Lovastatin enhances the photocytotoxicity of UVA radiation towards cultured N.C.T.C. 2544 human keratinocytes: prevention by cholesterol supplementation and by a cathepsin inhibitor

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The effect of the hydroxymethylglutaryl-CoA (HMG-CoA) inhibitor lovastatin on the UVA-induced photocytotoxicity has been investigated in cultured human N.C.T.C. 2544 keratinocytes. In the absence of irradiation, 5×10^{-7} M lovastatin did not exhibit any significant cytotoxic effect towards this cell line. Although the drug cannot act as a photosensitizer, because it does not absorb in the UVA range, it markedly increased the UVA-induced cellular damage (about 70% reduction in cell viability at 5×10^{-7} M). This effect was not accompanied by an increase in the lipid peroxidation product content of cells as compared with treatment with UVA alone. Medium supplementation with 0.01 mg/ml free cholesterol totally prevented the enhancement of UVA photocytotoxicity induced by lovastatin. A protective effect was also observed when cells were supplemented with an amount of low-density lipoprotein giving the same cholesterol concentration in the culture medium. Finally, E64 [L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane], a lysosomal cathepsin inhibitor, also prevents the cell death induced by UVA in cells treated with lovastatin. These results suggest that HMG-CoA reductase inhibitors could increase the sensitivity of skin cells to UVA radiation, and that this phenomenon is related to lysosomal enzyme release.

INTRODUCTION

Ultraviolet (UV) radiations are involved in degenerative processes of the skin such as photoaging and photocarcinogenesis [1,2]. DNA photochemistry plays a major role in photodegenerative processes of the skin produced by solar UVB (290–320 nm) [3]. However, it is now well established that UVA (320–400 nm) radiation potentiates the carcinogenic action of UVB [4,5], and induces *per se* metabolic damage in various cell types [6–12]. Some of the deleterious effects of UVA seem to be related to photosensitized lipid peroxidation [6,7]. The main endogenous UVA chromophores involved in this phenomenon are flavins, NADH/NADPH and the porphyrin ring [13].

The role of free cholesterol in maintaining the barrier properties of the skin has been stressed by several authors [14,15]. Skin treatment with competitive inhibitors of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the key enzyme of sterol synthesis, results in marked alteration of the structure and barrier properties of skin in animal models [16,17]. We thus investigated the effect of lovastatin, a well-known HMG-CoA reductase inhibitor [18], on UVA-induced cellular damage in the human keratinocyte cell line N.C.T.C. 2544. We showed that lovastatin, although not being a chromophore of UVA and having no cytotoxic action by itself under our conditions, strongly potentiated the UVA-induced damage as assessed by measuring cell viability. This effect was prevented by supplementation of the culture medium with cholesterol, or with low-density lipoprotein (LDL). Since lysosomal membrane destabilization and lysosomal enzyme release have been shown to be involved in cell death induced by exogenous photosensitizers absorbing in the UVA range, such as porphyrins [19,20], we also investigated the effect of a preincubation of N.C.T.C. 2544 keratinocytes with lovastatin and L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64), a powerful inhibitor of lysosomal cathepsins [21]. The addition of E64 to the culture medium completely inhibited the potentiation of the UVA-induced phototoxicity by lovastatin, suggesting that lysosomal damage is involved in the latter phenomenon.

MATERIALS AND METHODS

Materials

The N.C.T.C. 2544 human keratinocyte cell line [22] was purchased from Flow (Paris, France). Dulbecco's modified Minimum Essential Medium (DMEM) with Earle's salts, Hanks' salts solution and fetal-calf serum were from Gibco (Grand Island, NY, U.S.A.). Lovastatin was a generous gift from Specia Laboratories. The cathepsin inhibitor E64 and all the other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.) and were of the purest available grade.

