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# Ultraviolet radiation stimulates a biphasic pattern of 1,2-diacylglycerol formation in cultured human melanocytes and keratinocytes by activation of phospholipases C and D

Catherine J. CARSBERG,*‡§ Jacqueline OHANIAN† and Peter S. FRIEDMANN*

*Department of Dermatology, University of Liverpool, Liverpool, U.K. and †Department of Medicine, University of Manchester, Manchester, U.K.

Ultraviolet radiation (UVR) induces melanin synthesis by human epidermal melanocytes, and phospholipid-derived 1,2-diacylglycerols (DAGs) have been implicated in mediating this response. In previous experiments, addition of the synthetic DAG 1-oleoyl-2-acetylglycerol to cultured pigment cells stimulated melanogenesis. The purpose of the present study was to analyse the effects of UVR on the endogenous generation of DAGs. It was found that in a number of cultured cell types, including human melanocytes and B16 mouse melanoma cells, but also human keratinocytes and Swiss 3T3 fibroblasts, exposure to a single dose of UVR stimulated a biphasic increase in endogenous DAG formation. An early transient rise, over seconds, was followed by a more sustained delayed rise over minutes. The early rise in DAG levels was accompanied by a transient rise in inositol trisphosphate formation, indicating activation of phosphatidylinositol-specific phospholipase C. The delayed rise was accompanied by activation of phospholipase D. This endogenous DAG formation by pigment cells is further evidence for the involvement of DAGs in UVR-induced epidermal melanin synthesis. Since DAG formation is also seen in other cells types, it is possible that DAGs may be involved in an array of UVR-induced responses.

## INTRODUCTION

Exposure to solar ultraviolet radiation (UVR) is known to induce a number of effects in human skin, including melanogenesis, and also erythema. There is some previous evidence that plasma membrane phospholipid-derived second messengers may be important mediators of both these responses. Phospholipid-derived second messengers are released by activation of phospholipases, including types $A_2$, C and D [for review see (Liscovitch, 1992)]. Previously, UVR has been shown to activate phospholipase $A_2$ in human skin (Sondergaand et al., 1985) and cultured human keratinocytes (DeLeo et al., 1984), thereby releasing arachidonic acid, the fatty acid precursor of proinflammatory eicosanoids. Although eicosanoids are important in the vascular responses underlying erythema, they do not appear to mediate UVR-induced melanogenesis (Friedmann et al., 1990). However, a further class of second messengers released from the plasma membrane, the 1,2-diacylglycerols (DAGs) may be important in melanogenesis. We and others have demonstrated that adding the synthetic DAG 1-oleoyl-2-acetylglycerol exogenously to cultured human melanocytes (Gordon and Gilchrest, 1989; Friedmann et al., 1990) increases their basal melanin content and augments their melanogenic responses to UVR. However, whether UVR induces endogenous DAG formation in pigment cells has not previously been investigated.

The aim of the present study was, therefore, to determine whether UVR induces endogenous DAG formation in cultured human melanocytes and B16 mouse melanoma cells, and for comparison also in human keratinocytes and Swiss 3T3 mouse fibroblasts.

Phosphatidylinositol (PI)- and phosphatidylcholine (PC)-specific phospholipase C (PLC) and phospholipase D (PLD) have been shown to generate DAGs from different membrane phospholipids [for review see (Exton, 1990)]. In order to determine the probable source of generated DAGs, they can be separated by a technique that utilizes the total number of double bonds in their fatty acids (Kennerly, 1987). DAGs contain two fatty acid groups linked to glycerol, and DAGs from PI, PC or phosphatidylethanolamine (PE) can be distinguished by analysis of their fatty acid composition. Phospholipids tend to contain a saturated fatty acid at the C-1 position, usually palmitate ($C_{16:0}$) or stearate ($C_{18:0}$). In phosphoinositides, the predominant fatty acid at the C-2 position is arachidonate ($C_{20:4}$), whereas in PC and PE unsaturated fatty acids, oleate ($C_{18:1}$) and linoleate ($C_{18:2}$) predominate at C-2, with a smaller contribution of arachidonate (Exton, 1990; Billah and Anthes, 1990; Pettitt and Wakelam, 1993). Thus, DAGs from PI contain mainly four double bonds and those from PC or PE, zero, one or two, with only a minor component of four, double bonds.

DAGs are generated from phospholipids by a number of mechanisms. PI-specific PLC cleaves phosphatidylinositol bisphosphate to release both DAGs and inositol trisphosphate ($IP_3$) (Berridge and Irvine, 1989). Thus activation of PI-specific PLC can be determined by measuring $IP_3$ formation. In addition to generation of phosphatidic acid, PLD catalyses transphosphatidylation of phospholipids, whereby alcohol groups are added to phosphatidyl groups (Dawson, 1967; Kobayashi and Kanfer, 1987; Cook et al., 1991). This reaction is the basis of an assay for measuring PLD activity (Cook et al., 1991).

