription is regulated by SP-1[24] and NF-κB transcription factors.[23] In addition to that, the present study indicates that the transcriptional regulator STAT3 appears to be a key player for HIF1α transcription in response to OSM. STAT3 was also found to be involved in HIF1α mRNA expression in tumor cells and tumor-associated myeloid cells.[25]

Our study provides evidence that HIF1α is important for OSM signal transduction. Experiments with HIF1α siRNA showed that the expression of the OSM target genes VEGF and PAI1 involves regulation by HIF1α. OSM and other IL-6–type cytokines have been shown to induce expression of the HIF1 target gene VEGF.[10,12,26-29] The VEGF gene is also considered a STAT3 target gene, because a dominant negative STAT3 reduced[10,27,30,31] and constitutively active forms of

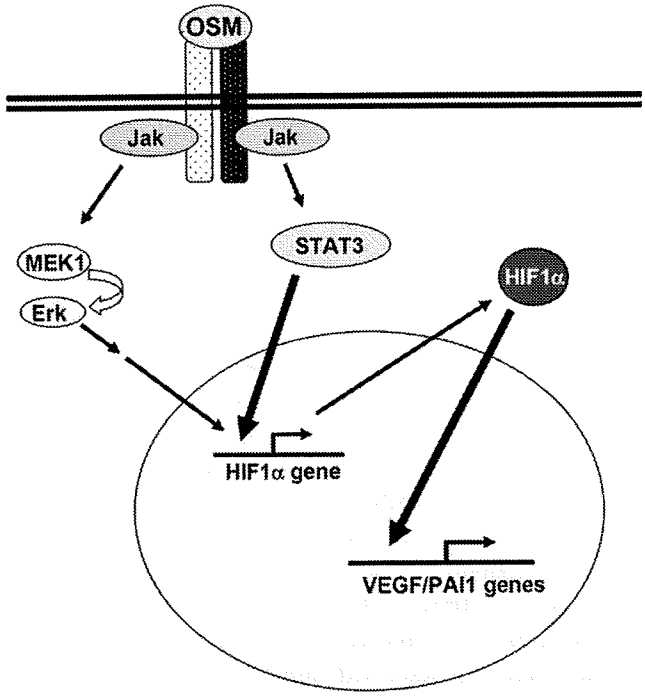

Fig. 6. Schematic representation of VEGF gene regulation by OSM.

STAT3 induced the VEGF promoter,[27,30,31] and a STAT3 binding element was detected at site −848.[30,31] We found that mutation of the described STAT3 binding site in the VEGF promoter at −848 did not affect OSM-dependent induction of reporter gene activity. These data are consistent with those of another study in which deletion of this site also did not affect IL-6–induced reporter gene activity.[27] Instead, our results with the VEGF reporter gene constructs clearly demonstrate a relevance of the HIF1 and AP1 binding elements for OSM-mediated regulation, because the constructs mutated at the HRE and the AP1 sites displayed significantly reduced induction in response to OSM. Thus, we conclude from our data that STAT3 regulation of the VEGF gene may rather be mediated indirectly via Erk1/2 and STAT3-dependent induction of HIF1α transcription. HIF1α then regulates VEGF and PAI1 transcription (Fig. 6), which leads to increased secretion of VEGF and PAI1 proteins (Supporting Fig. 7).

Although the present study was performed with hepatoma cells and nontransformed hepatocytes, it will be interesting to further clarify the role of HIF1α in OSM-mediated signal transduction and regulation of secreted factors involved in tissue remodeling (such as VEGF and PAI1) in processes such as liver development, regeneration, and inflammation.

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