Abbreviations used: CPL, choline-phospholipid; DMEM, Dulbecco's modified Minimal Essential Medium; E64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; HMG-CoA, hydroxymethylglutaryl-CoA; LDL, low-density lipoprotein; TBARS, thiobarbituric acid-reactive substances (lipid peroxidation products); UVA, ultraviolet A radiation; UVB, ultraviolet B radiation.

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Cell culture

Cells were seeded at a density of $1.5 \times 10^4/cm^2$, and cultured in 35-mm-diam. Nunc Petri dishes in DMEM supplemented with 10% (v/v) fetal-calf serum and 10 mM Hepes buffer. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere. All experiments were carried out on subconfluent cells.

Experimental conditions for cell irradiation

Before UVA irradiation, cells were washed three times with Hanks salt solution. Irradiation at 365 nm was performed in 1 ml of Hanks salts devoid of any additive, using a Vilber Lourmat (Torcy, France) table equipped with TF-20L tubes and appropriate filters. A glass window (4 mm thickness) was placed 20 mm above the lamp to absorb remaining short-wavelength UV light of the commercial equipment (transmittance < 0.01% at 320 nm). Samples (plastic Petri dishes) were placed on the glass window and irradiated from the bottom [23]. An average light intensity of 3.0 ± 0.2 mW/cm² was measured through the dish bottom with a Vilber Lourmat UVR 365 photometer. After irradiation, cells were left in the dark for 1 h at 37 °C. Sham-irradiated cells were left under similar conditions but without UVA exposure. It must be emphasized that in order to test the effect of the various agents tested, we used a mild UVA dose (about 13 J/cm²), which caused only moderate cellular damage (80–85% of cells were found to be alive 24 h after irradiation).

LDL preparation

LDL, taken as the $d = 1.024$–1.050 fraction, was prepared from the sera of healthy volunteers by sequential ultracentrifugation [24]. After preparation, the LDL solution was extensively dialysed against a 5×10^{-3} M Tris/0.04% EDTA, pH 7.4, buffer and stored at 4 °C before use.

Cell treatment with lovastatin, free cholesterol, LDL and E64

The effect of lovastatin on the UVA-induced photocytoxicity was studied in cells precultured for 24 h before irradiation in the presence of either lovastatin (1×10^{-7} or 5×10^{-7} M) or free cholesterol (0.01 mg/ml) [25], both in ethanolic solution (final ethanol concentration 0.5%), or with 0.01 ml of a human LDL solution corresponding to 0.01 mg of cholesterol-LDL. Combinations of these conditions (lovastatin + free cholesterol, lovastatin + LDL) were also studied. In experiments designed to investigate the role of lysosomal damage in lovastatin potentiation of the UVA photocytotoxicity, the lysosomal cathepsin inhibitor E64 was added concomitantly with 5×10^{-7} M lovastatin, at a final concentration of 0.01 mg/ml. Since this compound was introduced into the culture medium as a concentrated solution in DMSO, a control with DMSO alone (1% final concentration) was also prepared. It has also been verified by spectrophotometric studies that E64 has no significant absorption in the UVA range, and therefore cannot act as a photosensitizer (results not shown).

Cholesterol and choline-phospholipid (CPL) determination

The cellular lipids were first extracted [26]. The cholesterol mass was measured with the HiCo colorimetric kit from Boehringer–Mannheim, based on the cholesterol oxidase reaction after cholesteryl ester hydrolysis. The CPL content was determined using the PAP 150 colorimetric kit from BioMérieux (Marcy l'Etoile, France).

Thiobarbituric acid-reactive substances (TBARS) determination

The lipid peroxidation products (TBARS) were measured at the end of the 1 h dark period following cell irradiation. Measurements were performed on an aliquot of the irradiated medium, as it has previously been shown that most of the TBARS produced after UVA exposure are secreted by cells [6]. The TBARS were determined by the fluorometric method described by Yagi [27]. Results, calculated as malondialdehyde equivalents produced/mg of cell protein, were expressed as percentages of controls (unirradiated cells, at the beginning of the experiments).

