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Atorvastatin inhibition of cytokine-inducible nitric oxide synthase expression in native endothelial cells *in situ*

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1 Animal experimental studies have demonstrated that inducible nitric oxide synthase (iNOS) expression correlates with neointima formation and is prevented by HMG-CoA reductase inhibitors (statins). In the present study we have investigated the underlying mechanism of action of these drugs in isolated segments of the rat aorta.

2 Western blot analysis and immunohistochemistry revealed that tumour necrosis factor α (TNFα) plus interferon-γ (IFNγ) synergistically induce iNOS gene expression in the endothelium but not in the smooth muscle of these segments while constitutive endothelial NO synthase (eNOS) abundance was markedly reduced.

3 Pre-treatment with 1–10 μM atorvastatin, cerivastatin or pravastatin decreased TNFα plus IFNγ stimulated iNOS expression in the endothelium irrespective of the presence of the HMG-CoA reductase product mevalonate (400 μM).

4 Electrophoretic mobility shift assay experiments confirmed that the combination of TNFα plus IFNγ causes activation of the transcription factors STAT-1 and NF-κB in native endothelial cells. Neutralization of these transcription factors by employing the corresponding decoy oligonucleotides confirmed their involvement in TNFα plus IFNγ stimulated iNOS expression. Translocation of both transcription factors was attenuated by atorvastatin, and this effect was insensitive to exogenous mevalonate.

5 The present findings thus demonstrate a specific HMG-CoA reductase-independent inhibitory effect of statins, namely atorvastatin, on cytokine-stimulated transcription factor activation in native endothelial cells *in situ* and the subsequent expression of a gene product implicated in vascular inflammation. This effect may be therapeutically relevant and in addition provide an explanation for the reported rapid onset of action of these drugs in humans.
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Abbreviations: AP-1, activator protein-1; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility shift analysis; IFNγ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible isoform of NO synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; SMC, smooth muscle cells; STAT-1, signal transducer and activator of transcription-1; TNFα, tumour necrosis factor α

Introduction

Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, collectively referred to as statins, exert therapeutic effects beyond that of simply lowering plasma cholesterol (for review see Laufs & Liao, 2000a). HMG-CoA reductase is responsible for the conversion of HMG-CoA to mevalonate and products of mevalonate metabolism are critical for many cellular processes in eukaryotic cells. In particular, small GTP-binding proteins (G-proteins) which require post-translational isoprenylation or farnesylation for full functional activity may play an important role in mediating the effects of statins in the cardiovascular system (Laufs & Liao, 2000b).

Thus, statins inhibit pro-inflammatory cytokine formation in leukocytes and adhesion molecule expression in endothelial cells (for review see Koh, 2000). Moreover, statins have been shown to down-regulate inducible NO synthase (iNOS) expression in the endothelium and media of small vessels in the kidney of transgenic rats expressing human renin and angiotensinogen (Park *et al*., 2000), and *via* the same route prevent neointima formation in hyperlipidemic rabbits (Alfon *et al*., 1999).

Sustained production of large amounts of nitric oxide (NO) is induced in blood vessels by inflammatory stimuli as a result of iNOS expression. Induction of iNOS expression is frequently observed in systemic inflammatory reactions such as septic shock, but also locally due to denudation of the endothelium or during atherosclerosis (Chen *et al*., 2000). Injury-related expression of this enzyme in vascular smooth muscle cells may play a role in the development of atherosclerosis or postangioplasty restenosis by inhibiting smooth muscle cell proliferation and contraction, as well as by preventing leukocyte and platelet adhesion (Ikeda *et al*., 1998).

The objective of the present study was to investigate the mechanism of action by which the HMG-CoA reductase inhibitors, namely atorvastatin, affect cytokine-induced iNOS

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expression in native endothelial and smooth muscle cells *in situ*.

Methods

Cell culture

Rat aortic smooth muscle cells (SMC) were isolated and cultured as described (Krzesz *et al*., 1999). All incubations were performed in Waymouth medium containing 10% foetal bovine serum (FBS; Life Technologies, Karlsruhe, Germany) with SMC grown to at least 80% confluence. Incubations were terminated by washing the cells with Hank's Balanced Salt Solution (HBSS) followed by protein extraction (Krzesz *et al*., 1999).

