# Wavelength specific activation of PI 3-kinase by UVB irradiation

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Received 24 September 1998; received in revised form 17 November 1998

**Abstract We investigated the effect of UVB irradiation on phospholipid metabolism using normal human diploid skin fibroblasts. When cells were exposed to monochromatic UV light, the intracellular $PIP_3$ level was increased within 1 min in a wavelength-specific manner. The increase was most marked in the UVB range and was inhibited by a PI 3-kinase inhibitor, tyrosine kinase inhibitor, or antioxidant reagents. Furthermore, the decrease of cell viability induced by UVB irradiation was significantly blocked by wortmannin. These results suggest that PI 3-kinase activation is involved in novel cellular responses specific for UVB that lead to cell death.**



*Key words:* Ultraviolet B; Phosphatidylinositol 3-kinase; Cell death; Tyrosine kinase; Reactive oxygen intermediate

## 1. Introduction

Ultraviolet (UV) irradiation accounts for most of the harmful biological effects of sunlight, including cancer in mammals and malformations in amphibians. UV is known to interact quite differently with cells according to the wavelength range. The UVB portion (290–320 nm) is thought to be important in the harmful effects [1,2]. It has been shown that UVB damages biological macromolecules including lipids, proteins, and nucleic acids, and the effect of UVB on DNA has been investigated in detail [3]. The most significant DNA lesions generated by UVB are cyclobutane pyrimidine dimers, which constitute 70–90% of aberrant DNA photoproducts. The production of DNA lesions increases linearly with UVB exposure, and is the major factor responsible for the decrease in cell viability. This effect is caused by slowing transcription and mitosis and by expending extra energy for DNA repair, resulting in various abnormalities such as cancers [4]. In addition to these DNA lesions, UVB also generates various reactive oxygen intermediates (ROIs) [5] by biochemical processes including lipid peroxidation [6]. ROIs affect cellular activities and frequently induce the decrease of cell viability. Antioxidants, such as glutathione and dimethyl sulfoxide (DMSO), block UVB-induced apoptosis [7]. Furthermore, UVA (320–400 nm), which produces ROIs but not cyclobutane pyrimidine dimers, induces significant cell death [8]. These results suggest that the UVB-dependent pathways required for ROI-induced cell death are distinct from those associated with DNA lesions.

It has been reported that UV irradiation activates various signal transduction pathways and induces the expression of specific genes [9–11]; UV irradiation triggers the activation of cell surface receptors, such as EGF receptor and TNF receptor [12], *src* family tyrosine kinases [13], and the small GTP binding proteins Ras [14] and Rac [15]. Particularly, UVC (200–290 nm) activates MAP kinase family proteins such as c-Jun N-terminal kinase (JNK), which in turn activates various transcription factors such as AP-1 [16] and ATF-2 [17]. Furthermore, UVC irradiation affects phospholipid metabolism; that is, the hydrolysis of sphingomyelin, which generates the second messenger ceramide, is enhanced specifically by UVC, followed by the stimulation of the JNK signaling pathway [18]. On the other hand, UVB activates phospholipase $A_2$, which generates inflammation mediators such as $PGE_2$ [19]. Here, we report the UVB-specific activation of phosphatidylinositol 3-kinase (PI 3-kinase), which plays an important role in the UVB-dependent decrease of cell viability. ROIs specifically generated by UVB mediate this PI 3-kinase activation.

## 2. Materials and methods

### 2.1. Cell culture and UV irradiation

Neonatal human skin fibroblast cells (NG1RGB) were obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in RITC 80-7 medium supplemented with 5% fetal calf serum. Sub-confluent cultures from passages 3–8 were used in this study. For UV irradiation of cell cultures, the culture medium was replaced with phosphate-buffered saline (PBS). The source of the irradiation was an overhead UV lamp [T20LN for UVA (peak at 365 nm), T20M for UVB (peak at 312 nm), T20C for UVC (peak at 254 nm); Vilber Lourmat, Marne La Vallee, France]. Irradiation was controlled by altering the distance between the lamp and the cells and monitored with a broad band energy meter (13PEM001, Melles Griot, USA). Alternatively, cells suspended in PBS at a concentration of $10^6$ cells/ml were used for monochromatic UV irradiation. Irradiation was carried out in quartz vessels using a spectrofluorophotometer (RF5300PC, Shimazu, Japan) as a UV source. The UV energy was controlled either by the distance or by the irradiation time. Under these conditions, there was negligible loss of cell viability over the longest irradiation periods employed (approximately 2 min). For serum starvation, cells were maintained for 48 h in RITC 80-7 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 1 mg/ml bovine serum albumin.