Abbreviations used: UVR, ultraviolet radiation; DAG, 1,2-diacylglycerol; PLC, phospholipase C; PLD, phospholipase D; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; $IP_3$, inositol trisphosphate; FCS, fetal calf serum; DMPA, dimethyl-[$^{32}$P]phosphatidic acid; HBS, Hepes-buffered saline.

‡ Present address: CRC Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 9BX, U.K.

§ To whom correspondence should be addressed.

## EXPERIMENTAL

### Cell culture

Cultures of human melanocytes and keratinocytes were established from foreskins (donors up to 10 years old). The skin was trimmed of fat, cut into small pieces, and incubated for 18 h in phosphate-buffered saline (PBS, ICN Flow) with Dispase (2 mg/ml, Boehringer–Mannheim) at 4 °C. The epidermis was removed with fine forceps and disaggregated with trypsin (0.05 %, ICN Flow), EDTA (0.02 %) and deoxyribonuclease (0.2 mg/ml) for 5 min at 37 °C. Both melanocytes and keratinocytes were cultured in MCDB153 medium, with a final calcium concentration of 70 $\mu$M, insulin (5 $\mu$g/ml), transferrin (5 $\mu$g/ml), hydrocortisone (0.5 $\mu$M) and amino acids (Wille et al., 1984; Pittelkow and Scott, 1986). In addition, melanocyte medium contained cholera toxin (1 nM), 3 % (v/v) fetal calf serum (FCS, Seralab) and a dialysed saline extract of bovine hypothalami (100 $\mu$g/ml) (Gilchrest et al., 1984; Wilkins et al., 1985), and keratinocyte medium contained epidermal growth factor (10 ng/ml) (Pittelkow and Scott, 1986).

B16 mouse melanoma cells were a gift from Dr. G. Hunt (Dermatology Department, University of Newcastle-upon-Tyne, Newcastle, U.K.) and Swiss 3T3 fibroblasts were a gift from Dr. G. Brooks (St. Thomas' Hospital, London, U.K.). Both cell types were cultured in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10 % FCS.

For all experiments cells were grown to confluence in 35-mm- or 60-mm-diam. culture dishes, and rendered quiescent by growth factor deprivation for 3 to 5 days. Cells were transferred to the experimental medium, Hepes-buffered saline (HBS: NaCl, 116 mM; KCl, 5.4 mM; $CaCl_2$, 70 $\mu$M; $MgCl_2$, 1 mM; glucose, 10 mM; Hepes, 20 mM) 30 min prior to all experiments.

All chemicals were obtained from Sigma, except where otherwise indicated.

### U.v. irradiation

Cells were irradiated at a distance of 35 cm below a bank of four Philips TL12 fluorescent sunlamps, without removing the plastic lid of the culture plate/dish. This filtered out a proportion of the shorter-wavelength emission and resulted in a spectral distribution of 56.6 % u.v.-A (320–400 nm), 43.3 % u.v.-B (290–320 nm), with less than 0.1 % of long-wavelength u.v.-C (280–290 nm), and an irradiance of 0.96 mW/cm² of total UVR. For all experiments, cells received a total UVR dose of 57 mJ/cm². This dose was non-toxic to all cell types tested.

### 1,2-Diacylglycerol formation

DAG was quantified using the method of Preiss et al. (1986). Cells were grown to confluence in 60-mm-diam. culture dishes and rendered quiescent. Cells were irradiated with UVR and at time points following (0, 15 s, 30 s, 1 min, 2 min, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h), the reaction was stopped by aspiration of the medium and addition of 0.5 ml of ice-cold methanol. All further procedures were carried out on ice, unless otherwise indicated. To extract the lipids from the samples, 0.3 ml of chloroform was added to the cells in methanol, which were then left on ice for 20 min. The extract was partitioned into two phases by adding 0.3 ml of chloroform and 0.4 ml of distilled water. The upper aqueous phase was discarded, and the lower organic phase evaporated to dryness under a stream of $N_2$. The residual pellet was dissolved in 2 M NaOH and the protein content determined using the Pierce Micro BCA assay kit.

