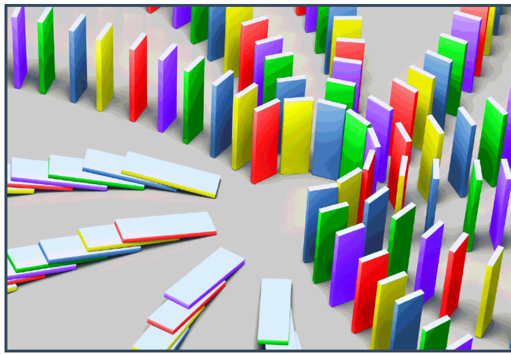
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# Simvastatin Modulates Cytokine-Mediated Endothelial Cell Adhesion Molecule Induction: Involvement of an Inhibitory G Protein[1]

**Mehran M. Sadeghi,* Mark Collinge,* Ruggero Pardi,† and Jeffrey R. Bender[2]***

**Endothelial cell adhesion molecules (CAMs) E-selectin, ICAM-1, and VCAM-1 play variably important roles in immune-mediated processes. They are induced by the proinflammatory cytokines IL-1 and TNF-$\alpha$, and NF-$\kappa$B is required for the regulated expression of all three genes. Regulators of this pathway could potentially be potent immune modulators. We studied the effect of a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, simvastatin, on cytokine-induced expression of CAMs in HUVEC. Unexpectedly, pretreatment with simvastatin potentiated the induction of all three endothelial CAMs by IL-1 and TNF, but not LPS or PMA, as detected by flow cytometry. Northern blot analysis demonstrated an increase in steady state IL-1-induced E-selectin mRNA levels in cells pretreated with simvastatin. This was associated with an increase in nuclear translocation of NF-$\kappa$B, as detected by EMSA. The effect of simvastatin was reversed by mevalonate and geranylgeranyl pyrophosphate but not squalene, indicating that an inhibitory prenylated protein is involved in endothelial responses to proinflammatory cytokines. Pertussis toxin mimicked the effect of simvastatin, and the G protein activator NaF inhibited the cytokine-induced expression of endothelial CAMs, indicating that a $G_{i\alpha}$ protein is involved. These results demonstrate that cytokine-mediated activation of the endothelium, and specifically CAM induction, can be modulated by a heterotrimeric G protein-coupled pathway. This may represent a "basal tone" of endothelial inactivation, which can either be disinhibited or amplified, depending on the stimulus.** *The Journal of Immunology,* **2000, 165: 2712–2718.**

Once thought to be a passive barrier, the endothelium is now recognized as a dynamic tissue, which, in part through regulation of adhesion receptors for circulating leukocytes, controls the traffic of cells between the blood and sites of inflammation and/or immune challenge. A sequential process occurs whereby leukocyte rolling is followed by firm, high-avidity adhesion and transmigration. Circulating leukocytes routinely display receptors for inducible endothelial cell adhesion molecules (CAMs)[3] (reviewed in Ref. 1). The CAMs E-selectin and VCAM-1 are only expressed on activated endothelium, stimulated by a wide array of proinflammatory mediators including IL-1 and TNF-$\alpha$. ICAM-1 is constitutively present on endothelial cells (EC) both in vitro and in vivo, but its membrane expression is greatly augmented by the same cytokines.

Molecular events leading to endothelial CAM induction have been extensively studied. The aforementioned stimuli trigger transcriptional activation of all three CAM genes in a NF-$\kappa$B-dependent fashion (2). TNF-$\alpha$ and IL-1 receptor engagement both result in activation of kinase(s) that phosphorylate the NF-$\kappa$B cytosolic anchor I$\kappa$B, thereby promoting its ubiquitination, dissociation, and proteasomal degradation, with consequent NF-$\kappa$B nuclear translocation and participation in transactivation. The complexities of signaling events resulting in I$\kappa$B phosphorylation are beginning to clarify (see *Discussion*). The diversity of receptor-mediated NF-$\kappa$B activation is imparted by differential signal complex formation and kinase activation.

Of the three CAMs mentioned above, E-selectin gene expression has been most extensively studied. Several promoter elements, also called positive regulatory domains (PDs), are necessary for cytokine responsiveness (3). Three of these elements (PDI, -III, and -IV) contain NF-$\kappa$B recognition sequences, whereas PDII is inducibly bound by activating transcription factor-2/c-Jun complexes, which is a target of the c-Jun N-terminal kinase/p38 mitogen-activated protein kinase signaling pathway. Small GTP-binding proteins Rac1 and CDC42 are upstream activators of this pathway (4), and mitogen-activated protein kinase activation can also lead to NF-$\kappa$B translocation (5). In the case of all three CAMs, efficient transcriptional activation requires the coordination of both the multiple of NF-$\kappa$B elements and the non-NF-$\kappa$B factors/*cis*-elements. Indeed, the positive regulatory influences imparted by various cytokines on individual CAM genes continues to be elucidated. However, there is little information about basal negative regulatory factors and/or parallel receptor-mediated pathways that modulate the aforementioned cytokine responsiveness.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, effectively reduce cardiovascular events in patients with coronary artery disease (6, 7). This benefit appears to exceed the cholesterol-lowering effect of this class of drugs, possibly through atherosclerotic plaque stabilization (8, 9). Because leukocyte adhesion to the endothelium plays an important role both in early atherogenesis and in plaque instability, the hypothesis that statins inhibit endothelial CAM expression in the inflammatory milieu was tested. We unexpectedly uncovered a