### 2.2. Cell viability

Cell viability was measured by MTT assay at 24 h post irradiation as described elsewhere [20]. Briefly, MTT (final concentration 0.5 mg/ml) was added to a culture dish and the cells were incubated for 30 min. Then, the medium was removed from the cultures and mixed with DMSO to solubilize the MTT-formazan product. The values of the absorbance at 595 nm were plotted as a measure of the relative number of cells.

### 2.3. Analysis of phospholipid

The production of phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) in intact cells was measured by $^{32}P$ labeling of the cellular phospholipids. Cells were seeded in 35-mm culture dishes at a density of $1\times10^5$ cells/dish and cultured for 3 days. To label the cells, the medium was replaced with phosphate-free medium containing 10 mM

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*Abbreviations:* UVB, ultraviolet B; PI 3-kinase, phosphatidylinositol 3-kinase; ROI, reactive oxygen intermediate; $PIP_3$, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B


PII: S0014-5793(98)01565-8

HEPES-NaOH (pH 7.5), 1% fetal calf serum (previously dialyzed against 0.9% NaCl), and [$^{32}$P]orthophosphate (0.1 mCi/ml), and the cells were incubated at 37°C for 2 h. UV irradiation was carried out as described above, and the reaction was stopped by adding of 1.8 ml chloroform/methanol/8% $HClO_4$ (50/100/5, by vol). Chloroform and 1% $HClO_4$ (each 0.5 ml) were added to each sample, and the resultant solutions were centrifuged at 10 000 × *g* for 1 min. The lower organic phase was collected and washed twice with 1% $HClO_4$/1 M NaCl solution (chloroform-saturated). After drying in vacuo, the lipid sample was dissolved in 50 μl of chloroform/methanol (5/1, by vol). For thin layer chromatography (TLC), the samples were spotted onto a silica gel 60 plate (20 × 20 cm, Merck) pretreated with potassium oxalate, and developed in chloroform/methanol/acetone/acetic acid/water (40/15/13/12/7, by vol). The radioactivity in the spots was quantitated with a Fuji BAS 1000 bioimaging analyzer. The $PIP_3$ spot was identified from the standard synthesized by PI 3-kinase in vitro as described previously [21].

*2.4. Phosphorylation analysis*

Cells were labeled and UV-irradiated as described above, and then lysed with RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxychloric acid, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 100 mM NaF, 10 mM β-glycerophosphate, 10 mM $Na_3VO_4$). Cell debris was removed by centrifugation. PI 3-kinase was immunoprecipitated with anti-PI 3-kinase antibody (Santa Cruz). After washed three times with RIPA buffer, the immunoprecipitates were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

## 3. Results

*3.1. Activation of PI 3-kinase in a wavelength-specific manner*

We first analyzed the wavelength-specific effect of UVB on phospholipid metabolism using normal human diploid skin fibroblasts, NG1RGB. [$^{32}$P]Orthophosphate-labeled cells in suspension were exposed to monochromatic UV light for 1 min (100–250 J/m$^2$), and phospholipids were analyzed by TLC and autoradiography. Among several changes observed in the phospholipid fractions, the most evident was in the $PIP_3$ fraction (Fig. 1A). Intracellular $PIP_3$ levels were elevated dramatically by irradiation with 300 nm UVB. Moderate increases in the $PIP_3$ level were observed in cells exposed to 260 nm, 280 nm, or 320 nm light. In contrast, the $PIP_3$ level was not altered by irradiation with 340 nm or 360 nm UVA. When cells were irradiated by UVB (300 nm, 250 J/m$^2$) for 1 min, the $PIP_3$ level was increased about 5-fold relative to non-stimulated cells (Fig. 1B). Cells stimulated by serum for 2 min exhibited a 20-fold increase in the $PIP_3$ level, indicating that the effect of UVB was less potent than that of serum.