Preparation and incubation of ring segments of rat thoracic aorta

Aortas (1.5 mm i.d.) were isolated from pentobarbitone-anaesthetized male Wistar rats (200–250 g body weight) as described previously (Wagner *et al*., 2000) and cleaned under sterile conditions of adherent fat and connective tissue. Segments of 5–7 mm in length were placed in 1 ml Waymouth medium containing 10% FBS and pre-incubated for 1 h in the absence (solvent control, 0.05% dimethylsulphoxide (DMSO), v v^{-1}) or presence of the test compounds followed by addition of the cytokines. All statins were dissolved in DMSO (0.05% (v v^{-1}) final concentration) to overcome their different membrane-permeating properties. After 20 h in an incubator (37°C, gassed with 5% CO_2 (v v^{-1}) the medium was removed for subsequent nitrite determination (Hecker *et al*., 1996) and the wet weight of the segments was measured. In some experiments, incubations were terminated after 30–60 min to prepare nuclear extracts (see below). If required, the lumen of the segments was carefully denuded with the aid of a roughened steel cannula. To verify that iNOS expression originated from the endothelium and was not due to adherent or infiltrated leukocytes, the mRNA level of CD62L, a specific marker for these cells, was monitored by RT–PCR analysis both in endothelium-intact and denuded segments as described (Wagner *et al*., 2000).

Western blot analysis

Homogenates of the isolated segments were prepared and the resulting protein extracts analysed as described previously (Wagner *et al*., 2000). For detection of iNOS, the immobilized proteins were consecutively exposed to a polyclonal rabbit anti-iNOS antibody (1 : 3000 dilution, BD Transduction Laboratories, Heidelberg, Germany), an anti-rabbit immunoglobulin (IgG) horseradish peroxidase (HRP) conjugate (1 : 3000 dilution; Sigma-Aldrich, Deisenhofen, Germany), the SuperSignal Blaze™ chemiluminescent reagent (Pierce Chemical, Rockford, IL, U.S.A) and finally an autoradiography film (Hyperfilm™ MP, Amersham Pharmacia Biotech, Freiburg, Germany). Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with a monoclonal anti-β-actin antibody from mouse ascites fluid and an anti-mouse IgG-HRP conjugate (both antibodies obtained from Sigma-Aldrich, 1 : 3000 dilution) followed by densitometry (Wagner *et al*., 2000).

Immunohistochemistry

Following exposure to the cytokines or vehicle for 20 h the rat aortic segments were embedded in tissue-tek® O.C.T™ compound (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Serial sections of 10 μm were made by using a cryostat (Leica Microsystems, Nussloch, Germany). Sections were fixed (acetone/methanol 1 : 1 (v v^{-1})) followed by blocking of endogenous peroxidase with 0.3% (v v^{-1}) hydrogen peroxide in phosphate buffered saline (PBS). After incubation in PBS containing 3% normal goat serum (DAKO, Hamburg, Germany) and 0.2% Triton X-100 (Sigma-Aldrich), sections were consecutively incubated with a rabbit anti-iNOS polyclonal antibody (1 : 5000 in PBS containing 3% normal goat serum and 0.2% Triton X-100; Chemicon International, Hofheim, Germany) and a mouse anti-CD31-IgG (1 : 100 dilution; Serotec, U.K., distributed by Biozol, Eching, Germany) or anti-eNOS antibody (1 : 2500 dilution, BD Transduction Laboratories). After several washes in PBS, sections were incubated with the corresponding secondary goat anti-rabbit or goat anti-mouse IgG-HRP-labelled dextran polymer (DAKO EnVision + ™). After washing with PBS, sections were incubated with a substrate-chromogen system (DAKO®AEC), and finally mounted with Aquamount (BDH Laboratory Supplies, Poole, U.K.). Positive immunoreactivity (brown coloured precipitate) was monitored with a SPOT RT colour CCD camera (Diagnostic Instruments, Burroughs St. Sterling Heights, MI, U.S.A.) coupled to an Axiovert S100 TV microscope (Zeiss, Goettingen, Germany) at 200× magnification.