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[3] Abbreviations used in this paper: CAM, cell adhesion molecule; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PMBS, polymyxin B sulfate; NIK, NF-$\kappa$B-inducing kinase; EC, endothelial cells; PD, positive regulatory domain.

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negative regulatory pathway in EC, disinhibited in the presence of the HMG-CoA reductase inhibitor simvastatin. That is, we found that simvastatin pretreatment potentiates cytokine-mediated endothelial CAM induction, in a NF-$\kappa$B-dependent fashion. Use of various intermediates in the cholesterol synthesis pathway indicated that inhibition of protein prenylation is responsible for the statin effect, and further pharmacologic analysis demonstrated that an inhibitory $G_i$ protein can modulate endothelial responses to IL-1. Thus, we describe a counterregulatory pathway, heterotrimeric G-protein-dependent, which influences the level of cytokine responsiveness (and CAM induction) in EC.

## Materials and Methods

### Reagents

All the reagents were from Sigma (St. Louis, MO) unless otherwise specified. Human recombinant IL-1$\beta$ was a kind gift from S. Gillis (Immunex, Seattle, WA). Human recombinant TNF-$\alpha$ was purchased from Collaborative Biochemical Products (Bedford, MA). Pertussis toxin and cholera toxin were purchased from Calbiochem (San Diego, CA). Simvastatin prodrug (Merck, West Point, PA) was activated to its active form as described (10, 11). In brief, 4 mg of simvastatin prodrug was dissolved in 100 $\mu$l of ethanol. Then, 150 $\mu$l of 0.1 N NaOH was added to the solution that was subsequently incubated at 50°C for 2 h. The pH was brought to 7.0 by HCl, and the final concentration of the stock solution was adjusted to 4 mg/ml. The stock solution was kept at 4°C.

### Cell culture

Single-donor HUVEC were isolated and cultured as previously described (12, 13) on 0.5% gelatin-coated tissue culture plastic (Falcon, Lincoln Park, NJ) in M199 containing 20% FBS (Life Technologies, Grand Island, NY), 50 $\mu$g/ml endothelial cell growth factor (Collaborative Biomedical Products), 100 $\mu$g/ml porcine heparin, 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. After confluence, HUVEC were harvested with trypsin-EDTA and were split 1:3. Cells were used within five passages and were identified as endothelial by their characteristic cobblestone morphology and immunocytochemical demonstration of factor VIII Ag. The cultures with >1% contaminating nonendothelial cells are routinely discarded.

### FACS analysis

HUVEC were grown in 24-well plates. After reaching confluence, they were pretreated with simvastatin or vehicle control for 12–72 h, as indicated, following which cytokine or vehicle was added to the medium. At the end of the indicated time, cells were harvested with trypsin-EDTA, immunostained, and membrane molecular expression was quantified by FACS analysis on a FACSort (Becton Dickinson, San Jose, CA). A total of 5000 events was analyzed per experimental sample. The primary mouse mAbs used were anti-E-selectin (H4/18; gift of Dr. Pober, Yale University, New Haven, CT), anti-ICAM-1 (RR1.1; gift of Dr. Springer, Harvard University, Cambridge, MA), and anti-VCAM-1 (E1/6; gift of Dr. Bevilacqua, Source Pharmaceuticals, Boulder, CO). The secondary Ab was a polyclonal FITC-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA). In experiments exploring the effect of various cholesterol synthesis pathway intermediates, these intermediates were added to the medium at the same time as simvastatin.

### Northern blot

Total RNA was prepared from cells by extraction with Trizol (Life Technologies), according to the method of Chomczynski and Sacchi (14). Then, 20 $\mu$g of total RNA was subjected to Northern analysis as described (15). Signals were visualized by autoradiography, and densitometric analysis was performed on an ImageQuant densitometric scanner (Molecular Dynamics, Sunnyvale, CA). Steady-state E-selectin mRNA levels were normalized to that of GAPDH. To generate normalized values, E-selectin densitometric units were divided by corresponding GAPDH units. The ratio in the IL-1-activated experimental sample was defined as 1.0. The cDNA probes used were: ELAM/pi3HM (gift of Dr. Bevilacqua, Source Pharmaceuticals), and a 1.0-kb fragment of human GAPDH (Clontech Laboratories, Palo Alto, CA).