Since PI 3-kinase is a major enzyme that produces $PIP_3$, we tested wortmannin and LY294002, specific inhibitors of PI 3-kinase, on UVB-dependent $PIP_3$ formation. As expected,

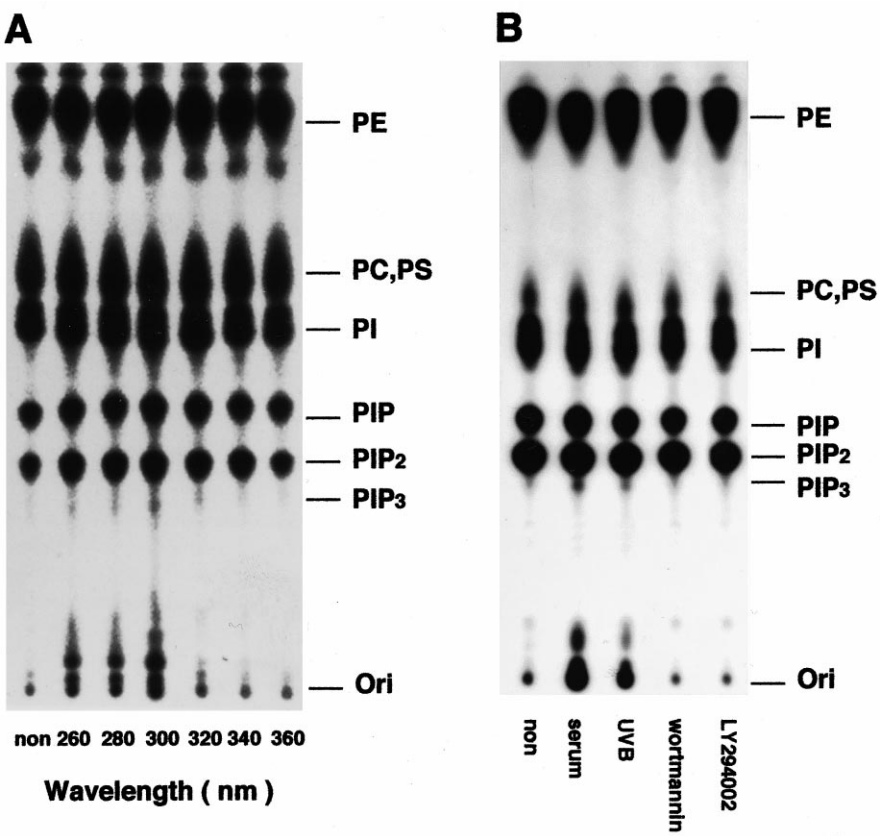


Fig. 1. Elevation of intracellular $PIP_3$ level by UV irradiation. A: Effect of monochromatic UV irradiation on phospholipid metabolism. $^{32}$P-labeled cells in suspension were exposed for 1 min to monochromatic UV irradiation ranging from 260 nm to 340 nm at intervals of 20 nm. The irradiated energy was varied from 100 to 250 J/m$^2$ with increasing wavelength. After irradiation, phospholipids were extracted and separated by TLC. An autoradiogram of the TLC plate used to detect $PIP_3$ is shown. The positions of phospholipids are indicated. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS; phosphatidylserine; PI: phosphatidylinositol; PIP: phosphatidylinositol monophosphate; $PIP_2$; phosphatidylinositol bisphosphate; $PIP_3$; phosphatidylinositol 3,4,5-trisphosphate. B: Effect of serum and PI 3-kinase inhibitors on $PIP_3$ levels. $^{32}$P-labeled cells in suspension were treated as follows; non: maintained in serum-free medium; serum: stimulated with serum for 2 min; UVB: irradiated by UVB (300 nm, 250 J/m$^2$) for 1 min; wortmannin and LY294002: pretreated with either $10^{-7}$ M wortmannin or $10^{-5}$ M LY294002 for 30 min, and then irradiated by UVB (300 nm, 250 J/m$^2$) for 1 min. Phospholipids were extracted and analyzed as described in A.