Electrophoretic mobility shift analysis (EMSA)

Preparation of nuclear extracts from endothelium-intact segments, subsequent non-denaturing polyacrylamide gel electrophoresis, autoradiography, and supershift analyses with the corresponding supershift antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) was performed as described previously (Lauth *et al*., 2000). The double-stranded gel shift oligonucleotides (ODN) for Sis-inducible element (SIE, 5′-GTGCATTTCCCGTAAATCTTGTCTACA-3′), NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′), activator protein-1 (AP-1, 5′-CGCTTGATGACTCAGCCGGAA-3′) and CCAAT/enhancer binding protein (C/EBP, 5′-TGCAGATTGCGCAATCTGCA-3′) were obtained from Santa Cruz Biotechnology and end-labelled with [γ-^{32}P]-ATP by using the 5′-end labelling kit from Amersham Pharmacia Biotech.

Decoy oligonucleotide (ODN) technique

Double-stranded decoy ODN were prepared from the complementary single-stranded phosphorothioate-bonded ODN obtained from Eurogentec (Köln, Germany) as described previously (Krzesz *et al*., 1999). The single-stranded forward sequences of the decoy ODN were as follows (underlined letters denote phosphorothioate-bonded bases,

mutated bases are indicated in bold): STAT-1cons, ATGTGAGTTCCCGGAAGTGAACT; NF-κBcons, AGTTGAGGGGACTTTCCCAGGC; NF-κBmut, AGTTGAGGTGAGTTTCACAGGC. On the basis of previous EMSA and RT–PCR analyses the maximal effective concentration and optimal pre-incubation time for all decoy ODN with the vessel segments were determined to be 10 μM and 4 h (Lauth *et al*., 2000). Transfer of the decoy ODN was achieved without using any cationic lipid or liposomal complex.

Data analysis

Unless indicated otherwise, results are expressed as means ±s.e.mean of n observations with aortic segments from different animals. One-way analysis of variance followed by a Dunnett's multiple comparisons test was used to determine differences between the means and the corresponding control value with $P<0.05$ considered statistically significant.

Results

Cytokine-induction of endothelial iNOS expression

Exposure of endothelium-intact segments to IL-1β (60 u ml^{-1}) but not TNFα (1000 u ml^{-1}) or IFNγ (200 u ml^{-1}) alone resulted in a significant induction of iNOS expression. Both combination of TNFα with IFNγ and in particular that of TNFα with IL-1β resulted in a supra-additive increase in iNOS expression (Figure 1a). Interestingly, iNOS expression in response to TNFα plus IFNβ stimulation appeared to be localized exclusively to the endothelium, as the 135 kDa iNOS protein band in the Western blot completely disappeared when the endothelium was removed before preparation of the protein extracts (Figure 1b, left panel). In contrast, exposure to TNFα plus IL-1β appeared to induce iNOS protein expression primarily in the vascular smooth muscle, for this was still evident in the denuded aorta (Figure 1b, right panel). In support of this assumption, Western blots analyses of rat aortic cultured SMC revealed a prominent iNOS band after 24 h exposure to TNFα plus IL-1β but not TNFα plus IFNγ (Figure 1c). Because it is technically impracticable to harvest sufficient endothelial cells from the isolated aortic segments for Western blot analysis, immunohistochemistry finally confirmed a marked induction of iNOS protein in the endothelium of segments incubated with TNFα plus IFNγ for 20 h while eNOS expression was concomitantly suppressed under these conditions (Figure 2).

Effects of atorvastatin on iNOS expression and activity

Exposure of the endothelium-intact segments to atorvastatin (10 μM) for up to 1 h before cytokine stimulation affected the subsequent iNOS protein expression only when this was induced by TNFα plus IFNγ but not TNFα plus IL-1β or IL-1β alone, pointing towards a selective inhibition of endothelial iNOS expresssion (Figure 3a). This effect of atorvastatin was concentration-dependent with a near maximum inhibition of TNFα plus IFNγ stimulated iNOS expression at a concentration of 1 μM (Figure 3b). In addition, atorvastatin (10 μM) pretreatment significantly