### EMSA

After treatments, HUVEC monolayers (~$4 \times 10^6$ cells/experimental sample) were harvested with trypsin-EDTA. A total of 10 $\mu$g of nuclear protein was used in EMSAs as previously described (16). The NF-$\kappa$B target sequence used was derived from the PDI domain of the E-selectin promoter region (5′-CATTGGGGATTTCCTCTTTA-3′). In some samples, a 50-fold excess of unlabeled oligonucleotide was added as competitor.

## Results

### Simvastatin effect on cytokine-mediated E-selectin induction

HUVEC were pretreated with various doses of simvastatin before cytokine exposure. Although some cell loss was observed, HUVEC tolerated up to 1 $\mu$g/ml of simvastatin for 48 h. Lower concentrations could be used for longer duration without significant cell loss. Therefore, the minimal dose of simvastatin required to consistently achieve the experimental effect was used. The effect of simvastatin pretreatment on E-selectin induction in response to several activating stimuli was assessed by flow cytometry. HUVEC were pretreated with simvastatin 300 ng/ml (600 nM) for 18 h, following which IL-1$\beta$ was added to the medium for 4 h, at a final concentration of 6 U/ml. Fig. 1*A* demonstrates a minor induction of E-selectin by this submaximal concentration of IL-1$\beta$. Unexpectedly, simvastatin pretreatment potentiated the IL-1 effect, whereas simvastatin alone did not induce E-selectin. A similar potentiating effect of simvastatin was observed when a low concentration of TNF-$\alpha$ (6 U/ml) was used as the cytokine stimulus (Fig. 1*B*). In parallel experiments, pretreatment with mevastatin, another HMG-CoA reductase inhibitor, yielded similar results (data not shown).

To evaluate whether all E-selectin-inducing stimuli are potentiated by simvastatin, LPS and PMA were tested in identical assays. In contrast to the consistently reproducible simvastatin-potentiating effect on IL-1-and TNF-$\alpha$-mediated E-selectin induction, simvastatin had either a marginal (Fig. 1*C*) or no effect on LPS responses, and none on PMA responses (Fig. 1*D*). These findings suggest that simvastatin affects the noted cytokine (IL-1 and TNF-$\alpha$) signaling pathways, as opposed to primarily affecting downstream transcriptional responses.

Because LPS has been shown to synergistically promote IL-1- and TNF-$\alpha$- mediated CAM induction, the LPS-inactivator polymyxin B sulfate (PMBS) was added to the activation experiments described above. Whereas PMBS completely abrogated LPS-induced potentiation of IL-1-mediated HUVEC E-selectin induction, it had no effect on simvastatin-mediated potentiation (not shown), indicating that contaminating LPS is not responsible for this effect.

### Simvastatin effect on ICAM-1 and VCAM-1 induction

If simvastatin alters cytokine signaling pathways, modulatory effects on other molecular inductions would be expected. Therefore, similar experiments were performed to address whether simvastatin potentiates IL-1$\beta$ induction of ICAM-1 and VCAM-1. After an 18-h simvastatin (300 ng/ml) pretreatment, HUVEC were IL-1 (3 U/ml)-treated for 6 h, following which VCAM-1 and ICAM-1 membrane expression were assessed by flow cytometry. Fig. 1, *E* and *F*, demonstrates that IL-1-mediated ICAM-1 and VCAM-1 induction, respectively, are also potentiated by simvastatin. The magnitude of the potentiating effect, which can be seen at concentrations as low as 25 ng/ml, is dose-dependent. Fig. 1*G* demonstrates that the potentiation of IL-1$\beta$-mediated ICAM-1 induction is greater at 200 than 25 ng/ml. The maximal effect is observed at concentrations in the 500 ng/ml (1 $\mu$M) range, with a required 12- to 72-h pretreatment. Higher concentrations and more prolonged simvastatin exposure resulted in EC detachment and cell death. Time points for CAM analysis were chosen based upon easily interpretable expression levels. Analysis over multiple time points demonstrated that simvastatin does not affect kinetics of CAM