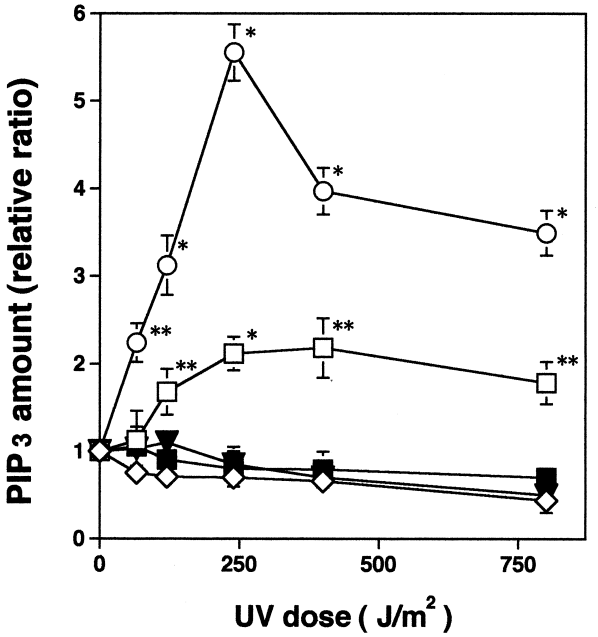


Fig. 2. Dose-dependent change in intracellular $PIP_3$ levels after UV irradiation. Fibroblast cells on culture dishes were irradiated directly by overhead UVA (◇), or UVB (○) lamp for 2 min, or UVC (□) lamp for 1 min. The UV energy doses were controlled by the distance. The effect of PI 3-kinases was evaluated using cells pretreated with $10^{-7}$ M wortmannin (■) or $10^{-5}$ M LY294002 (▼) for 30 min before UVB irradiation. $PIP_3$ was separated by TLC and quantified by an image analyzer. The amounts of $PIP_3$ were plotted relative to the amounts in non-irradiated cells. Data shown are the means ± S.D. of three independent experiments. Statistical significances between UVB- or UVC-irradiated and the non-irradiated control groups were calculated by Student's *t*-test; $^{**}P < 0.05$, $^{*}P < 0.01$.

treatment of fibroblasts with wortmannin or LY294002 abolished the elevation of $PIP_3$ levels induced by UVB irradiation (Fig. 1B). These results show that UVB irradiation enhances the enzyme activity of PI 3-kinase in a wavelength-specific manner.

To confirm whether the UVB-associated increase in $PIP_3$ occurs under adherent conditions, cell cultures maintained on dishes were exposed directly to UV light using overhead UV lamps, and the phospholipids were analyzed by the same method as for the cell suspensions. The effect on $PIP_3$ formation varied among UVA, UVB, and UVC (Fig. 2). The $PIP_3$ levels were increased by UVB irradiation up to 250 J/m$^2$ and gradually decreased at energies over 800 J/m$^2$. The $PIP_3$ formation was rapid and transient; the level reached a maximum at 2 min following UVB irradiation, and then decreased to the basal level (data not shown). The UVB-dependent $PIP_3$ formation was completely suppressed by PI 3-kinase inhibitors at all energy levels (Fig. 2). In contrast, UVC moderately enhanced the $PIP_3$ level up to 250–400 J/m$^2$, but the effect was less potent than that of UVB. UVA did not induce $PIP_3$ formation at any energy level examined. These results on monolayer cultures are similar to those of cell suspensions (Fig. 1).

### 3.2. Tyrosine kinase and ROIs in UVB-induced PI 3-kinase activation

In order to identify upstream factors involved in PI 3-kinase activation, we examined tyrosine kinase activity in cells exposed to UVB. Fig. 3 shows the effect of a tyrosine kinase inhibitor, genistein, on intracellular $PIP_3$ levels. As is the case with PI 3-kinase inhibitors (Fig. 2), genistein completely abolishes the elevation of $PIP_3$ levels. In this case, the phosphorylation of the p85 subunit of PI 3-kinase is induced by UVB irradiation, and this is inhibited by genistein (Fig. 3, inset). On the other hand, $AlF_4^-$, a G-protein activator, has no effect the increase in the $PIP_3$ levels (data not shown). These results indicate that tyrosine phosphorylation of the p85 subunit may be important in UVB-dependent PI 3-kinase activation, and that G-proteins are unlikely to mediate this pathway.

It has been shown that UVB irradiation induces the formation of ROIs. We also examined the possibility that ROIs induced by UVB participate in PI 3-kinase activation. Vitamin E, a fat-soluble antioxidant that suppresses the formation of organic ROIs, completely inhibits the elevation of $PIP_3$ levels (Fig. 3). On the other hand, nitroarginine methyl ester hydrochloride (NAME), a general inhibitor of NO synthase, does not significantly affect the activation of PI 3-kinase, indicating that free radicals rather than NO species participate in PI 3-kinase activation.