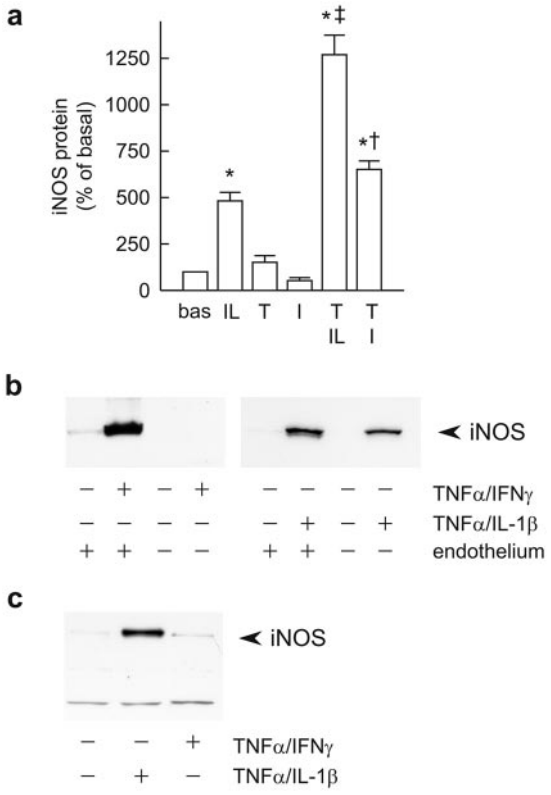

Figure 1 Detection of endothelial iNOS protein expression in the isolated rat aortic segments. (a) Statistical summary of iNOS protein expression in endothelium-intact segments incubated with TNFα (1000 u ml^{-1}), IFNγ (200 u ml^{-1}), IL-1β (60 u ml^{-1}), TNFα (1000 u ml^{-1}) plus IL-1β (60 u ml^{-1}) or TNFα (1000 u ml^{-1}) plus INFγ (200 u ml^{-1}), calculated as percentage of basal iNOS expression. ($n=3$, $*P<0.05$ vs basal, $\dagger P<0.05$ vs TNFα or IFNγ, $\ddagger P<0.05$ vs TNFα or IL-1β). (b) Western blot analysis of iNOS protein expression in endothelium-intact or denuded rat aortic segments incubated with IFNγ (200 u ml^{-1}) plus TNFα (1000 u ml^{-1}) or IL-1β (60 u ml^{-1}) plus TNFα (1000 u ml^{-1}). Results are representative of five and two separate experiments, respectively. (c) Representative Western blot analysis demonstrating iNOS protein expression in cultured rat aortic smooth muscle cells incubated with IFNγ (200 u ml^{-1}) plus TNFα (1000 u ml^{-1}) or IL-1β (60 u ml^{-1}) plus TNFα (1000 u ml^{-1}). Qualitatively identical results were obtained with at least one further batch of cells.

inhibited iNOS activity (estimated on the basis of the nitrite concentration in the conditioned medium after 20 h incubation; Figure 3c).

Moreover, the inhibition of TNFα plus IFNγ stimulated endothelial iNOS expression was not restricted to atorvastatin, but mimicked by both cerivastatin (1 μM) and pravastatin (10 μM) at comparable concentrations (Figure 3d). Interestingly, the inhibitory effect of atorvastatin on endothelial iNOS expression was not altered in the presence of the HMG-CoA reductase product, mevalonate, at a concentration of 400 μM (Figure 4).

Effects of atorvastatin on transcription factor activation

Next the transcription factors putatively involved in TNFα plus IFNγ stimulated iNOS expression in the native endothelial cells were investigated by employing the decoy ODN technique. TNFα plus IFNγ stimulated iNOS expres-

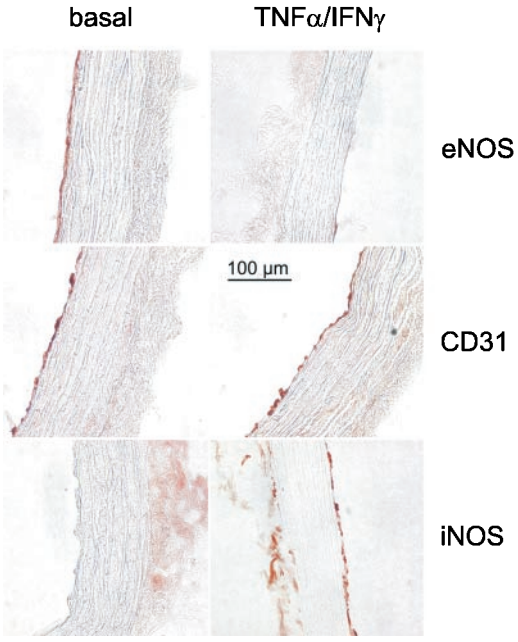

Figure 2 Immunohistochemical localization of iNOS and eNOS protein in the endothelium of segments incubated with IFNγ (200 u ml^{-1}) plus TNFα (1000 u ml^{-1}) for 20 h. Co-staining for the endothelial cell marker CD31 (PECAM-1) shows that iNOS and eNOS immunoreactivity is confined to the endothelium. Qualitatively identical results were obtained with at least one further set of segments.