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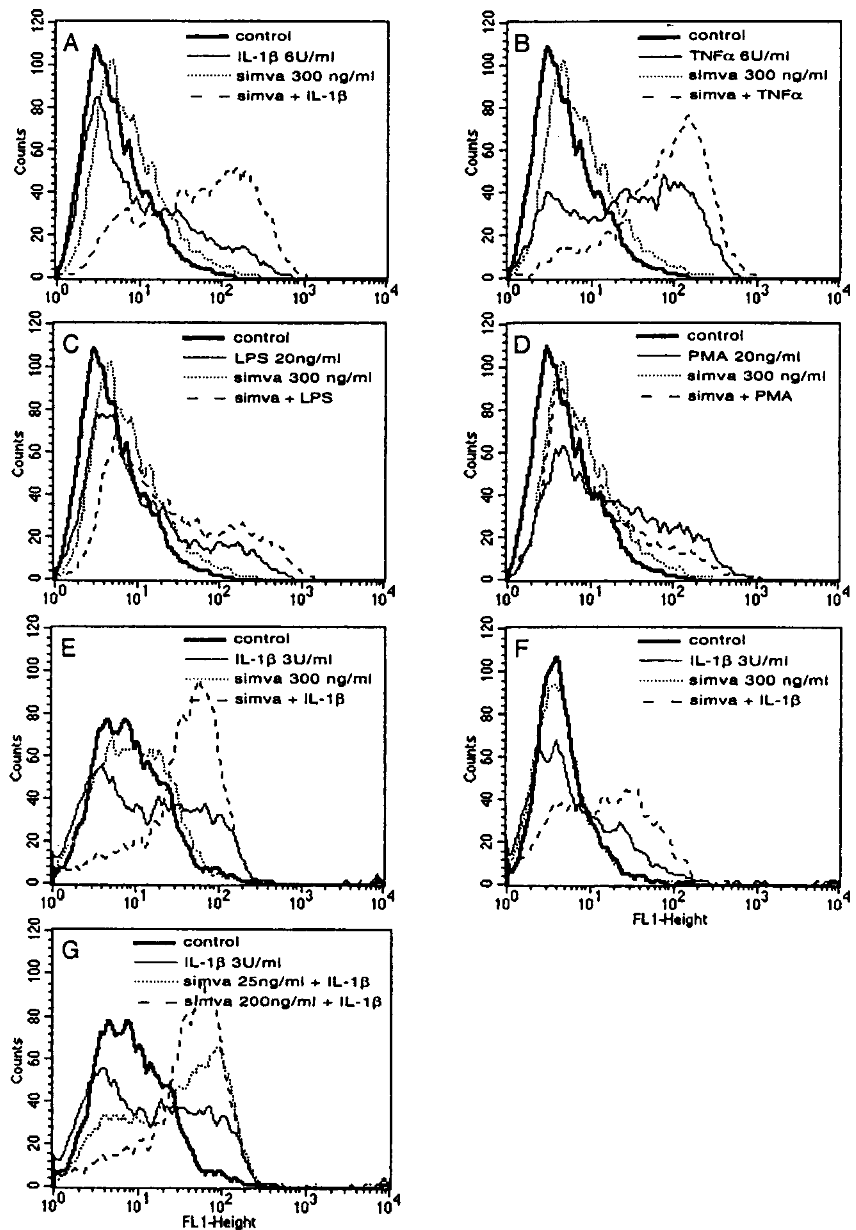


**FIGURE 1.** Flow cytometric analysis of the simvastatin effect on adhesion molecule up-regulation in response to cytokines. Confluent HUVEC in 24-well plates were pretreated with simvastatin for 18 h, following which the indicated cytokine was added to the medium for 4 h (*A–D*) or 6 h (*E–G*). The cells were then harvested and stained for E-selectin (*A–D*), ICAM-1 (*E* and *G*), and VCAM-1 (*F*). "Control" in this, and all, experiments denotes no cytokine, vehicle control. A total of 5000 cytometer-acquired events was analyzed per sample, and the histograms are representative of 15 separate experiments.

expression (data not shown). At the high tolerable range, simvastatin alone (in the absence of cytokine) promoted HUVEC ICAM-1 hyperinduction (data not shown), indicating common regulatory pathways of basal and cytokine-induced EC ICAM-1 expression, affected by HMG-CoA reductase inhibition.

### *Simvastatin effect on E-selectin steady-state mRNA levels*

Induction of endothelial CAMs by the aforementioned proinflammatory cytokines is known to occur through gene activation. To address whether the simvastatin-mediated increase in surface E-selectin expression is reflective of greater mRNA levels, Northern blot analyses were performed on RNA harvested from IL-1-activated HUVEC, pretreated for 18 h with simvastatin or carrier control. Fig. 2 demonstrates the expected increase in detectable E-selectin mRNA in IL-1-activated cells (*lane 3*), and an augmentation of the increased levels in the setting of simvastatin pretreatment (*lane 4*). This represents a 2.5-fold potentiation of mRNA expression, as determined densitometrically with GAPDH-normalized signals (Fig. 2). E-selectin mRNA was undetectable in HUVEC treated with simvastatin alone (*lane 2*).

