Statin-induced apoptosis of vascular endothelial cells is blocked by dexamethasone

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Abstract

Statins block *de novo* synthesis of cholesterol by inhibiting the enzyme, HMG CoA reductase. The product of this reaction, mevalonic acid, is also a precursor of isoprenoids, molecules required for the activation of signalling G-proteins, such as Ras. Signal transduction pathways involving Ras are important for cell survival and this may be why statins induce apoptotic death of several cell types. Given that statins are used to treat vascular disease, it is surprising that no studies have been conducted on vascular endothelial cells. For this reason, we have tested the effect of fluvastatin (FS) on the endothelial cell line EA.hy 926. Here we show that FS, at concentrations from 1 to 2 μ M, blocks growth and induces apoptosis of the endothelial cell line, EA.hy 926. As considerable redundancy exists in cell signalling pathways for cell survival, toxicity of FS under more physiological conditions might be prevented by pathways that do not require Ras, such as those activated by adrenal or sex steroids. To test this hypothesis, first RT-PCR analysis was performed for nuclear receptor mRNA expression. This revealed the presence of mRNA

for the androgen receptor (AR) and glucocorticoid receptor (GR). The effect of the AR agonist, dihydrotestosterone (DHT), and the GR agonist, dexamethasone (Dex), was then tested. Whilst DHT (100 nM) had no effect on FS-induced cell death, Dex (1 μ M) blocked FS-induced apoptosis. Cell cycle analysis revealed that 24 h exposure to FS prevented cells from leaving G₁ and 24–48 h later a marked sub-G₁ peak was observed. Dex was able to reduce the sub-G₁ peak, but it failed to reduce accumulation of cells in G₁. Further studies revealed that, in addition to blocking FS-induced apoptosis, Dex was able to block apoptosis of EA.hy 926 cells induced by serum deprivation, tumour necrosis factor- α , oxidants, DNA damage and mitochondrial disruption. This study strongly suggests that glucocorticoids have a role to play in preventing vascular injury and they may provide a reason why statins are apparently not toxic to vascular endothelial cells *in vivo*.

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Introduction

Statins are a class of compound that inhibit HMG-CoA reductase and result in the inhibition of cholesterol biosynthesis (Grundy 1998). Due to this property, statins are now one of the most widely used agents for the treatment of high cholesterol-related vascular disease (Gould *et al.* 1998). Several reports over the last 5 years have indicated that statins induce apoptosis of a number of cell types, including vascular smooth muscle cells (Stark *et al.* 1998, Buemi *et al.* 1999, Guijarro *et al.* 1999). To a large extent this may be explained by the fact that mevalonic acid, the product of the enzyme HMG-CoA reductase, is not only a precursor for cholesterol biosynthesis but is also a building block for isoprenoids (Edwards & Ericsson 1999). As a component of one of the major cell signalling pathways, the small Ras G-protein is activated by iso-

prenylation (Sinensky 2000) and isoprenylation inhibitors have been widely reported to induce apoptosis (Perez-Sala & Mollinedo 1994). Indeed, the covalent modification of Ras is currently a major target in cancer drug development (Rowinsky *et al.* 1999, Perrin & Hill 2000, Sebt & Hamilton 2000). Recently, confirmation that the inhibition of Ras prenylation is the mechanism by which statins induce apoptotic cell death has come from studies on rat brain neuroblasts (Garcia-Roman *et al.* 2001). Here, it was shown that lovastatin-induced apoptosis was associated with a decrease in the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-x_L (Adams & Cory 2001) and a decrease in the prenylation of Ras. These effects were shown to be fully reversed by the addition of mevalonate.

A notable absence in the medical research literature is reference to articles on the effect of statins on vascular endothelial cells. For this reason the current report

describes experiments where the effect of fluvastatin (Langtry & Markham 1999) has been tested on immortalised human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Reagents

All reagents for cell culture were obtained from Life Technologies (Paisley, Strathclyde, UK). Fluorescent probes for flow cytometry were obtained from Molecular Probes (Cambridge Bioscience, Cambridge, UK). Other flow cytometry reagents were obtained from Beckton Dickinson UK Ltd (Cowley, Oxfordshire, UK). The cell death ELISA was obtained from Roche Diagnostics Ltd (Lewes, E. Sussex, UK). Reagents for reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Life Technologies and MWGAG Biotech (Ebersberg, Germany). Fluvastatin was provided as a gift from Novartis Ltd. All other reagents were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Mevalonic acid was added to the cells as the lactone (Sigma). In solution, mevalonic acid lactone spontaneously converts to mevalonic acid. Cholesterol was prepared from a stock ethanolic solution of 100 mM. This was then diluted to 2 mM in Dulbecco's modified Eagle's medium (DMEM) prior to homogenisation with an Ultra-Turrax T25 and filtration with a 2- μ m sterile filter. Following this procedure, cholesterol determinations revealed that there was a 50% loss of cholesterol due to the filtration.