Assessment of cell viability

The cell viability was determined by the Neutral Red assay [28], slightly modified as follows: at the end of the 4 h incubation with the dye at 37 °C, cells were washed three times with a phosphate-buffered solution, pH 7.4, and then dissolved with 1 ml of SDS (5%, w/v) in water added to each well. After homogenization of the solution, the wells were further incubated for 30 min at 37 °C. The absorbances of the resulting solutions were read at 535 nm using a Perkin–Elmer L3 spectrophotometer. Protein determination was carried out on an aliquot of the cell homogenate by the method of Peterson [29].

All experiments were performed at least in triplicate. Statistical analysis was done by Student's t test.

RESULTS

Table 1 displays the effect of 1×10^{-7} or 5×10^{-7} M lovastatin on the photocytotoxic effect of the UVA radiation. It can be observed that, as mentioned above (see the Materials and methods section), the low UVA dose used in our experiments resulted in only a moderate loss of cell viability (85–90% of the cells remained alive 24 h after irradiation). In contrast, cells precultured for 24 h in the presence of lovastatin appeared to be much more sensitive to UVA damage, with about 30 and 70% reduction of Neutral Red uptake for 1×10^{-7} and 5×10^{-7} M lovastatin respectively.

In order to investigate the mechanism(s) by which lovastatin could enhance cell photodamage induced by UVA radiation, we

Table 1 Effect of lovastatin on the UVA-induced photocytotoxicity in cultured human N.C.T.C. 2544 keratinocytes

Cells were pretreated with the drug for 24 h in medium supplemented with 2% Ultroser G. After washing and irradiation at a UVA dose of 13 J/cm², the cells were further incubated for 1 h in the dark, then replaced in fresh medium. The cell viability was measured 24 h after irradiation by the Neutral Red assay (see the Materials and methods section). Abbreviations: NI, sham-irradiated cells; I, UVA-irradiated cells. Control, cells cultured in the absence of lovastatin. Results are means of six experimental values ± S.D.

	Cell viability (%)	
	NI	I
Control	100	79 ± 6
Lovastatin		
1×10^{-7} M	96 ± 6	61 ± 5
5×10^{-7} M	92 ± 4	25 ± 3

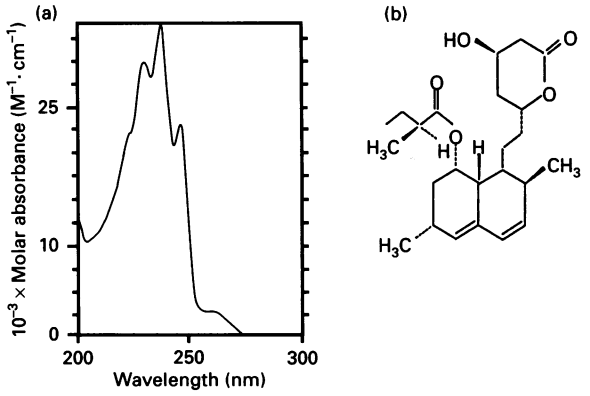

Figure 1 Absorption spectrum of lovastatin in ethanol (a) and chemical structure of the drug (b)

It is clearly shown that lovastatin has no significant absorption in the UVA range (320–400 nm).

Table 2 Effect of antioxidants on the UVA-induced TBARS formation and cell viability determined 24 h after irradiation by the Neutral Red assay

The cells were precultured for 24 h with or without antioxidants (10^{-5} M vitamin E + 10^{-3} M vitamin C) combined or not with 5×10^{-7} M lovastatin. Other experimental conditions are the same as described in Table 1 legend. Abbreviations: NI, sham-irradiated cells; I, UVA (13 J/cm^2)-treated cells. Control, cells cultured in standard conditions. Results are means of six experimental values ± S.D.