### *Effect of simvastatin on IL-1-induced NF-κB translocation*

Because simvastatin does not potentiate all endothelial CAM-inducing stimuli, its effect on mRNA levels is more likely to occur at the level of cytokine-mediated signaling and consequent transcription than mRNA stabilization. When comparing the promoter regions of E-selectin, ICAM-1, and VCAM-1, the induced expression of all of which are affected by simvastatin, both distinct and common regulatory elements can be identified. NF-κB binding sites are the common *cis* elements found in all three genes. Therefore, EMSAs were performed with nuclear extracts obtained from IL-1-activated HUVEC, pretreated with simvastatin or carrier control. The NF-κB PDI domain of the E-selectin promoter was used

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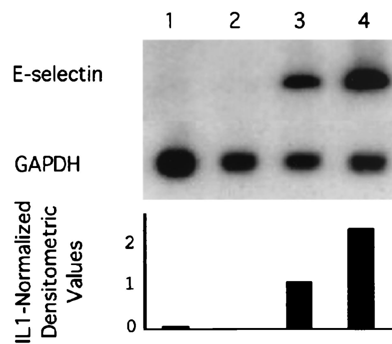


**FIGURE 2.** Northern blot analysis of the simvastatin effect on E-selectin mRNA levels. Confluent HUVEC were pretreated with simvastatin 300 ng/ml for 18 h, followed by IL-1$\beta$ 3 U/ml. After 90 min, total RNA was isolated and used for Northern blot analysis using E-selectin and GAPDH-specific probes, and normalization done as per *Materials and Methods*. The figure is representative of three separate experiments. *Lane 1*, Control cells; *lane 2*, simvastatin; *lane 3*, IL-1$\beta$; *lane 4*, simvastatin plus IL-1$\beta$.

as the target oligonucleotide. As expected, there is an easily detectable gel mobility shift, i.e., PDI binding, observed using IL-1-activated extracts (Fig. 3, *lane 3*). This IL-1-induced binding activity is greater in nuclear extracts from simvastatin-pretreated cells (*lane 4*), whereas simvastatin itself does not induce NF-$\kappa$B translocation (*lane 2*). This suggests that the potentiating effect of simvastatin occurs, at least in part, through amplifying NF-$\kappa$B translocation and consequent gene activation.

*Critical intermediaries in the cholesterol synthesis pathway*

In cholesterol synthesis from Acetyl CoA, in which HMG-CoA reductase is a proximal rate-limiting enzyme, there are several potentially critical metabolic intermediaries with multiple byproducts, some of which themselves have important cellular functions (Fig. 4) (17). Most notably, farnesyl pyrophosphate and geranylgeranyl pyrophosphate are isoprenoids that can contribute to important lipid modifications of a variety of proteins (18). To determine whether the critical modulatory event resulting in augmented CAM induction is reduction in cholesterol or the lipid

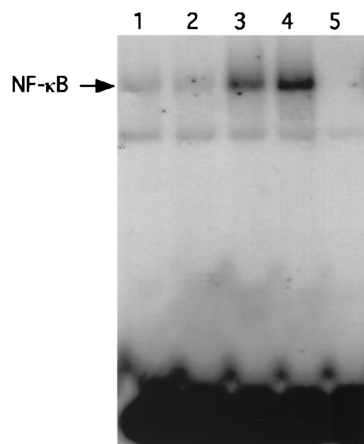


**FIGURE 3.** EMSA of induced nuclear NF-$\kappa$B in the presence of simvastatin. HUVEC were pretreated with simvastatin 300 ng/ml for 18 h, followed by IL-1$\beta$ 3 U/ml for 1 h. Nuclear protein extracts were incubated with poly(dI-dC) and a $^{32}$P-labeled E-selectin PDI domain NF-$\kappa$B oligonucleotide. The data are representative of three separate experiments. *Lane 1*, Control cells; *lane 2*, simvastatin; *lane 3*, IL-1$\beta$; *lane 4*, simvastatin plus IL-1$\beta$; *lane 5*, IL-1$\beta$ in the presence of 50-fold excess cold competitor.

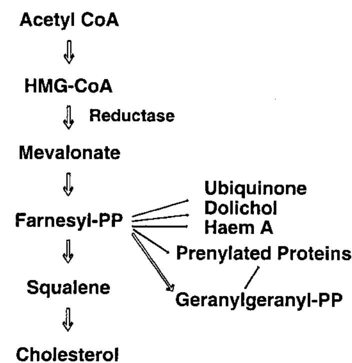


**FIGURE 4.** Cholesterol synthesis pathway. In addition to cholesterol, a number of other biologically important molecules are produced by this pathway. HMG-CoA reductase (reductase) is the target for simvastatin, which prevents the formation of mevalonate. Farnesyl pyrophosphate (farnesyl-PP) and geranylgeranyl pyrophosphate (geranylgeranyl-PP) are involved in protein prenylation.