Cell culture

Cells of the line, EA.hy 926, originally derived from HUVECs (Claise *et al.* 1997), were maintained in DMEM with phenol red, penicillin (50 U/ml), streptomycin (50 mg/ml) and 10% fetal calf serum (FCS) (Life Sciences). For the majority of studies reported here, experiments were conducted on cells seeded into 48-well culture plates at densities over the range 2000 to 10 000 cells per cm². Cells were kept at 37 °C in an atmosphere of 5% CO₂ in air. For RT-PCR, flow cytometry experiments and caspase 3 assays, cells were harvested from 75-cm² culture flasks by the addition of trypsin/EDTA, centrifuged at 200 *g* and the cell pellet was either frozen immediately at -80 °C for RT-PCR and caspase 3 activity or suspended in PBS for flow cytometry. For all experiments, cells were treated in medium containing 10% FCS.

Flow cytometry for cell cycle distribution

Cell cycle analysis was performed on control and treated cells by the method of Ormerod (1998). In brief, a single cell suspension was prepared in 200 μ l PBS to which was

added 2 ml ice-cold, 70% ethanol. This suspension was then left for 30 min at 4 °C. Cells were harvested by centrifugation at 200 *g* and were re-suspended in 800 μ l PBS. Propidium iodide (PI; 20 μ l, 0.5 mg/ml) was then added and the labelled cells were kept at 37 °C for 30 min before analysis with a FACS^{CALIBUR} (Becton-Dickinson UK Ltd) flow cytometer with an argon laser tuned to 488 nm. Forward and orthogonal light scatter and red fluorescence (FL-2) were then determined and histogram plots were analysed for cell cycle distribution using the program ModFit (Becton-Dickinson).

Determination of cell numbers and mode of cell death

Cell numbers were directly determined using a Coulter Counter. Prior to measurement, cell monolayers (in 48-well culture plates) were washed with PBS before the addition of trypsin/EDTA (400 μ l). After 10 min at 37 °C, 50 μ l FCS were added and the detached cells were re-suspended in 20 ml Isoton (Beckman-Coulter UK Ltd) prior to counting. An indirect estimate of live cell numbers was also made by the addition of 50 μ l of a 1 mg/ml solution (in PBS) of the diazo salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). After 4 h, the blue crystals were solubilised by the addition of sodium dodecyl sulphate in HCl at final concentrations of 10% and 10 mM respectively as previously described (Newton *et al.* 1995).

The mode of cell death was determined by normal light and fluorescence microscopy in combination with the measurement of biochemical indicators of apoptosis. Microscopic analysis was made using a Leica DMIL fitted with a SPOT^{JUNIOR} digital camera (Diagnostic Instruments, CA, USA), using a combination of Image Modulation Contrast for normal light and u.v. exposure (380 nm) with a wide band pass filter for fluorescence. Photo-quality pictures were produced with a Hewlett Packard 970 Cxi and Kodak Premium paper. The integrity of cell membranes was determined under u.v. light following the addition of a mixture of PI and Hoechst 33342 to give final concentrations of 10 μ g/ml and 1 μ g/ml respectively. Cells with disrupted membranes gave red nuclear fluorescence due to the uptake of PI, whilst cells with intact membranes gave blue nuclear fluorescence.

The mode of cell death was further characterised by determining caspase 3 activity with the ApoAlert colorimetric assay kit from Clontech Labs (Palo Alto, CA, USA) and fragmentation of DNA with an ELISA (Roche Diagnostics Ltd) that we have previously shown to be specific for the detection of DNA fragmentation that occurs in apoptosis (Newton *et al.* 1999).

RT-PCR protocol

RNA extraction and cDNA synthesis were performed as previously described (Speirs *et al.* 1997). Substitution of

Figure 1 Response of low density EA.hy 926 endothelial cells to 24-h exposure to 10 μ M FS (b and d) in comparison with control, untreated cells (a and c), shown as image modulate contrast microscopy ($\times 200$, a and b) and flow cytometry (c and d).

reverse transcriptase with distilled water served as a control to ensure that RNA, rather than genomic DNA, was being extracted and subsequently amplified. In further experiments, this control was uniformly negative.