	TBARS formation		Cell viability (%)	
	NI	I	NI	I
Control	0.25 ± 0.04	1.10 ± 0.15	100	77 ± 4
+ Antioxidants	0.22 ± 0.05	0.34 ± 0.06	100 ± 6	94 ± 5
+ Lovastatin	0.27 ± 0.06	1.15 ± 0.12	91 ± 4	24 ± 3
+ Antioxidants and lovastatin	0.25 ± 0.03	0.39 ± 0.04	94 ± 5	35 ± 4

first checked that the drug does not simply act as a photosensitizer. Figure 1(a) effectively shows that lovastatin had no significant absorption in the UVA range, as could be expected from its chemical structure (Figure 1b).

The photocytotoxic action of UVA radiation is thought to be due to the production of active oxygen species, primarily singlet oxygen, by endogenous photosensitizers (porphyrins, flavins, NADH/NADPH) [13]. Among various mechanisms, lipid peroxidation has been suggested to play an important role in the cytotoxic effect of UVA by propagation of oxidative damage via free radical formation [6]. Indeed, cellular damage induced by UVA is strongly protected against by preculturing cells with antioxidants such as vitamin E [6]. It may thus be hypothesized that lovastatin could indirectly influence the UVA-induced photodamage by enhancing lipid peroxidation. Table 2 shows that the enhancement of the cytotoxic effect of UVA radiation by lovastatin did not induce a marked increase in the TBARS content of cells as compared with irradiated controls cultured without the drug. Moreover, a 24 h preculture with antioxidants (5×10^{-5} M vitamin E + 1×10^{-3} M vitamin C), which resulted in almost total prevention of the UVA-induced lipid peroxidation in the absence or in the presence of lovastatin, only partially protected cells against the damage induced by UVA in cells precultured with lovastatin (Table 2).

Table 3 Effect of lovastatin, cholesterol, or both compounds, on the cholesterol content and on the cholesterol/CPL (C/CPL) ratio in N.C.T.C. 2544 keratinocytes

Cells were precultured for 24 h with either 5×10^{-7} M lovastatin, or 0.01 mg/ml cholesterol, or both compounds. The cellular cholesterol content and the cholesterol/choline-phospholipid (C/CPL) ratio were determined as specified in the Materials and methods. Means of four experimental values ± S.D.

	Cholesterol (μg/mg of cell protein)	C/CPL (μg/mg of cell protein)
Control	65 ± 9	0.72 ± 0.05
Lovastatin	60 ± 7	0.69 ± 0.08
Cholesterol	82 ± 8	1.05 ± 0.14
Lovastatin + cholesterol	81 ± 10	0.94 ± 0.12

Table 4 Prevention by cholesterol or LDL of the stimulatory effect of lovastatin on the phototoxicity of UVA towards N.C.T.C. 2544 keratinocytes

The cells were precultured for 24 h in the presence of 5×10^{-7} M lovastatin alone, or 5×10^{-7} M lovastatin + 0.01 mg/ml cholesterol in ethanolic solution, or 5×10^{-7} M lovastatin + 0.05 mg/ml LDL protein. Other experimental conditions are the same as described in the legend to Table 1. The viability of N.C.T.C. 2544 keratinocytes was assessed by the Neutral Red test. Abbreviations: NI, sham-irradiated cells; I, UVA (13 J/cm^2)-treated cells. Results are means of six experimental values ± S.D.