intermediates, attempts were made to reverse simvastatin's effect by the addition of various downstream byproducts at the onset of simvastatin treatment, followed by cytokine activation and cell harvest for flow cytometric CAM analysis. Fig. 5*A* demonstrates that mevalonate (400 $\mu$M) completely reversed the ICAM-1 potentiating effect of simvastatin, indicating that HMG-CoA reductase inhibition is responsible for the noted affect. However, squalene, the immediate precursor to cholesterol, which has been used extensively in cellular studies to reverse cholesterol-mediated effects of statins (19), had no effect on the potentiation of IL-1-induced HUVEC ICAM-1 expression (Fig. 5*B*), demonstrating that cholesterol synthesis inhibition, per se, does not result in this response. In contrast, partial inhibition and complete reversal of the simvastatin potentiating effect were observed with farnesyl pyrophosphate (23 $\mu$M, Fig. 5*C*) and geranylgeranyl pyrophosphate (20 $\mu$m, Fig. 5*D*), respectively. This indicates that a prenylated protein, perhaps of an inhibitory class, is involved in CAM regulation, and its inhibition is required for the potentiating effect of simvastatin. Mevalonate, squalene, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate had no effect on ICAM-1 expression in control or IL-1$\beta$-treated (no simvastatin) cells (data not shown). Similar results were obtained for E-selectin (data not shown). This effect on protein prenylation explains the necessary extended period of pretreatment with simvastatin. That is, sufficient time is required for turnover of preexisting prenylated proteins and neosynthesis with inhibition of posttranslational modification.

*Role of an inhibitory $G_i$ protein in IL-1 signaling*

Prenylated proteins include small GTP-binding proteins (e.g., ras and rho) and the $\gamma$ subunit of heterotrimeric G proteins (18). To further investigate the role of heterotrimeric G proteins in cytokine-mediated CAM induction, the effects of cholera and pertussis toxin, which ADP-ribosylate and modulate $G_{s\alpha}$ and $G_{i\alpha}$, respectively, on IL-1$\beta$-mediated HUVEC ICAM-1 were evaluated. Fig. 5*E* demonstrates that pretreatment with cholera toxin (100 ng/ml, 18 h, dotted line) did not affect ICAM-1 expression. In contrast, pertussis toxin (100 ng/ml, 18 h, Fig. 5*E*, dashed line) potentiated ICAM-1 induction by IL-1$\beta$, at a level similar to that seen with simvastatin. This suggests that $G_{i\alpha}$ modulates cytokine responses in HUVEC. Cholera or pertussis toxin alone had no effect on ICAM-1 expression (data not shown). Similar results were obtained when evaluating E-selectin induction, and in response to TNF-$\alpha$ rather than IL-1$\beta$ (data not shown).

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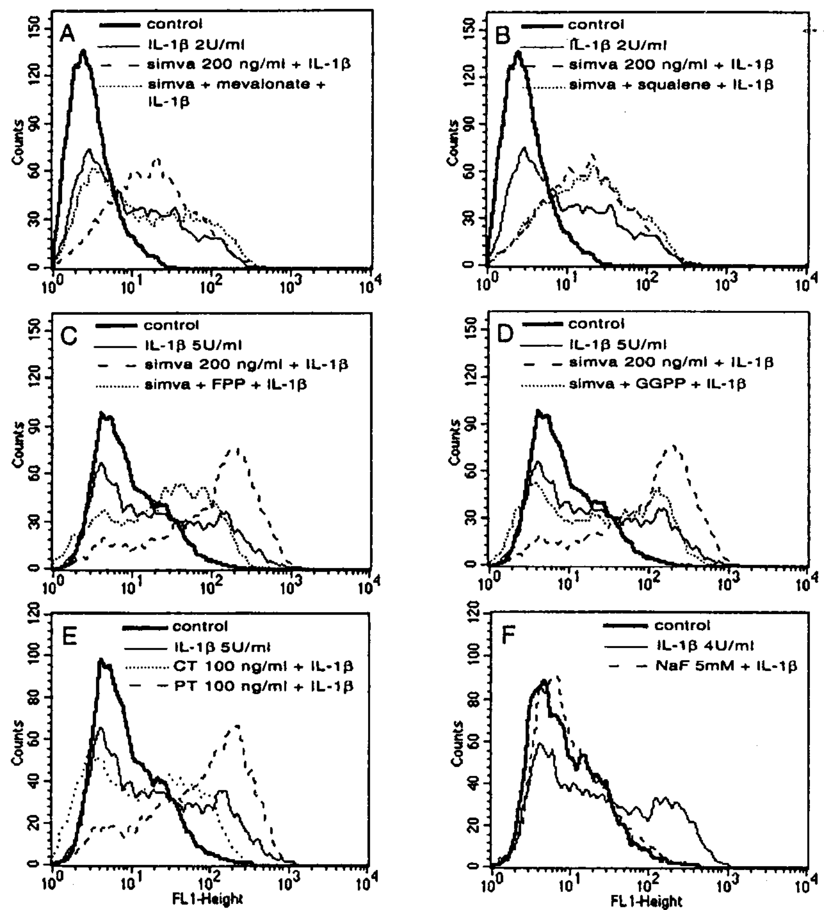