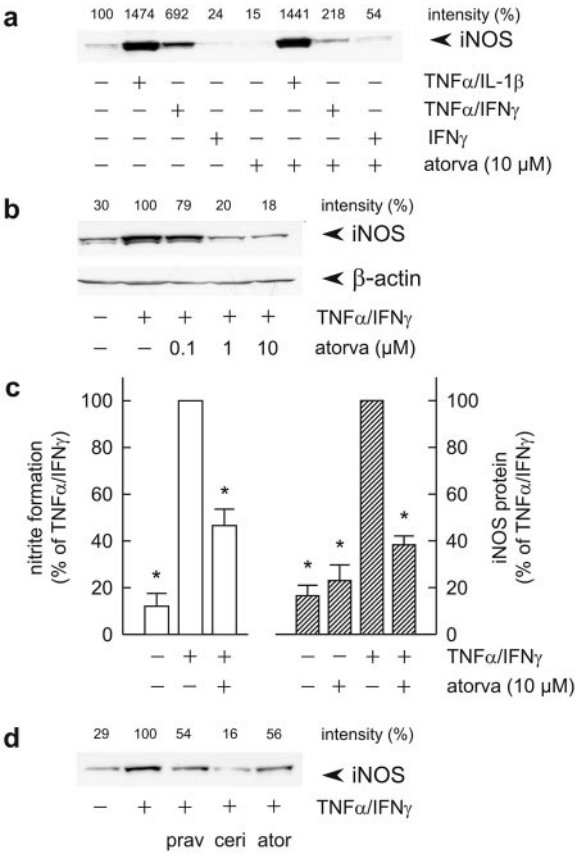

Figure 3 Inhibition by statins of iNOS expression in endothelium-intact segments. (a) Representative Western blot analysis (with the relative intensities (%), as judged by densitometry, indicated at the top) demonstrating that the inhibitory effect of atorvastatin is cytokine-dependent. Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with an anti-β-actin antibody. Segments were pre-incubated with atorvastatin (10 μM) for 1 h followed by an incubation with IFNγ (200 u ml^{-1}), IL-1β (60 u ml^{-1}) or TNFα (1000 u ml^{-1}) alone or in combination for 20 h. Results are representative of two separate experiments. (b) Concentration-dependent inhibition by atorvastatin (1 h pre-incubation) on TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}) stimulated iNOS protein expression for 20 h. The Western blot analysis is representative of two separate experiments. (c) Statistical summary of iNOS protein expression (dashed columns) and activity (open columns) in segments pre-incubated with atorvastatin (10 μM) for 1 h followed by 20 h incubation with TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}). Enzyme activity was estimated on the basis of the nitrite concentration in the conditioned medium. Results are calculated as percentage of cytokine-induced iNOS expression ($n=8$) or nitrite formation ($n=5$) (*$P<0.05$ vs TNFα/IFNγ). (d) Effects of 10 μM atorvastatin (ator), 1 μM cerivastatin (ceri) or 10 μM pravastatin (prav) on iNOS protein expression after 1 h pre-incubation and 20 h incubation with TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}). Typical Western blot analysis, results are representative of two separate experiments.

sion was markedly inhibited by consensus decoy ODN directed against the TNFα-dependent transcription factor NF-κB and the IFNγ-dependent transcription factor STAT-1 (Figure 5a) while neither the NF-κB mutant control ODN nor a decoy ODN directed against IRF-1, another IFNγ-sensitive transcription factor (not shown), revealed such an effect.