	Cell viability (%)	
	NI	I
Control	100	79 ± 6
Lovastatin	97 ± 6	22 ± 3
Lovastatin + cholesterol	96 ± 8	77 ± 5
Lovastatin + LDL	95 ± 10	72 ± 4

HMG-CoA reductase inhibitors have been shown in some experimental models to reduce the cellular free cholesterol content [30] and to induce alterations in the lipid metabolism of cells [17,31]. Since cholesterol is one of the important factors in the regulation of the activity of various membrane-bound enzymes and transporters [32], it might be speculated that perturbation of the cellular cholesterol content could occur under our experimental conditions, leading to an increased sensitivity to UVA radiation without significant modification of lipid peroxidation. Consequently, we investigated the effect of a 24 h cell preculture under the following conditions: lovastatin alone (5×10^{-7} M), cholesterol alone (0.01 mg/ml), and lovastatin + cholesterol, on the cellular cholesterol content and on the cholesterol/CPL (C/CPL) ratio. Table 3 shows that lovastatin alone had no significant effect on the cellular cholesterol content, while, as expected, cholesterol supplementation increased it by about 25–30%. This increase was also observed in cells cultured in the presence of both cholesterol and lovastatin. Table 3 also indicates that cholesterol alone significantly increased the C/CPL ratio, while lovastatin had no significant effect on this parameter; a similar increase in the C/CPL ratio was observed in cells pretreated with both lovastatin and cholesterol.

We then investigated the effect of a 24 h preculture of N.C.T.C. 2544 keratinocytes with 0.01 mg/ml exogenous free cholesterol, or an equivalent amount of LDL-cholesterol, on the viability of lovastatin-treated cells after UVA exposure. Table 4 shows that cholesterol did not significantly affect cell viability in the absence

Table 5 Prevention by the cathepsin inhibitor E64 of the stimulatory effect of lovastatin on the phototoxicity of UVA towards N.C.T.C. 2544 keratinocytes

Experimental conditions are similar to those specified in Table 1 legend, except that E64 (0.01 mg/ml) was added concomitantly with 5×10^{-7} M lovastatin (see the Materials and methods section). The viability of N.C.T.C. 2544 keratinocytes was assessed by the Neutral Red test. Abbreviations: NI, sham-irradiated cells; I, UVA (13 J/cm^2)-treated cells. Results are means of six experimental values ± S.D.

	Cell viability (%)	
	NI	I
Control	100	79 ± 6
Lovastatin	96 ± 4	25 ± 4
E64	96 ± 6	98 ± 5
Lovastatin + E64	97 ± 5	99 ± 4

of lovastatin, and almost completely prevented the potentiating effect of lovastatin on the UVA-induced cellular damage. A similar effect was observed when cells were precultured in the presence of an amount of LDL giving about the same final concentration of cholesterol (0.01 mg/ml) in the culture medium. The cell viability of lovastatin + UVA-treated cells rose from about 25% to about 65% whatever the mode of cholesterol delivery (Table 4).

Finally, Table 5 indicates that the cathepsin inhibitor E64 completely prevented the enhancement by lovastatin of the UVA-induced photocytotoxicity.

DISCUSSION

Taken all together, these results demonstrate that the HMG-CoA reductase inhibitor lovastatin induced a dramatic increase in the sensitivity of N.C.T.C. 2544 human keratinocytes to moderate doses of UVA radiation, as assessed by the Neutral Red viability assay. This effect is almost totally prevented by either free or LDL-cholesterol supplementation of the culture medium. It is of note that the range of lovastatin concentration used in our experiments is close to that observed in the plasma of hypercholesterolaemic patients treated with the drug [18]. Thus our observation could be of physiological importance and draw attention to possible new side-effects of HMG-CoA reductase inhibitors. In the solar spectrum, UVA radiations have recently been shown to induce photodamage in animal models or in cultured cells [6–12]. It must be stressed that if the currently described effect of lovastatin (e.g. increased UVA-induced damage to keratinocytes) also takes place *in vivo*, this could have consequences not only for the barrier properties of the skin, but also possibly for skin photoaging and photocarcinogenesis.

The mechanisms by which lovastatin and possibly other HMG-CoA reductase inhibitors could enhance the UVA-induced cellular damage are as yet not clearly elucidated, but some lines of evidence appear from our data: these are outlined below.

(i) The drug is not a photosensitizer, since it does not absorb in the UVA range (see Figure 1b).