Primers for PCR of the androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), oestrogen receptor alpha (ER α), and oestrogen receptor beta (ER β) were designed from published gene sequences and were synthesised by Life Technologies and MWG Biotech. To check the integrity of the cDNA, fragments of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or pyruvate dehydrogenase (PDH), standard housekeeping genes, were amplified in parallel. The PCR reaction comprised 2 units of BIOTAQ, 10 $\times$ PCR buffer (Bioline, London, UK), 1.5 mM MgCl₂, 0.5 μ g of each oligonucleotide primer, 200 μ M each of dATP, dCTP, dGTP and dTTP, 1 μ l nascent cDNA and sterile distilled water to bring the volume to 50 μ l. Appropriate positive and negative controls were set-up in parallel. Negative

controls included substitution of RNA or cDNA with distilled water, or substitution of cDNA with an irrelevant cDNA (human tibialis anterior muscle). Transcripts were analysed in parallel in a thermal cycler (Hybaid OmniGene, Teddington, UK) with the following cycle: a denaturation step at 94 $^{\circ}$ C for 2 min, followed by 35 cycles of 94 $^{\circ}$ C for 30 s (denaturing), 55 $^{\circ}$ C for 30 s (annealing), 72 $^{\circ}$ C for 30 s (extension) and a final primer extension step of 72 $^{\circ}$ C for 5 min. PCR for ER β used an alternative annealing temperature of 58 $^{\circ}$ C. PCR products were analysed by electrophoresis through a 1.2% or 1.5% agarose gel and visualised by ethidium bromide staining under u.v. light.

Restriction digests were performed on representative PCR products to confirm their identity. Five microlitres of the amplified product were digested with the appropriate restriction enzyme for 2 h at 37 $^{\circ}$ C. The digested products were then electrophoresed through a 2% agarose gel and visualised as described above.

Figure 2 (a) The growth curve for endothelial cells exposed to a dose range of FS. Normal light and fluorescent microscopy ($\times 400$) for control cells and cells exposed to 10 μM FS for 72 h is shown in (b) and (c) respectively. All values in this and the following figures are given as means $\pm$ S.D. * $P < 0.05$, day 4 and 5, 5 μM FS compared with zero time cell numbers; ** $P < 0.01$, day 2, 5 μM FS compared with control.

Statistics

Statistical analysis was performed on a minimum of three replicates using ANOVA, but in some cases figures are only marked with significance values where a particular aspect of the work requires emphasis. With the exception of Table 1, where values are a composite from three identical experiments, all figures are reproduced as representative experiments from at least five studies. All values given in the figures are expressed as means $\pm$ S.D.

Results

Cell cycle effects of short-term exposure to fluvastatin (FS)

Following a 24-h exposure of cells at a density of around 25 000/cm² to 10 μM FS, microscopic observation of cells revealed a rounding of the cell body with many cells showing filamentous projections as indicated in Fig. 1b compared with untreated cells (Fig. 1a). Cells similarly treated with FS for 24 h in 75-cm² flasks were subjected to flow cytometry analysis after fixing and labelling nuclei with PI as described in Materials and Methods. The corresponding histogram profiles for one of three experiments following cell cycle distribution analysis are shown in Fig. 1c and d. The insets on (c) and (d) give the combined statistics for all three experiments. This analysis reveals that the proportion of cells in G₀-G₁ increased from 69 $\pm$ 8% for control untreated cells to 93 $\pm$ 6% for cells

treated with 10 μM FS (control vs FS, $P < 0.01$). In parallel, the number of cells in G₂-M and S phases decreased from 12 $\pm$ 5% and 19 $\pm$ 6% respectively for controls to 1.6 $\pm$ 0.8% and 6 $\pm$ 6% for cells treated with FS (control vs treatment; G₂-M, $P < 0.01$ and S phase, $P < 0.05$). These observations strongly suggest that FS prevented cells from leaving the G₁ phase, allowing accumulation in this phase of the cell cycle.