**FIGURE 5.** Flow cytometric analysis of the affect of various cholesterol synthesis pathway intermediates and G protein modulators. Confluent HUVEC in 24-well plates were treated with simvastatin for 18 h (*A–D*), followed by IL-1$\beta$ for 4 h. Cells were then harvested and immunostained for ICAM-1. Mevalonate 400 $\mu$M (*A*), squalene 100 $\mu$M (*B*), farnesyl pyrophosphate (FPP) 23 $\mu$M (*C*), or geranylgeranyl pyrophosphate (GGPP) 20 $\mu$M (*D*) was added to the medium at the same time as simvastatin. HUVEC were treated with cholera toxin (CT) 100 ng/ml, or pertussis toxin (PT) 100 ng/ml for 18 h (*E*), or NaF 5 mM for 2 h (*F*), followed by IL-1$\beta$ for 4 h, before they were immunostained for ICAM-1. A total of 5000 cytometer-acquired events was analyzed per sample, and the histograms are representative of three separate experiments.

To confirm that G proteins are involved in HUVEC cytokine responses, cells were pretreated with NaF (a potent activator of several G proteins) before IL-1 exposure. NaF pretreatment (5 mM, 2 h) completely abrogated IL-1-mediated HUVEC ICAM-1 induction (Fig. 5*F*), also observed for E-selectin analysis (data not shown). Although NaF effect on protein tyrosine phosphatase activity cannot be excluded, these results strongly support a modulatory effect of G protein signaling on cytokine responses in EC.

## Discussion

While studying the effects of HMG-CoA reductase inhibitors on endothelial function, it was unexpectedly determined that this pharmacologic class potentiates induction of the endothelial CAMs E-selectin, ICAM-1, and VCAM-1, in vitro, in response to IL-1$\beta$ and TNF-$\alpha$. The plasma levels obtained with a single dose of simvastatin (up to 125 ng/ml) (20) are comparable to lower concentrations used in this series of experiments. However, because statins are thought to impart antiinflammatory influences in vivo, and have been shown to reduce cardiovascular events in the setting of atherosclerotic heart disease, this in vitro effect is an apparent paradox. Leukocyte-endothelial interactions are the result of complex phenomena involving both the endothelium and leukocytes. Statins have been shown to reduce integrin expression on leukocytes (21), and augment endothelial NO mRNA levels and NO release (22). Both of these effects can be considered antiinflammatory, with regard to leukocyte adhesion to the endothelium and the resultant vascular pathology. It is likely that the net effect of these inhibitors in vivo is, in fact, vasoprotective. Nevertheless, the observed potentiation of CAM induction provides a potentially important tool with which to dissect previously unappreciated components of cytokine-mediated signaling in the endothelium.

The reversal of simvastatin's potentiation by mevalonate but not squalene demonstrates that HMG-CoA reductase, but not cholesterol synthesis, inhibition is responsible for this effect. More specifically, reversal achieved by the addition of the metabolic intermediates geranylgeranyl pyrophosphate and farnesyl pyrophosphate indicates that a prenylated (geranylgeranylated) protein is involved in endothelial responses to cytokines, and disruption of this posttranslational modification modulates the noted receptor-mediated signaling. Potentially relevant, candidate prenylated proteins include the $\gamma$ subunit of heterotrimeric G proteins and small GTP binding proteins. Members of the Rho family of small GTP binding proteins (Rho A, Rho B, Rho C, CDC 42, and Rac) and at least eight $\gamma$ subunits of heterotrimeric G proteins are known to be posttranslationally modified by geranylgeranylation (23).

To further dissect the level at which the simvastatin effect occurs, thereby attempting to pinpoint a role(s) for G proteins in cytokine-mediated endothelial activation, general features of CAM induction were considered. Cytokine (IL-1 and TNF-$\alpha$) receptor engagement triggers CAM gene activation and new mRNA synthesis. We have determined by flow cytometry that the EC surface expression of neither the p55 nor p75 TNF receptors is affected by simvastatin pretreatment (data not shown), demonstrating that the statin effect is not simply the consequence of enhanced membrane receptor levels. The simvastatin-mediated augmentation in steady-state E-selectin mRNA levels induced by IL-1 could be a consequence of enhanced transcription or RNA stabilization. Indeed, HMG-CoA reductase inhibition has been demonstrated to prolong