DAG in the samples was then phosphorylated to [$^{32}$P]phosphatidic acid using DAG kinase (Lipidex, U.S.A.) and [$\gamma$-$^{32}$P]ATP (Amersham, U.K.), for 30 min at 4 °C. Such DAG kinase results in 100 % conversion up to 500 pmol of DAG (J. Ohanian, unpublished work). To allow quantification of DAG, a known amount (5 pg) of 1,2-dioleoylglycerol was also converted into [$^{32}$P]phosphatidic acid. Aliquots of the resultant radioactive extracts were removed and resolved by t.l.c. The samples dissolved in chloroform were spotted on to a Silica Gel 60 t.l.c. plate (Whatman) and developed with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.) (Wright et al., 1988). The t.l.c. plates were dried and labelled phospholipids visualized by autoradiography (Figure 1). The radioactive spot corresponding to phosphatidic acid was located, scraped from the plate and radioactivity quantified in a liquid scintillation counter (Cocktail: Ultima Gold MV; Counter: TR2500, Canberra Packard U.K.). Results are expressed as pmol of DAG/mg of protein, as previously described (Ohanian et al., 1990).

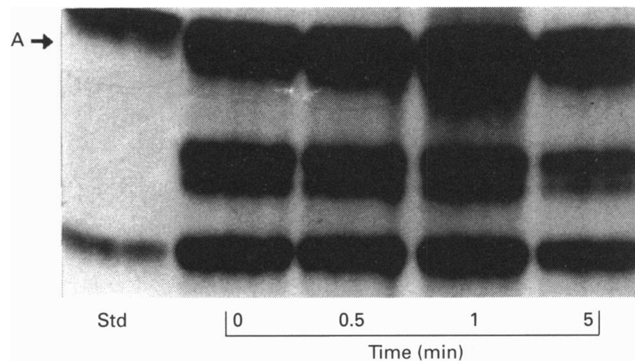


**Figure 1 Time course of DAG formation in Swiss 3T3 murine fibroblasts**

Following a single UVR exposure DAGs were phosphorylated by addition of DAG kinase and [$\gamma$-$^{32}$P]ATP, separated from other lipids by t.l.c., and visualized by autoradiography, as described in the Experimental section. Band A, [$^{32}$P]phosphatidic acids. The further phosphorylated bands represent ceramide phosphates (middle pair of bands) and lysophosphatidic acids (lower band). The left-hand lane contains standard (Std) 1,2-dioleoylglycerol; other lanes show the time course following exposure to UVR.

Following conversion into phosphatidic acid the remainder of the lipid extract was separated into subgroups of phosphatidic acid.

### Separation of subgroups of phosphatidic acid

Lipid extracts were converted into dimethyl esters by the method of Kennerly (1987), using a solution of diazomethane in ether. The resulting dimethyl-[$^{32}$P]phosphatidic acids (DMPA) were resolved by t.l.c. Extracts were spotted on to Silica Gel 60 t.l.c. plates and developed in ethyl acetate. The radioactive spot corresponding to DMPA was located using autoradiography and scraped. The DMPA was eluted from the silica gel by twice washing with 1 ml of chloroform/methanol (3:1, v/v). The eluate was dried under a stream of $N_2$, and samples in chloroform were spotted on to a Silica Gel 60 t.l.c. plate that had been impregnated with 5 % (w/v) $AgNO_3$ (Kennerly, 1987). The plate was developed to 120 mm above the origin with chloroform/methanol (98:6, v/v), air dried and then developed to within 10 mm of the top of the plate with chloroform/methanol (98.5/1.5, v/v). This double development system gave good separation of the different species of DMPA, according to their degree of unsaturation. A mix of known standards was used to identify different bands as located by autoradiography (Figure

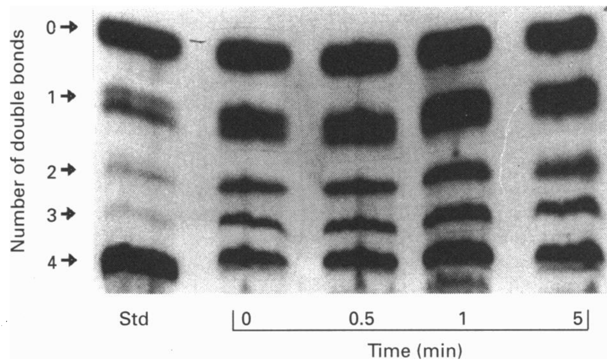


**Figure 2 Argentation t.l.c. of the dimethyl phosphatidic acid esters of DAG formed in Swiss 3T3 fibroblasts following a single exposure to UVR**

[$^{32}$P]DMPA was prepared from aliquots of DAG, separated according to the total number of double bonds in DAG by argentation t.l.c., and visualized by autoradiography, as described in the Experimental section. Left-hand lane, known mix of standards; other lanes, time course following exposure to UVR.

2). The amount of radioactivity in each band was quantified and expressed as pmol of DAG/mg of protein.