Long-term response of EA.hy 926 cells to FS

To determine how the apparent cell cycle blockade imposed by FS affected cell numbers, the dose-response to FS was monitored over a 5-day treatment period. Figure 2a shows that at 5 μM FS, cell numbers were significantly lower ($P < 0.01$) than the corresponding controls after 48 h of treatment. By day 3, there was widespread appearance of cells with numerous small blebs as shown in Fig. 2c. Some of these contained blue fluorescent fragments indicating that nuclear fragmentation had occurred, a classical sign of apoptosis. This response is quite distinct from necrosis, as nuclear fragmentation is never a hallmark of the necrotic process. Therefore, cell numbers after 4 days of treatment with 5 μM FS had fallen significantly below the pretreatment values ($P < 0.05$) due to the loss of cells by apoptosis. By visual observation under u.v. and normal light, Fig. 3 shows the proportion of the cell population with apoptotic nuclei from an experiment where cells

Figure 3 Response of cells to a dose range of FS (72-h treatment) measured as the proportion of apoptotic cells determined after addition of Hoechst 33342 and PI. ** $P < 0.01$, *** $P < 0.001$ compared with control.

Figure 4 Time course for the activation of caspase 3 and the appearance of DNA fragments in response to treatment of cells with 5 μM FS. *** $P < 0.001$ compared with control. abs, absorbance.

were treated with a dose range of FS. After 72-h exposure to FS, a significant increase ($P < 0.01$) in the proportion of the population undergoing apoptosis was observed from 1 μM with a maximum response observed at around 5 μM. It should be noted that necrosis was never observed in any experiment with FS.

Biochemical parameters of cell death in response to FS

Over a time course of 72 h, caspase activity and DNA fragmentation was measured after exposure to 5 μM FS. Figure 4 shows that a significant ($P < 0.001$) rise in caspase 3 activity was noted after 24-h exposure to 5 μM FS. In

contrast, DNA fragmentation measured with the ELISA was not observed to rise significantly above control values until the 48-h time point of exposure.

Cellular response to FS in combination with products of the HMG-CoA pathway

To determine the specificity of the response to FS for inhibition of HMG-CoA reductase, experiments were performed where FS was combined with mevalonic acid and also with cholesterol over a dose range of 5 to 50 μM. Over a time period of 72-h treatment, mevalonic acid completely blocked the FS-induced loss of cellular metabolic activity measured by the addition of the diazo dye, MTT (Fig. 5a). As the end product of the HMG-CoA pathway, the effect of cholesterol was also determined. Over the dose range 0.5 to 2 μM FS and with cholesterol at final concentrations of 5, 10, 25 and 50 μM, the effect on cellular metabolic activity was determined after 72-h exposure. Figure 5b shows that, in contrast to mevalonic acid, cholesterol at concentrations of 10 μM and above significantly enhanced ($P < 0.001$) the response to FS.

The effect of the endocrine environment on response to FS

Prior to exposure to a dose range of various steroid hormones, the expression of receptor mRNA was determined by RT-PCR with selected primer sequences for ERα, ERβ, PR, AR and GR. Figure 6 shows that both the AR and the GR were expressed in these EA.hy 926 endothelial cells. Experiments were then conducted on the dose-response to FS in the presence of both the GR agonist, dexamethasone (Dex), and the AR agonist, dihydrotestosterone (DHT), compounds shown to fully transcriptionally activate the receptors (Newton *et al.* 2001). Prior to treatment with FS, cells were pretreated for 24 h with 100 nM DHT or 1 μM Dex. After a further 72-h exposure to a dose range of FS, DNA fragmentation was measured by ELISA. Whilst DHT had no effect on the response to FS, Dex completely blocked the ability of FS to induce DNA fragmentation (Fig. 7). For high density cells exposed to FS for 72 h in combination with a dose range of Dex (1 nM–1 μM), Fig. 7b shows the light microscope appearance of cells. Clear apoptotic morphology is observed in the presence of FS and low concentrations of Dex (1 nM) and this is progressively blocked as the concentration of Dex is increased. It should be noted from Fig. 7b that even at 1 μM, Dex failed to block the FS-induced change in cell morphology.

Further analysis by flow cytometry of the effect of Dex on the cell cycle distribution in response to 48-h and 72-h exposure to FS (5 μM) revealed that at 48 h there was a substantial sub- G_1 peak with FS alone and that this was absent in the presence of Dex (1 μM) (Fig. 8). In contrast to the profile for untreated cells, cells still appeared to be accumulating in the G_1 phase when treated with FS and

Figure 5 (a) Reversal of the effect of FS (5 μM) by the inclusion of mevalonic acid lactone (Mev, 200 μM). (b) The enhancement of low dose FS-induced loss of cellular metabolic activity (MTT absorbance, 540 nm) by the inclusion of cholesterol (ch) in the culture media. *** $P<0.001$ compared with FS alone.