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the half-life of endothelial NO synthase mRNA, in a Rho inhibition-dependent fashion (24). Although we cannot exclude a CAM RNA stabilization mechanism, the increase in NF-κB translocation, as demonstrated by augmented E-selectin PDI-binding activity within nuclear extracts of simvastatin-pretreated cells, strongly supports that gene activation is enhanced. An effect on NF-κB activation is consistent with the simvastatin-mediated potentiation of all three CAMs evaluated, E-selectin, ICAM-1, and VCAM-1. Although each of the three genes has a specific set of 5′ regulatory elements and induced transcription factors, NF-κB is an important regulator of all three genes (2). Recently, the signaling events leading to phosphorylation and degradation of the cytosolic anchor IκB, which are key triggers of NF-κB translocation, have been better defined. A "signalsome" protein complex must be activated for NF-κB translocation to occur. This signalsome includes the kinases IκB kinase-α and -β (IKK-α and IKK-β), NF-κB-inducing kinase (NIK), the NF-κB essential modulator IKK-γ, and the IKK complex-associated protein, as well as the IκBs, which must dissociate for proteasomal degradation (25–34). IKK-α, IKK-β, and NIK are differentially regulated by distinct inflammatory mediators, e.g., TNF and LPS, and, in turn, the IκB proteins α, β, and ε are consequently differentially phosphorylated and targeted to proteasomes (35). That is, all NF-κB-activating stimuli do not follow the same signal transduction cascade. This allows for simvastatin to affect IL-1 and TNF-mediated signal transduction, but not that of LPS or phorbol esters. Furthermore, the TNF-α (via TRAF2) and IL-1 (via TRAF6 and IL-1 receptor-associated kinase) proximal signaling pathways merge at NIK activation (30, 36). Therefore, the specificity of the statin-mediated potentiation likely lies within the complexity of these signaling cascades.

Although cholera toxin had no effect on cytokine-induced endothelial CAM expression, pertussis toxin mimicked the simvastatin effect, indicating that a $G_{i\alpha}$ protein is involved in this phenomenon, likely in an inhibitory fashion (37). This is consistent with the effect of the direct G protein activator NaF, which inhibited IL-1-mediated CAM induction, although a tyrosine phosphatase inhibitory effect of NaF cannot be excluded. A role for heterotrimeric G proteins in the modulation of endothelial responses to the cytokines IL-1 and TNF has not been previously described. The human IL-1RI cytoplasmic domain does contain a motif at positions 428–431, which predicts G protein coupling (38). Pertussis toxin has been reported to affect a range of IL-1 responses in various cell (nonendothelial) types, including IL-2, PG, and macrophage CSF production, and IL-2 receptor induction (39). Pertussis toxin inhibits all these IL-1 responses. Similar to our findings, there are established examples of cross-talk between IL-1 signaling and inhibitory responses, such that modulating these pathways could either disinhibit or prevent inhibition. Low dose IL-1 inhibits KCl-induced increases in cortical synaptosome intracellular calcium (40). This inhibition is abrogated by pertussis toxin. Also, although not identified as heterotrimeric G protein-dependent, inhibition of protein geranylgeranylation, either by a specific inhibitor or lovastatin, promotes superinduction of IL-1-induced NO synthase-2 expression (41). Thus, IL-1 responses can be either positive or negative, and associated signaling molecules/pathways can either positively or negatively affect IL-1 responses, depending on the cell type and function evaluated.

It is possible that, although not known as such, there could exist a G protein-coupled IL-1 receptor. That is, IL-1 could initiate two parallel responses, one traditionally stimulatory, as described above, and a second that is G protein-coupled and inhibitory, which, if inhibited, allows the stimulatory pathway to be unopposed. Alternatively, there could be a non-IL-1-responsive, distinct inhibitory $G_{i\alpha}$-coupled receptor that could modulate IL-1 responses and, as such, confer a basal "inhibitory tone" for endothelial CAM expression in vitro. This inhibitory pathway could be sequentially coupled to a downstream kinase, such as NIK, which is crucial for both IL-1 and TNF-α-mediated NF-κB translocation and consequent gene activation.

The toxin and NaF experiments indicate the involvement of a heterotrimeric G protein. However, it remains possible that a small GTP binding protein also plays a role in negatively modulating endothelial responses to cytokines. CDC42 and Rac are involved in TNF-α-induced E-selectin transcription, although in a positive regulatory fashion (4). Experiments are currently underway to determine whether, in addition to $G_{i\alpha}$, small GTP-binding proteins can contribute to the inhibitory tone described above.

Although this HMG-CoA reductase-mediated potentiation of cytokine signaling was unexpected, it has uncovered a potentially novel component of such responses, and will allow a deeper molecular understanding of control on endothelial cell adhesion molecule expression, both stimulatory and inhibitory.

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