### 3.3. Effect of UVB on cell viability

We analyzed the apoptotic effects of UV using NG1RGB cells. The effect was significantly evident in the UVB-UVC region. When cells in suspension were irradiated by mono-

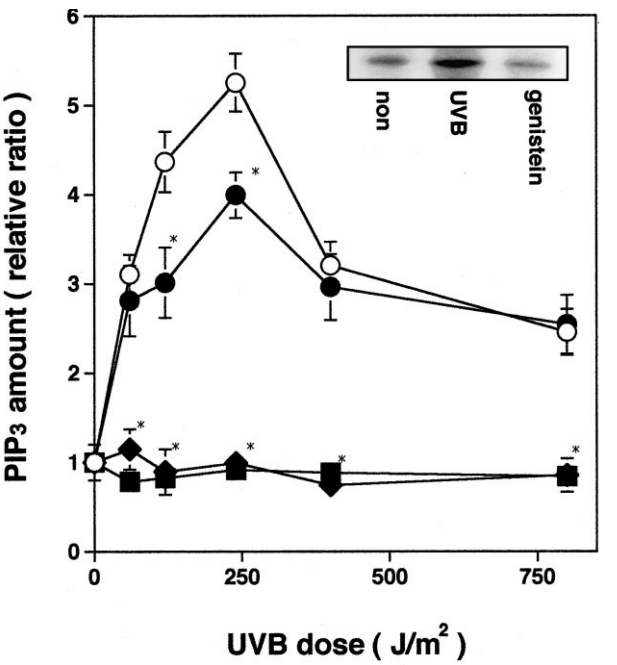


Fig. 3. Effect of antioxidants and tyrosine kinase inhibitor on $PIP_3$ formation induced by UVB irradiation. Intracellular levels of $PIP_3$ after UVB irradiation were measured as described in Fig. 2. $^{32}$P-labeled cells were preincubated with 100 μg/ml genistein (■) or 1 mM vitamin E (◆), 100 μg/ml NAME (●), or vehicle (○) for 30 min, and then irradiated with the indicated doses of UVB. Statistical significances between the antioxidant- or kinase inhibitor-treated and the non-treated control groups were calculated by Student's *t*-test; $^{**}P < 0.05$, $^{*}P < 0.01$. Inset: Phosphorylation of the p85 subunit of PI 3-kinase. $^{32}$P-labeled cells pretreated with or without the tyrosine kinase inhibitor genistein were exposed to UVB irradiation (250 J/m$^2$). Then, p85 was recovered in the immunoprecipitates and its phosphorylation was analyzed by SDS-PAGE and autoradiography.

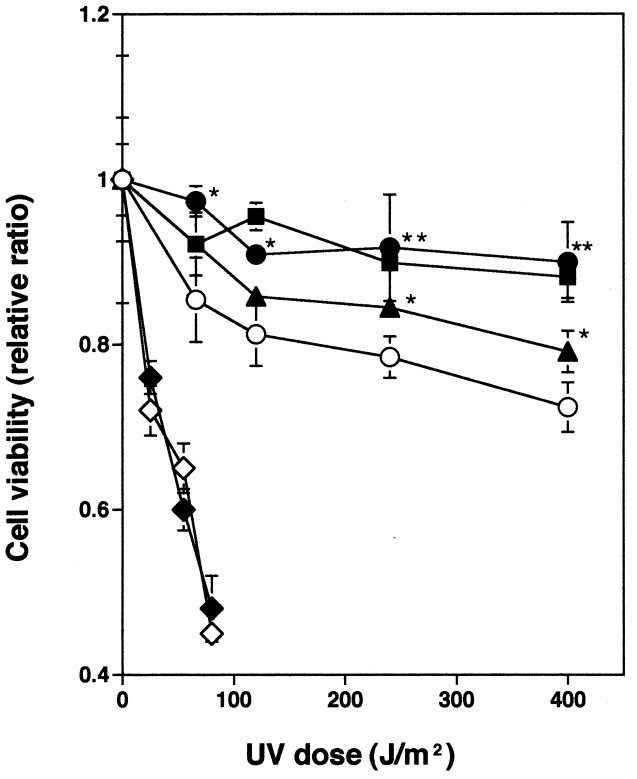


Fig. 4. Effect of PI 3-kinase inhibitors or vitamin E on UV-induced cell death. Fibroblasts maintained on culture dishes were pretreated with $10^{-7}$ M wortmannin (●), $10^{-5}$ M LY294002 (■), 1 mM vitamin E (▲), or vehicle (○) for 30 min, and then irradiated by overhead UVB at the indicated doses. Similarly, cells pretreated with $10^{-7}$ M wortmannin (◆), or vehicle (◇) for 30 min were irradiated by UVC lamps at the indicated doses. After 24 h, cell viability was examined by MTT assay. Data shown are the means ± S.D. of three independent experiments. Statistical significances between the treated and non-treated groups were calculated by Student's *t*-test; $^{**}P < 0.05$, $^{*}P < 0.01$.