Subsequent electrophoretic mobility shift assay analysis with nuclear extracts from endothelium-intact and denuded segments revealed that transcription factor activation detected by this technique is confined to the endothelium (exemplary shown for STAT-1 in Figure 5b). Moreover, EMSA confirmed that TNFα plus IFNγ stimulation leads to an activation of NF-κB and STAT-1 in the native endothelial cells (*cf.* Figure 6). In the presence of atorvastatin (10 μM, 1 h pre-incubation), TNFα plus IFNγ activation of these transcription factors, namely that of STAT-1, was clearly attenuated (Figure 6). Again this inhibitory effect of atorvastatin on cytokine-mediated transcription factor activation was not reversed by exogenous mevalonate (Figure 6).

Discussion

The present findings demonstrate that iNOS expression in the endothelium of isolated rat aortic segments is selectively induced by the cytokine combination TNFα plus IFNγ through activation of the transcription factors NF-κB and STAT-1, presumably inducing *de novo* transcription of the iNOS gene in a synergistic manner. They further show that different HMG-CoA reductase inhibitors, namely atorvastatin, specifically attenuate this cytokine-induced endothelial iNOS expression by interfering with the activation of both NF-κB and STAT-1. Finally the inhibitory effect of atorvastatin on both transcription factor activation and gene expression appears to be independent of a blockade of HMG-CoA reductase.

The primary cell type responsible for iNOS gene expression in the vessel wall during a pro-inflammatory response continues to be a matter of debate (for review see Muller *et al.*, 2000). While some studies have identified the adventitia as

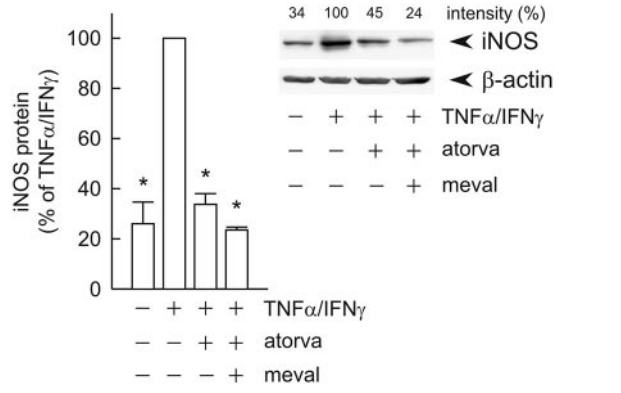

Figure 4 Effect of 1 h pre-incubation of 10 μM atorvastatin (atorva) alone or in combination with 400 μM mevalonate (meval) on TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}) stimulated iNOS protein expression (20 h incubation in the presence of a solvent control) in endothelium-intact segments ($n=4$; $^*P<0.05$ vs TNFα/IFNγ). The inset shows a representative Western blot analysis with the relative intensities, as judged by densitometry, indicated at the top.