Figure 6 RT-PCR analysis for the expression of ERα, ERβ, PR, AR and GR within EA.hy 926 vascular endothelial cells. L, 100 bp ladder; 1, EA.hy ERα; 2, ERα +ve control; 3, ERα -ve control; 4, EA.hy ERβ; 5, ERβ +ve control; 6, ERβ -ve control; 7, EA.hy PR; 8, PR +ve control; 9, PR -ve control; 10, EA.hy AR; 11, AR +ve control; 12, AR -ve control; 13, EA.hy GR; 14, GR +ve control; 15, GR -ve control.

Dex. Over three experiments, the proportion of the cell population in the various phases of the cell cycle is given in Table 1. From this Table it is clear that Dex significantly ($P<0.01$) prevents the appearance of sub- G_1 nuclei with a reduced DNA content. It is also apparent that in comparison with the controls, there are still significantly fewer cells in S and G_2 -M after treatment with both FS and Dex.

After a further 24-h exposure to FS, the majority of cells were sub- G_1 and Dex was still capable of providing some protection against FS-induced apoptosis (Fig. 8). It should also be pointed out that when added alone, Dex failed to change cell cycle parameters (Fig. 8) and although data are not shown, this was reflected by an inability of Dex to alter cell numbers over a period of 5 days from first treatment.

Figure 7 (a) Response of high-density endothelial cells to a dose range of FS (72-h exposure) in the presence of the androgen, DHT (100 nM) and the glucocorticoid, Dex (1 μM). (b) Image modulate contrast microscopy pictures ($\times 200$) of cells exposed to FS (5 μM) and a dose range of Dex.

Dex response with other inducers of apoptosis

To test whether Dex blocked the apoptotic pathway within these endothelial cells, the effect of Dex on several agents known to induce apoptosis through different cellular targets was tested. Table 2 shows that Dex (1 μM) prevented an increase in DNA fragmentation and an increase in the proportion of the cell population with apoptotic nuclei in response to all agents tested.

Discussion

Considering that statins are one of the most widely used classes of cholesterol lowering agents, an omission in the medical research literature would appear to be studies addressed to the effect of statins on vascular endothelial cells. We now show in this paper that statins are toxic to endothelial cells derived from the human umbilical vein. It should be noted that although a cell line derived from HUVECs has been used for the studies presented here, FS was also toxic to primary HUVEC cultures. These studies are therefore consistent with the growing number of reports indicating that statins are toxic to a range of cell types (Stark *et al.* 1998, Buemi *et al.* 1999, Guijarro *et al.* 1999).

The current study concurs with other reports showing that statins prevent cells from entering S phase of the cell cycle (Wu & Gilbert 2000). As a consequence of this effect on cell cycle or by a mechanism involving the blockade of

survival pathways, apoptotic cell death follows later. The ability of FS to induce apoptosis has been confirmed by the morphological appearance of cells, the presence of a sub- G_1 peak, induction of caspase 3 activity and the presence of extensive DNA fragmentation. The belief that FS causes these dramatic changes by interfering with the HMG-CoA pathway is confirmed by our observation showing that the product of HMG-CoA, mevalonic acid, completely reverses the effect of FS. As cholesterol, the final product of the HMG-CoA pathway, is unable to prevent FS-induced cell death, it is highly likely that blockade of the pathway of isoprenylation (Garcia-Roman *et al.* 2001) is the mechanism by which FS induces cell death.

It is significant that (as shown using flow cytometry) the glucocorticoid, Dex, is only able to block apoptosis, unlike mevalonic acid which blocks apoptosis and restores growth. This finding is clearly demonstrated in Fig. 8 and Table 1 where, particularly for 48-h exposure to FS, the accumulation of sub- G_1 cells is blocked by Dex, but there are still very few cells entering S and G_2 . Therefore, it would appear that the action of Dex is not specific to modulating the effect of statin on the cell cycle, rather it would seem that Dex blocks endothelial cell apoptosis. This finding is consistent with some of the recent literature including our own (Newton *et al.* 2001) that glucocorticoids block apoptosis in other cell systems (Messmer *et al.* 2001, Mikosz *et al.* 2001, Sasson *et al.* 2001, Zhang *et al.* 2001). Table 2 provides data that strongly support the idea