chromatic UV, almost half the cells were eliminated by 280 nm light at an energy level of 300 J/m$^2$, while only 5–10% of the cell population were killed by 320 nm light, even at 4000 J/m$^2$ (data not shown). Similar results were obtained from adherent cells exposed to UV lamps. As shown in Fig. 4, when cells were irradiated by UVB or UVC light, the cell viability was significantly decreased in a dose-dependent fashion: 20–25% and 50% of the cell population were killed by 250 J/m$^2$ UVB and 75 J/m$^2$ UVC, respectively. To examine the effect of PI 3-kinase activation on the UVB-induced cell lethality, cell viability was measured in the presence or absence of wortmannin. The addition of wortmannin partially but significantly blocks UVB-dependent decrease of cell viability; e.g. 65% inhibition at 400 J/m$^2$, and ~70% at 250 J/m$^2$ (Fig. 4). The addition of wortmannin had little effect on UVC-dependent cell death. Similar results were obtained from experiments using LY294002. Treatment with vitamin E also blocked the UVB-dependent decrease of cell viability.

## 4. Discussion

Stratospheric ozone has decreased significantly over the past several decades. Atmospheric ozone strongly and selectively absorbs solar UVB; thus decreased stratospheric ozone levels directly affect UVB irradiation on the earth's surface, which certainly results in harmful effects to biological and ecological systems [4]. Under these circumstances, it is quite important to understand the effects of UVB-specific biological and biochemical reactions induced in normal tissues or cells. In this report, we demonstrate the activation of PI 3-kinase and the resultant accumulation of $PIP_3$ induced specifically by UVB irradiation in normal human skin fibroblasts. It is noteworthy that this PI 3-kinase activation is induced by UVB at relatively low levels (~250 J/m$^2$), those usually found in nature. Since this alteration occurs within 1 min after irradiation, the effect on PI 3-kinase seems to be primary. That is, UVB light directly activates signaling systems in which PI 3-kinase is involved. Multiple forms of PI 3-kinase family proteins have been identified in mammalian cells, and these are activated by different mechanisms [22]; PI 3-kinase α and β have been identified as primary targets of various protein tyrosine kinases [23], while the γ subtype is activated by βγ subunits of trimeric GTP binding proteins [24]. Our results indicate that tyrosine kinases rather than GTP binding proteins are required for the PI 3-kinase activation induced by UVB. Indeed, phosphorylation of the p85 subunit of PI 3-kinase is detected by UVB irradiation, and inhibited by a specific protein tyrosine kinase inhibitor, genistein (Fig. 3). These findings suggest the possible involvement of the α or β subtype. In addition, various ROI species, but not NO species, play important roles in this PI 3-kinase activation. The mechanisms underlying how ROIs control protein tyrosine kinases and PI 3-kinase are so far unknown. Further careful studies are required to address this issue.

PI 3-kinase is implicated in biologically diverse processes such as mitogenesis, gene expression, and membrane trafficking [22]. The present study also indicates that PI 3-kinase activation induced by UVB is important in cell lethality. Recent studies indicate that PI 3-kinase is also involved in the signaling pathway that regulates cell survival. PI 3-kinase mediates the type-1 insulin-like growth factor-dependent survival of Rat-1 cells and granule neurons [25,26]. In these survival systems, Akt/PKB, a serine/threonine protein kinase, plays an important role as a downstream effector of PI 3-kinase. On the other hand, we could detect no significant activation of Akt/PKB under our experiment conditions in which the activation of PI 3-kinase is evident (manuscript in preparation). Consequently, a decrease of cell viability, but not survival, is induced in our cell systems. Although this discrepancy may be explained by differences in the experimental conditions, such as cell types and growth factor stimulation, it is also possible that the PI 3-kinase pathway is required, but insufficient, for Akt/PKB activation. Moreover, it is possible that UVB irradiation may lead to both cell death and survival under different conditions. That is, in the absence of growth factors, as in the present study, UVB irradiation induces the relatively small activation of PI 3-kinase (Fig. 1B), leading to cell death. When survival factors are present in the system, PI 3-kinase may be activated strongly, leading to the overcoming of cell death and cell survival.

In summary, we demonstrate using normal human skin fibroblasts that PI 3-kinase is activated specifically by UVB irradiation. The energy level required for the induction of maximal activation is relatively low, and occurs normally at the earth's surface. This PI 3-kinase activation leads to cell death rather than the survival of these normal cells.

*Acknowledgements:* This work was supported by a grant from Toray Research Foundation.

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