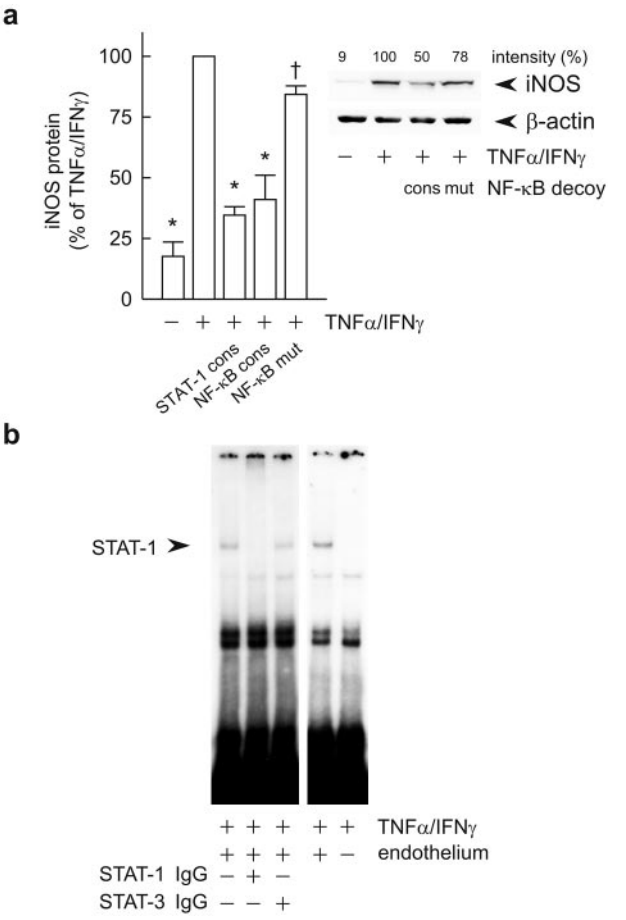

Figure 5 (a) Statistical summary of the effects of a NF-κB or STAT-1 consensus (cons) decoy ODN as compared to that of a NF-κB mutant (mut) control ODN (4 h pre-incubation, 10 μM each) on stimulated iNOS protein expression in endothelium-intact segments after 20 h incubation with TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}) ($n=3-7$; $^*P<0.05$ vs TNFα/IFNγ, $\dagger P<0.05$ vs TNFα/IFNγ + NF-κB cons). The inset shows a representative Western blot analysis with the relative intensities, as judged by densitometry, indicated at the top. (b) Identification of a STAT-1-DNA complex (indicated by the arrow) in nuclear extracts of rat aortic segments with or without endothelium incubated for 30 min with TNFα (1000 u ml^{-1}) plus IFNγ (200 u ml^{-1}) The figure also depicts a supershift analysis, revealing that only STAT-1 but not STAT-3 is bound to the SIE gelshift oligonucleotide employed.

the main site of iNOS expression, e.g. in response to lipopolysaccharide (Kleschyov *et al*., 2000), others have demonstrated iNOS expression in rat aortic cultured endothelial cells upon exposure to IL-1β (Kanno *et al*., 1994). The present data confirm expression of this enzyme in the luminal endothelial cells of this blood vessel, but only in response to IFNγ plus TNFα treatment. Cytokine alone did not significantly affect iNOS expression. Such a synergistic effect of the two cytokines on iNOS gene expression has been described previously for human alveolar epithelial cells (Kwon & George, 1999) and hepatocytes (Taylor *et al*., 1998), but not for rat aortic cultured smooth muscle cells (Wileman *et al*., 1995; this study).

It is well known that exposure of vascular cells to TNFα enhances the activity of NF-κB while IFNγ increases that of STAT-1 and subsequently IRF-1 (for review see Mantovani *et al*., 1997). While the IRF-1 specific decoy ODN had no effect on TNFα plus IFNγ stimulated iNOS expression in the native endothelial cells, this was markedly reduced by decoy ODN directed against NF-κB and STAT-1. Moreover, EMSA revealed that both transcription factors translocated to the nucleus of the native endothelial cells upon cytokine exposure. It would appear, therefore, that NF-κB and STAT-1 induce transcription of the rat iNOS gene in a synergistic manner, as recently proposed for the human enzyme (Ganster *et al*., 2001). MatInspector analysis (Quandt *et al*., 1995) of the first 1713 bp of the promoter of the rat iNOS gene (GenBank accession no. X95629; Eberhardt *et al*., 1996) indeed reveals binding sites for NF-κB at positions -964, -900 and -106 and for STAT-1 at positions -1293, -934 and -872. Thus, the two overlapping NF-κB and STAT-1 motifs located at position -964 to -872 may provide the molecular basis for the synergistic effect of TNFα and IFNγ on iNOS expression in the endothelium of the rat aorta.

Statins have been shown to enhance IL-1β-induced iNOS expression in rat aortic cultured SMC (Muniyappa *et al*., 2000) and cardiac myocytes (Ikeda *et al*., 2001). In contrast, iNOS expression in the endothelium of hypercholesterolaemic rabbits (Alfon *et al*., 1999) and transgenic rats (Park *et al*., 2000) is inhibited by HMG-CoA reductase inhibitors. The present data confirm that statins, namely atorvastatin, inhibit cytokine-induced iNOS expression in the endothelium but not in the smooth muscle of the rat aorta. In addition to this cell-specific inhibitory effect of the statin there also appears to be a cytokine, i.e. stimulus-specificity. Expression of the enzyme in the endothelium, as mentioned before, required the exposure to both TNFα and IFNγ, but not, e.g. IL-1β. Moreover, EMSA revealed that atorvastatin pre-treatment attenuates the nuclear translocation of both NF-κB and in particular STAT-1 in the native endothelial cells, hence providing a molecular basis for the inhibitory effect of the statin on iNOS gene expression. The atorvastatin-insensitive IL-1β-induction of iNOS expression in the smooth muscle, on the other hand, suggests that this must involve the activation of other transcription factors or a combination thereof (Ortego *et al*., 1999).