## $IP_3$ formation

For $IP_3$ measurement, cells were grown to confluence in 35-mm-diam. culture dishes and rendered quiescent. Cells were irradiated with UVR and at time points following (0, 15 s, 30 s, 1 min), the reaction was stopped by aspiration of the medium and addition of 0.5 ml of ice-cold methanol. Cell lipids were extracted from the samples as described in the section on DAG quantification. The upper aqueous layer, containing $IP_3$, was removed and the $IP_3$ content determined using the Amersham [$^3$H]D-*myo*-inositol 1,4,5-trisphoshate assay system. Results are expressed in c.p.m. as a percentage of unirradiated control cells.

## PLD activity

PLD activity was determined in an assay which utilizes its ability to catalyse transphosphatidylation of phospholipids. In this assay the phosphatidyl moiety is transferred to butan-1-ol to form phosphatidylbutanol (Cook et al., 1991). Cells were grown to confluence in 35-mm-diam. dishes and rendered quiescent. They were then cultured for a further 48 h in their normal culture medium containing additionally 4 $\mu$Ci of [$^3$H]palmitic acid (Amersham) per dish. For measurement of PLD activity, cells were incubated in HBS for 30 min at 37 °C prior to incubation for a further 10 min in 1 ml of HBS containing 30 mM butan-1-ol (0.3 %, v/v). Cells were then irradiated with UVR, and the reaction stopped by addition of 0.5 ml of ice-cold methanol at the times indicated. The number of cells present per dish was determined by counting cells in replicate dishes in a Coulter counter (model ZM).

Cell lipids were extracted as described in the section on DAG quantification. The lower organic phase was dried under $N_2$, samples in chloroform were spotted on to Whatman LK5DF t.l.c. plates, and the t.l.c. plates were developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.). [$^3$H]Phosphatidylbutanol, identified by its co-migration with an authentic [$^{14}$C]phosphatidylbutanol standard (a gift from Dr. R. Randall, Wellcome Research Laboratories, Beckenham, Kent, U.K.), was scraped from the plates and its radioactivity determined by scintillation counting. Results are expressed either as c.p.m. per $10^6$ cells or as c.p.m. as a percentage of control sham-irradiated cell counts.

## [$^3$H]Choline release

Cells were grown to confluence in 35-mm-diam. dishes and rendered quiescent. PC in the cells was labelled by addition of [$^3$H]choline (Amersham, U.K.) to their normal culture medium for 24 h. Prior to u.v.-irradiation, cells were incubated for 30 min in HBS. To measure the amount of [$^3$H]choline-labelled metabolites released, a 100 $\mu$l sample was taken from the HBS 1 h post-irradiation and the radioactivity determined by scintillation counting (Punnonen and Yuspa, 1992). Results are expressed in c.p.m. as a percentage of sham-irradiated controls.

# RESULTS

## DAG formation

UVR induced a biphasic pattern of DAG formation in all the cell types examined (Figures 3a–3d). An early transient rise, peaking between 15 and 30 s, was followed by a decrease, then a further delayed more sustained rise peaking between 15 and 30 min (Figures 3a–3d).

In order to attempt to identify the phospholipid source of the DAGs generated in the two peaks, DAGs were separated according to their degree of unsaturation. The percentages of the resting levels of DAG containing a total of zero, one, two, three,

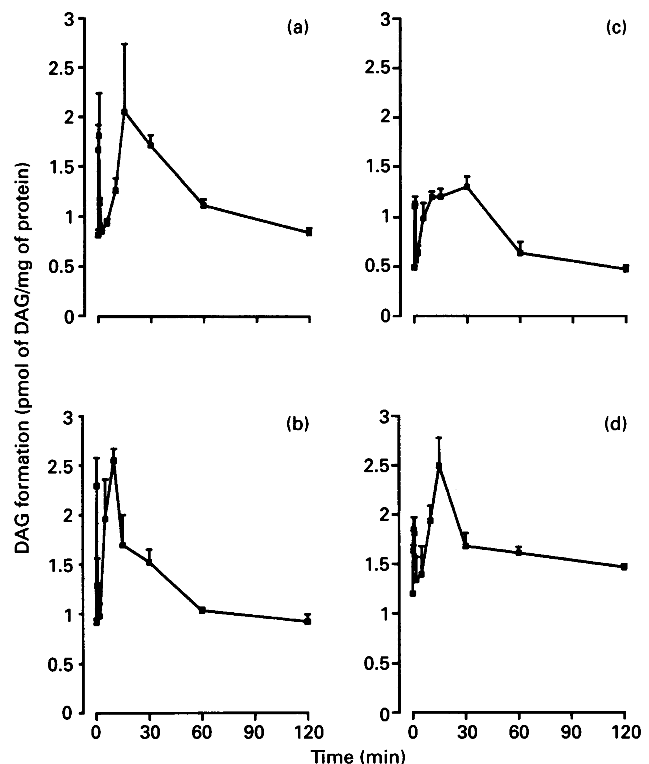


**Figure 3 Time course of DAG formation in response to a single dose of UVR**

Cells were cultured in 60-mm-diam. culture dishes and exposed to a single dose of UVR. DAGs were extracted and quantified, as described in the Experimental section. UVR induced a biphasic time-dependent increase in DAG formation in (**a**) human melanocytes, (**b**) human keratinocytes, (**c**) B16 murine melanoma cells and (**d**) Swiss 3T3 murine fibroblasts. Data are expressed as mean ± S.E.M. from two experiments per cell type.