Figure 8 Flow cytometry profiles for cells exposed for 48 and 72 h to FS (5 μ M) alone and in combination with Dex (1 μ M).

that the effect of Dex is to block apoptosis at a common element in the pathway. These agents were chosen to cover a range of cellular targets, from signal transduction pathways (serum withdrawal, tumour necrosis factor- α (TNF α)), to DNA damage with the topoisomerase 1 inhibitor, camptothecin. Of particular note are the observations with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, a compound that collapses the mitochondrial membrane potential (Newton *et al.* 2001), and with hexadecyl triphenyl phosphonium bromide, a compound that also targets mitochondria (unpublished observations, Newton CJ). By collapsing the membrane potential, both agents would be expected to result in the release of cytochrome *c* and the activation of the Apaf-1

complex of the apoptotic pathway (Zou *et al.* 1999). This strongly suggests that Dex blocks apoptosis down-stream of changes in mitochondrial function. Based on these findings, studies are being conducted to determine the precise point in the apoptotic pathway that is disrupted by Dex.

The past 5 years have seen a high degree of interest in the effect of oestrogens on the vasculature (Linder *et al.* 1998, Makela *et al.* 1999, Scheidegger *et al.* 2000, Andersson *et al.* 2001). This is now the first report to indicate that glucocorticoids are effective antiapoptotic agents for vascular endothelial cells. Using powerful, new quantitative PCR techniques, we are now showing the low level expression of the ER β in these endothelial cells. Despite this, we have been unable to demonstrate a direct

Table 1 Combined data from three flow cytometry experiments, where cells were exposed for 48 h to FS (5 µM) alone and in combination with the glucocorticoid, Dex (1 µM). All values for cell cycle phase, calculated by ModFit as percentage of the total number of expressed cells, are expressed as means ± S.D.

	Control	Dex	FS	FS+Dex
Cell cycle phase				
Sub G ₁	1.2 ± 0.9	2.3 ± 1.3	39 ± 12***	7 ± 4***
G ₀ –G ₁	73 ± 7	85 ± 7	56 ± 7*	88 ± 4
S	15 ± 4	11 ± 3	3.5 ± 1.3**	2.9 ± 1.5**
G ₂ –M	11 ± 4	9 ± 4	1.2 ± 1.7**	3.1 ± 2.3*

*P<0.05, **P<0.01, ***P<0.001 compared with control; ***P<0.01 compared with FS.

Table 2 The response of EA.hy 926 cells to a range of known inducers of apoptosis in the absence and presence of 1 µM Dex. With the exception of serum withdrawal, where cells remained without serum for 4 days prior to assay, the other agents were added to cells for 24 h prior to assay. Values are means ± S.D.

	DNA fragmentation (ELISA units)			% Apoptosis	
	Control	Statin	Statin + Dex	Statin	Statin + Dex
Apoptosis inducer					
Serum withdrawal	0.29 ± 0.05	1.78 ± 0.21	0.35 ± 0.12*	29 ± 7	2 ± 2*
TNFα (100 ng/ml)	0.29 ± 0.11	0.98 ± 0.08	0.25 ± 0.09*	12 ± 3	2 ± 1*
H ₂ O ₂ (400 µM)	0.31 ± 0.07	2.37 ± 0.18	0.46 ± 0.13*	34 ± 6	7 ± 3*
Camptothecin (1 µM)	0.27 ± 0.05	2.37 ± 0.31	0.34 ± 0.04*	49 ± 7	9 ± 4*
mCICCP (100 µM)	0.31 ± 0.14	2.46 ± 0.21	0.52 ± 0.03*	41 ± 6	7 ± 3*
HDTP (5 µM)	0.36 ± 0.05	2.27 ± 0.19	0.41 ± 0.10*	43 ± 4	5 ± 2*

mCICCP, carbonyl cyanide m-chlorophenylhydrazone; HDTP, hexadecyl triphenyl phosphonium bromide.

*P<0.001 compared with statin alone.

antiapoptotic effect of a range of oestrogens on these vascular endothelial cells. This finding raises the possibility that *in vitro* it is the glucocorticoid, cortisol, that protects vascular endothelial cells against apoptosis induced by oxidised low density lipoprotein (Claise *et al.* 1997) and other toxic agents such as TNFα. In addition to this potential new physiological significance of glucocorticoids, these hormones may also provide a 'fail safe mechanism', whereby vascular endothelial cells are protected against the toxic side effects of statins, agents shown clinically to lower circulating cholesterol concentrations and to improve vascular function.

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