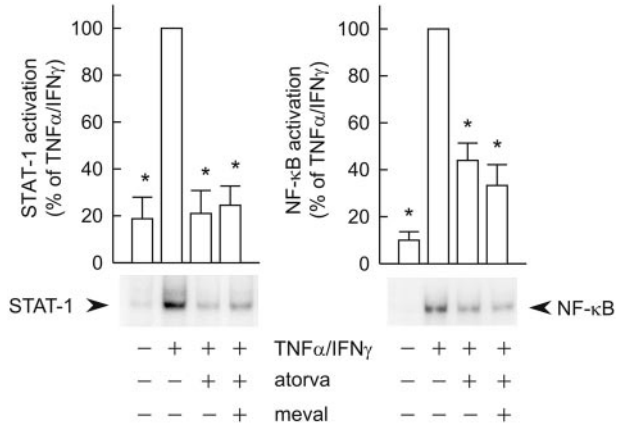

Figure 6 Effect of 1 h pre-incubation with 10 μM atorvastatin (atorva) on the nuclear translocation of STAT-1 and NF-κB and its sensitivity to exogenous mevalonate (meval, final concentration of 400 μM) in endothelium-intact rat aortic segments incubated for 0.5 h with IFNγ (200 u ml^{-1}) plus TNFα (1000 u ml^{-1}). The figure depicts the statistical summary ($n=3-4$, $^*P<0.05$ vs TNFα/IFNγ) and a typical EMSA at the bottom for each transcription factor, respectively.

Perhaps the most striking finding of the present study was that the inhibitory effect of atorvastatin on both cytokine-induced transcription factor activation and the subsequent increase in gene expression was not reversed by exogenous mevalonate, suggesting that it is brought about independently of the concomitant HMG-CoA reductase blockade. This notion is supported by the fact that the onset of the inhibitory effect of atorvastatin was rather rapid, unlike its mevalonate-reversible hence HMG-CoA reductase and small G-protein dependent effects on eNOS expression or phorbol ester-stimulated O_2^- formation in the same vascular preparation (Wagner *et al*., 2000). What could be the mechanism underlying this HMG-CoA reductase-independent effect of the statins on gene expression? There is a recent report (Weitz-Schmidt *et al*., 2001) suggesting that statins selectively block leukocyte function antigen-1 (LFA-1), a β2 integrin, *via* binding to a novel allosteric site within LFA-1. This effect was unrelated to the inhibition of HMG-CoA reductase and suppressed the inflammatory response to thioglycollate in a murine model of peritonitis. Perhaps the effect of the statins on cytokine-induced iNOS gene expression is likewise mediated by their binding to another (presumably different) integrin-like receptor on the surface of the rat aortic endothelial cells. In this context, it may be of interest that an integrin-dependent modulation of gene expression through LFA-1 has been described, leading to an increased expression of a reporter gene construct in Jurkat T cells (Bianchi *et al*., 2000).

The role of iNOS in mediating different aspects of cardiovascular pathophysiology in the vessel wall is still controversial (for review see Kibbe *et al*., 1999). For example, it is not clear whether long term overproduction of NO plays a beneficial or detrimental role (Dusting *et al*., 1998). In this regard, it is noteworthy that pro-inflammatory cytokines such as TNFα and IFNγ down-regulate rather than up-regulate eNOS gene expression (Zhang *et al*., 1997; this study) so that the parallel induction of iNOS gene expression in the endothelium (this study) may represent a compensatory mechanism (Binion *et al*., 2000). On the other hand, the development of atherosclerosis appears to be associated with increased expression of iNOS hence exaggerated synthesis of NO both in humans (Dusting *et al*., 1998) and in experimental animals (Detmers *et al*., 2000; Niu *et al*., 2001; Alfon *et al*., 1999). It thus remains to be determined whether cytokine-induced iNOS expression in the vascular endothelium is beneficial or detrimental, and this will ultimately define whether the inhibitory effect of the HMG-CoA reductase inhibitors in this context constitutes a therapeutic or an adverse side effect. Nonetheless, the present findings demonstrate that in addition to the HMG-CoA reductase-dependent effects of statins on plasma cholesterol or the activity of small G-proteins, they also exert a HMG-CoA reductase-independent effect on the transcription of potentially harmful pro-inflammatory genes.

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