**Table 1 Subgroups of DAG comprising the resting level of DAG**

Prior to exposure to UVR, the species of DAG present in each cell type were separated according to the total number of double bonds present in their fatty acid side chains, as described in the Experimental section. Values are given as percentages of the total resting levels of DAG due to each subgroup of DAG, expressed as means from two experiments.

| | Number of double bonds in DAGs* (% of each group) | | | | | |
|---|---|---|---|---|---|---|
| | 0* | 1 | 2 | 3 | 4 | 4+ |
| Human melanocytes | 42.2 | 32.1 | 13.5 | 4.5 | 6.4 | 1.3 |
| Human keratinocytes | 36.5 | 28.6 | 15.1 | 6.3 | 9.4 | 4.1 |
| B16 melanoma cells | 42.2 | 37.3 | 7.5 | 5.4 | 5.4 | 2.2 |
| Swiss 3T3 fibroblasts | 36.9 | 27.7 | 10.1 | 6.0 | 15.7 | 3.6 |

* Total number of double bonds in two fatty acid chains in DAGs.

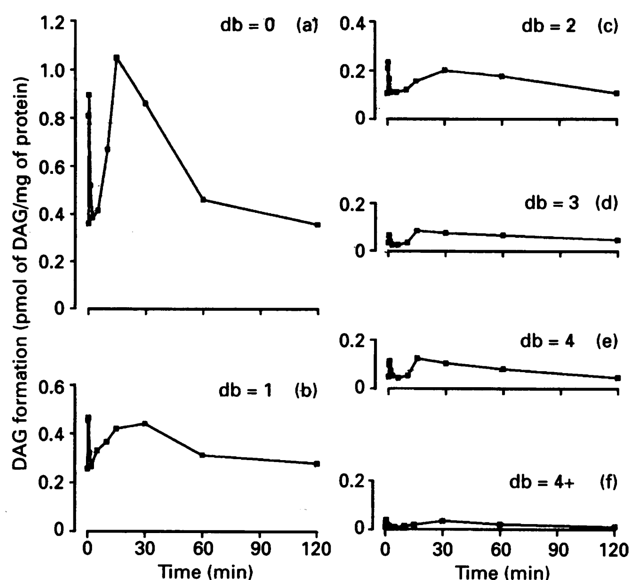


**Figure 4 Separation of subgroups of DAG formed by human melanocytes in response to a single exposure to UVR**

Subgroups of DAG were separated according to the number of double bonds present in their fatty acid chains, as described in the Experimental section. (**a**) 0, (**b**) 1, (**c**) 2, (**d**) 3, (**e**) 4, (**f**) more than 4 double bonds. Data are expressed as the mean from two experiments.

four and more than four double bonds are shown in Table 1. In all four cell types the predominant subgroups of DAG contain a total of zero, one or two double bonds.

In human melanocytes all the subgroups of DAGs followed a similar pattern to total DAG, but the major DAGs contributing to the total levels were the subgroups of DAG containing zero or one double bonds (Figures 4a–4f). The early rise peaked at 30 s, with an increase in DAG level from 0.36 to 0.90 pmol of DAG per mg of protein at 30 s. A total of 87.5 % of this rise was due to the subgroups of DAGs with two or less double bonds in their fatty acid chains (Table 2). Only 6.5 % of the rise was due to DAGs with four double bonds. Similarly 87.0 % of the delayed rise, peaking at 15 min, was due to subgroups of DAGs with two or less double bonds and only 7.2 % due to DAGs with four double bonds.

Similarly, in human keratinocytes and B16 melanoma cells, at least 80 % of the DAGs formed had two or less double bonds in both the early and delayed peaks, and less than 5 % of generated DAGs contained four double bonds (Table 2).

In Swiss 3T3 fibroblasts, a proportion of the DAGs generated also had two or less double bonds, 48.2 % in the early peak and 54.5 % in the delayed peak. However, 18 % of DAGs in the early peak and 20 % in the delayed peak contained four double bonds.

## $IP_3$ formation

In a response to a number of agents in a variety of cell types, early DAG increases are due, at least in part, to activation of PI-specific PLC. All four cell types tested responded to UVR with a significant rise (Student's *t*-test, $P < 0.01$) in $IP_3$, which peaked at 30 s before decreasing to control levels (Figure 5).