(ii) The enhancement of cell photosensitivity to UVA by lovastatin is not due to an effect on lipid peroxidation, because only a slight increase in TBARS formation (+10%) occurred in irradiated cells pretreated with lovastatin as compared with irradiated controls. Moreover, antioxidants failed to prevent cell death in UVA-irradiated keratinocytes pretreated with lovastatin. However, it must be noted that although they did not entirely protect lovastatin-treated cells against the UVA-induced damage, antioxidants exhibited a significant effectiveness in reducing cell death after UVA exposure (the cell viability rose from about 25 to 35%; Table 2). Thus if the enhancement of the sensitivity of cultured keratinocytes to UVA by lovastatin cannot be directly attributed to an effect on lipid peroxidation, the influence of the drug on the enzymic and non-enzymic antioxidant cellular defences which could affect the oxidation of other molecular targets (for example proteins) remains to be investigated.

(iii) The prevention of the lovastatin-induced enhancement of the photocytotoxic effect of UVA by either free or LDL-cholesterol, which markedly increased the cellular cholesterol content while the latter was not significantly affected by the drug, suggests that physico-chemical characteristics of cell membranes might be of importance in the observed phenomenon. Although lovastatin by itself did not significantly modify the overall cellular cholesterol content, we suspected that the drug could modify the local cholesterol concentration or/and distribution at the level of the membranes of intracellular organelles. The possible role of the lysosomal membrane in the observed phenomenon was suggested by previous studies from our group demonstrating a particular sensitivity of lysosomes towards photosensitization by exogenous porphyrin derivatives, which induced the release of lysosomal proteinases into the cytosolic compartment, causing irreversible cellular damages [20,21]. The prevention of the slight decrease in cell viability after exposure to UVA alone by the cathepsin inhibitor E64 (in the absence of lovastatin) first suggests that lysosome destabilization could be involved in the cytotoxic effect of UVA radiation. Moreover, the striking protective effect of E64 against the potentiating action of lovastatin on the UVA photocytotoxicity demonstrates that lysosomal membrane destabilization and release of cathepsins is one of the primary events leading to cell death in this experimental model. It can be supposed that cell supplementation with either free or LDL-cholesterol results in an increased resistance of the lysosomal membrane to UVA photosensitization and therefore in the protection against the cytotoxicity of UVA radiation in lovastatin-treated cells. The hypothesis of lysosomal membrane 'fragilization' by lovastatin is presently under investigation in our laboratory, using microspectrofluorometry which allows the study of specific organites at the level of a single living cell [33]. Studies on the lipid composition of the lysosomal membrane after cell fractionation in control and lovastatin-treated keratinocytes could also be of use to specify the mechanism by which the drug enhances the UVA photocytotoxicity.

As a conclusion, our findings bring some new insights into the potential effect of HMG-CoA reductase inhibitors on the properties of skin cells. Previous studies demonstrated important alterations of the epidermis in lovastatin-treated rats, without significant modification (as in the present study) of the cholesterol content of epidermal cells [17]. The drug also induces a marked delay in the recovery of the barrier function of the skin after solvent treatment [16]. Our data suggest that, in addition, HMG-CoA reductase inhibitors might increase the sensitivity of skin cells to UVA. Experiments on animal models are planned in our laboratory in order to explore the latter hypothesis. At the present time, there is no clear explanation for either the alterations of the barrier function of the skin by lovastatin or the increased sensitivity of lovastatin-treated keratinocytes to UVA radiation. However, in the latter case lysosomal damage seems to be involved in the observed phenomenon. Whether the deleterious action of HMG-CoA reductase inhibitors is due to alterations in the cholesterol content and/or distribution in membrane microdomains without a detectable action on the overall cholesterol content of cells, or to unknown specific effects of these drugs on

cellular metabolism unrelated to cholesterol but in any case prevented by cholesterol supplementation, remains to be specified.

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