## PLD activity

Delayed DAG rises have been shown in a number of cell types to be from activation of PLD and cleavage of PC (Exton, 1990). In human melanocytes, UVR stimulated a small but significant increase in PLD activity by 15 min (Student's *t*-test, $P < 0.01$), as assessed by its ability to catalyse a transphosphatidylation reaction (Figure 6a). [$^3$H]Phosphatidylbutanol continued to accumulate for 1 h post u.v.-irradiation (Figure 6a, Table 2), after which the levels plateaued (Figure 6a). Similar results were observed following exposure of Swiss 3T3 fibroblasts to UVR (Figure 6b, Table 3).

Similarly, in human keratinocytes and B16 melanoma cells, a rise in PLD activity was observed 1 h post u.v.-irradiation (Table 3).

## [$^3$H]Choline release

UVR stimulated release of [$^3$H]choline metabolites from all the cell types, indicating that PC metabolism is altered (Table 2).

## DISCUSSION

In our previous studies of mechanisms by which UVR activates melanogenesis, we had observed that addition of the synthetic DAG 1-oleoyl-2-acetylglycerol would augment both basal and UVR-induced melanogenesis. Therefore the aim of the present study was to determine whether exposure to UVR results in formation of DAGs from endogenous sources, and if so to characterize the subgroups of DAG formed, their source and the mechanism of their formation. We found that in human melanocytes and B16 mouse melanoma cells, as well as human keratinocytes and Swiss 3T3 fibroblasts, exposure to a single non-lethal dose of UVR resulted in a biphasic pattern of endogenous DAG formation. An early rise, peaking between 15 and 30 s, was followed by a more delayed rise, peaking between 15 and 30 min and sustained for up to 1 h. The early rise appeared to reflect activation of PLC and was associated with an increase in $IP_3$ levels, while the delayed rise was associated with activation of PLD and was accompanied by release of PC metabolites.

Much previous work has focused on UVR-induced phospholipase $A_2$-mediated release of arachidonic acid (DeLeo et al., 1984; Sondergaand et al., 1985), the precursor of eicosanoids such as prostaglandins which are responsible, at least in part, for UVR-induced erythema. However, prostaglandins have little effect on either basal or UVR-induced melanogenesis in pigment cells (Friedmann et al., 1990). In previous studies, by ourselves and others, the synthetic DAG 1-oleoyl-2-acetylglycerol, when

**Table 2 Subgroups of DAG in early and delayed increases in DAG formation**

Cells were exposed to a single dose of UVR, and a biphasic pattern of total DAG formation was observed. At the time points representing the two peaks, the subgroups of DAG present were separated according to the total number of double bonds present in their fatty acids, as described in the Experimental section. The increases in amount of each of the subgroups are expressed as a percentage of the resting level of each subgroup (see Table 1).

| | | Number of double bonds in DAGs† | | | | | |
|---|---|---|---|---|---|---|---|
| | Time* | 0 | 1 | 2 | 3 | 4 | 4+ |
| Human melanocytes | 30 s | 53.5‡ | 21.0‡ | 13.0‡ | 3.0‡ | 6.5‡ | 3.0‡ |
| | 15 min | 66.3§ | 15.9§ | 4.8§ | 4.8§ | 7.2§ | 1.0§ |
| Human keratinocytes | 30 s | 31.1‡ | 37.0‡ | 13.0‡ | 12.7‡ | 4.2‡ | 2.0‡ |
| | 15 min | 29.1§ | 39.5§ | 22.1§ | 5.1§ | 3.5§ | 0.7§ |
| B16 melanoma cells | 15 s | 57.0‡ | 32.5‡ | 6.5‡ | 1.6‡ | 2.4‡ | 0‡ |
| | 15 min | 50.7§ | 33.8§ | 10.6§ | 2.1§ | 2.1§ | 0.7§ |
| Swiss 3T3 fibroblasts | 30 s | 23.6‡ | 10.4‡ | 14.2‡ | 15.1‡ | 19.8‡ | 16.9‡ |
| | 15 min | 22.1§ | 17.1§ | 15.3§ | 16.2§ | 22.5§ | 6.8§ |

* Time, from exposure to UVR, of peak in DAG formation.
† Total number of double bonds in two fatty acid chains in DAGs
‡ Early increase in DAG, percentage of rise due to subgroup of DAGs, expressed as mean from two experiments.
§ Delayed increase in DAG, percentage of rise due to subgroup of DAGs, expressed as mean from two experiments.

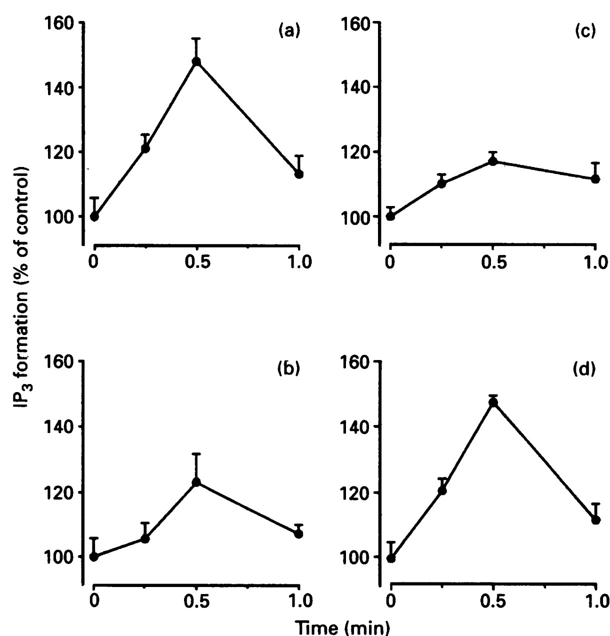


**Figure 5 UVR induces a transient increase in $IP_3$ formation in response to a single exposure to UVR**

Cells were cultured in 35-mm-diam. dishes and at time points following exposure to a single dose of UVR, $IP_3$ was extracted and quantified as described in the Experimental section. (**a**) Human melanocytes, (**b**) human keratinocytes, (**c**) B16 murine melanoma cells, and (**d**) Swiss 3T3 murine fibroblasts. Data are expressed as mean ± S.E.M. from two experiments per cell type.

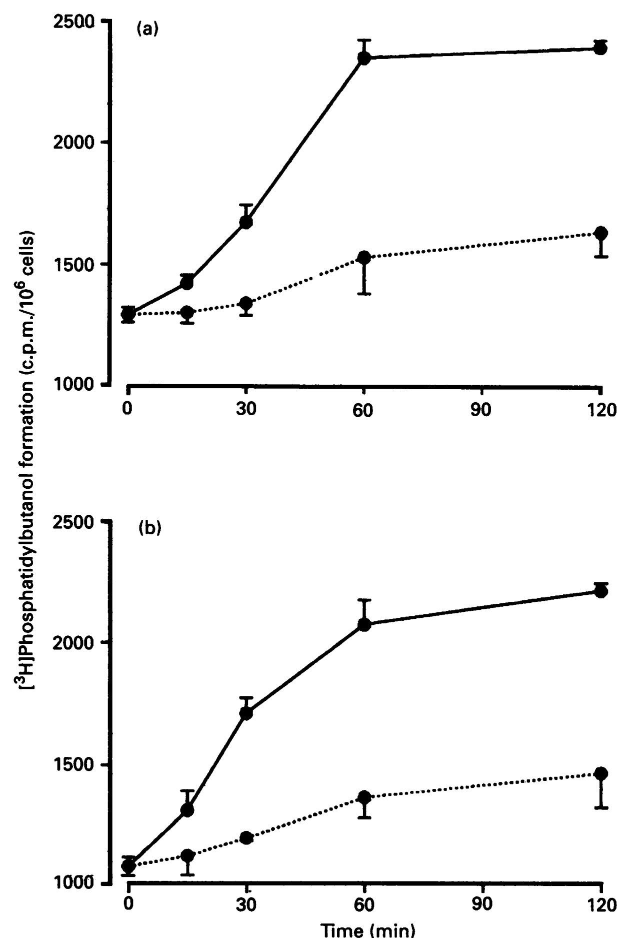


**Figure 6 UVR induces increased PLD activity**

Time-dependent increase in PLD activity, as measured by [$^3$H]phosphatidylbutanol formation, as described in the Experimental section, in sham-irradiated control cells (dotted line) and in response to a single exposure to UVR (full line), measured in (**a**) human melanocytes and (**b**) Swiss 3T3 murine fibroblasts. Data are expressed as mean ± S.E.M. from three experiments per cell type.

added to cultured pigment cells or to murine skin, both increased basal melanin content (Gordon and Gilchrest, 1989; Friedmann et al., 1990; Agin et al., 1991) and augmented UVR-induced melanogenesis (Friedmann et al., 1990; Agin et al., 1991). Here we have shown that UVR also stimulates endogenous formation of DAGs from phospholipids in pigment cells, as well as human keratinocytes and Swiss 3T3 fibroblasts. Taken together the

**Table 3 UVR-induced stimulation of PLD activity and [$^3$H]choline metabolites release**

PLD activity and [$^3$H]choline release following exposure to UVR were measured as described in the Experimental section. Values of [$^3$H]phosphatidylbutanol (PtdBut) accumulation 1 h post-u.v.-irradiation were determined as c.p.m. and then given as a percentage of control sham-irradiated cell c.p.m. [$^3$H]Choline metabolites accumulation in HBS 1 h post-u.v.-irradiation were also determined as c.p.m. and given as a percentage of control sham-irradiated cell c.p.m. All data are means ± S.E.M. from at least three experiments.

| Cell type | [$^3$H]PtdBut accumulation (% of control) | [$^3$H]Choline accumulation (% of control) |
|---|---|---|
| Human melanocytes | 142±11 | 147±10 |
| Human keratinocytes | 122±1 | 131±7 |
| B16 melanoma cells | 118±3 | 125±8 |
| Swiss 3T3 fibroblasts | 130±8 | 132±9 |

studies of pigment cells suggest that DAGs may be important in mediating UVR-stimulated melanin synthesis. DAGs exert their effects by modulating the activity of a number of enzymes, mainly protein kinase C (Nishizuka, 1988; Asaoka et al., 1992), but also PLC, PLD and DAG kinase (Billah and Anthes, 1990). A number of workers have investigated the role of protein kinase C in melanogenesis, but there are contradictions in their findings. Some studies suggest that the enzyme is important (Park et al., 1993), and others that it plays no part (Friedmann et al., 1990; Carsberg et al., 1994).

Biphasic patterns of DAG generation have been seen in a number of cells types in response to a variety of agents (Exton, 1990), including by Swiss 3T3 fibroblasts in response to bombesin (Cook et al., 1991). The initial DAG formation is transient, over seconds, and most often results from activation of PI-specific PLC, resulting in formation of $IP_3$ (Berridge and Irvine, 1989). However, activation of PC-specific PLC has also been demonstrated (Choudhury et al., 1991). This early transient rise is frequently followed by a more sustained elevation of DAG, over minutes. Extensive analyses of fatty acid compositions has indicated that the second phase of DAG formation is due to hydrolysis of PC in various stimulated cells, with PE a minor source (Billah and Anthes, 1990). Such sustained formation of DAG is thought to be essential for longer-term physiological responses such as cell proliferation and differentiation (Agwu et al., 1989). Several mechanisms release DAG from PC, including activation of PC-specific PLC and activation of PLD, resulting in the formation of phosphatidic acid which is then converted into DAG by phosphatidic acid phosphohydrolase (Exton, 1990; Billah and Anthes, 1990). PLD activity can result directly from receptor stimulation, or indirectly for example through protein kinase C, activated as a result of early DAG formation (Exton, 1990; Billah and Anthes, 1990). Phospholipase $A_2$-derived fatty acids and lysophosphatidylcholine have also been shown in some systems to enhance the delayed formation of DAGs, mainly through modulation of protein kinase C activity (Asaoka et al., 1992).

In this study we attempted to determine the phospholipid sources and mechanisms of the u.v.-induced biphasic DAG rise. To determine the source of the DAGs, whether from PI or PC, subgroups were separated according to the degree of unsaturation of their fatty acids. In all four cell types, DAGs containing two or less double bonds in their fatty acid chains are the major subgroups comprising both the early and delayed DAG peaks, although a small component of DAGs with four double bonds was present. Hence, since subgroups of DAG from PI mainly contain four double bonds, it appears that UVR-stimulated DAG formation is predominantly from sources other than PI. DAGs from PC and PE are mainly comprised of fatty acids with a total of two or less double bonds. PC is therefore the most likely major source, although purely from the results of the present study, the involvement of PE cannot be excluded.

Since PI-derived DAGs contain mainly four double bonds and PI is a minor constituent of the plasma membrane compared with PC, it is possible that the small quantities of DAGs with four double bonds may have been derived from either or both of these phospholipids. The early rise in DAGs in response to UVR was accompanied by a transient rise in $IP_3$, indicating that PI turnover had occurred and that, at least in this early rise, PI-specific PLC is activated (Berridge and Irvine, 1989). UVR has also been shown to induce $IP_3$ formation in lymphocytes, where PI-specific PLC is activated following phosphorylation by tyrosine kinase (Schieven et al., 1993). The mechanism underlying the source of the early DAGs not derived from PI has not been investigated in the present study, but PC-specific PLC is the likely pathway.

The mechanisms underlying the delayed rise in DAGs in response to UVR appeared to have been activation of PLD, which was demonstrated. Furthermore the release of PC metabolites provides further evidence of PC breakdown.

In conclusion, in this study we have demonstrated that UVR stimulates a biphasic rise in DAG production in a number of cell types, including human melanocytes and mouse B16 melanoma cells, as well as human keratinocytes and mouse Swiss 3T3 fibroblasts. These changes result from the action of phospholipases C and D on plasma membrane phospholipids. This endogenous DAG formation by pigment cells is further evidence for the involvement of DAGs in UVR-induced melanogenesis. Since similar increases in DAGs are seen in other cell types, it is possible that DAGs may also be involved in other UVR-induced events.

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