Stimulation of Prostaglandin Production by Quinolone Phototoxicity in Balb/c 3T3 Mouse Fibroblast Cells in Vitro

Kohji Shimoda, Nobuhiko Wagai, and Michiyuki Kato

Drug Safety Research Laboratory, Daiichi Pharmaceutical Co., Ltd., 1-16-13, Kita-Kasai, Edogawa-ku, Tokyo 134, Japan

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A number of quinolone antibacterial agents, including loxomoxacin (LFLX), sparfloxacin (SPFX), ofloxacin (OFLX), ciprofloxacin (CPFX), fleroxacin (FLRX), and pefloxacin (PFLX), have been reported to induce photosensitivity in humans at a very low incidence (Food and Drug Administration, 1993; Christ and Lehnert, 1990; Yamaguchi et al., 1993). We previously reported that ultraviolet-A (UVA) irradiation for 4 hr following single oral administration of SPFX at 50 or 100 mg/kg, enoxacin (ENX) at 400 or 800 mg/kg, or levofloxacin (LVFX) at 800 mg/kg induced auricular inflammation consisting of dermal edema and neutrophil infiltration, leading to increased thickness of the auricle in albino mice (Shimoda et al., 1993). Antioxidants such as catalase and dimethyl sulfoxide inhibited the auricular thickening induced by SPFX phototoxicity in the early stage, while anti-inflammatory drugs such as indomethacin, dexamethasone, and phenidone, which inhibit the production of arachidonic acid metabolites mediated by cyclooxygenase, inhibited this thickening in both the early and later stages. However, AA-861, a 5-lipoxygenase inhibitor, and pyrilamine malate and cimetidine, both histamine antagonists, had little or no exacerbating effect (Shimoda et al., 1996). These results suggested that reactive oxygen species and cyclooxygenase products initiated the skin inflammatory reactions, with the latter also contributing to augmentation of the inflammation in the later stage, whereas 5-lipoxygenase products and histamine did not involve in the mechanism of quinolone photosensitivity.

Arachidonic acid is released from cells by physical or chemical stimulation and metabolized by cyclooxygenase into prostaglandins (PGs) and thromboxanes (TXs), or by 5-lipoxygenase into leukotrienes (LTs). Both cyclooxygenase- and 5-lipoxygenase-mediated metabolites have a variety of physiologic and pathologic effects, including potentiation of vascular hyperpermeability and accumulation and activation of leukocytes (Williams, 1983).

In the present study, we investigated the role of fibroblasts, which constitute a major component of the skin dermis, in quinolone photosensitivity in vivo by examining the release of inflammatory mediators PGE2, a potentiator of vascular hyperpermeability, 6-keto-PGF1a, a metabolite of the potentiator of vascular hyperpermeability PG12, and LTB4, a chemoattractant of polymorphonuclear leukocytes, from Balb/c 3T3 mouse fibroblast cells after incubation with SPFX or LVFX under UVA irradiation because dermal edema and neutrophil infiltration were the main histological findings in the auricular inflammation in our previous in vivo study (Shimoda et al., 1993).

MATERIALS AND METHODS

Chemicals. SPFX and LVFX (Fig. 1) were synthesized at Daiichi Pharmaceutical Co. Ltd., Japan. RPMI medium 1640 with or without phenol red, penicillin, streptomycin, and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco BRL (U.S.A.), and indomethacin was from Sigma Chemical Co. (U.S.A.). SPFX and LVFX were dissolved at 1, 10, 100, and 1000 μM, 1, 10, 100, and 1000 μM, and 1, 10, and 100 μM concentrations of SPFX and LVFX, respectively, were used as a stock solution in dimethyl sulfoxide for administration to Balb/c mice. We used 1, 10, 100, and 1000 μM concentrations of SPFX and LVFX, respectively, were used as a stock solution in dimethyl sulfoxide for administration to Balb/c mice. We used 1, 10, 100, and 1000 μM concentrations of SPFX and LVFX, respectively, were used as a stock solution in dimethyl sulfoxide for administration to Balb/c mice.

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or 100 μM in RPMI medium 1640 containing 10% FBS without phenol red, and indomethacin at 0.1, 1, or 10 μM in the medium with phenol red and 10% FBS.

**Cell culture.** On the basis of our having previously demonstrated that auricular skin inflammation was induced by quinolone phototoxicity in Balb/c mice (Shimoda et al., 1993), Balb/c 3T3 mouse fibroblast cells obtained from American Type Culture Collection and maintained in our laboratory were used in the present study. The cells were seeded in 12-well culture plates coated with collagen type I (Iwaki Glass, Japan) at a uniform density of 5 × 10^5 cells/ml in RPMI medium 1640 with phenol red, supplemented with 10% FBS, penicillin (100 μg/ml), and streptomycin (100 units/ml), followed by incubation for 72 hr at 37°C in a humidified 5% CO_2_ incubator to approximately 100% monolayer confluence. Prior to use in the experiments, the cells were rinsed once with RPMI medium 1640 without phenol red.

**UVA source and irradiation.** Three FL20SBLB black light tubes (Toshiba Co., Ltd., Japan), emitting radiation from 300 to 400 nm, were used as UVA source. Immediately after the test compound solution was added to the well, the culture plates were covered with a 3-mm-thick pane of glass to eliminate wavelengths below 320 nm and irradiated with UVA at 1.5 mW/cm^2_ for 5 min (0.5 J/cm^2_). UVA intensity was measured at 365 nm using a UVX digital radiometer fitted with a UVX-36 sensor (UVP Inc., U.S.A.). This UVA dose was confirmed in a preliminary study not to induce the elevation of PGE_2_ concentration in the incubation medium or morphological changes in 3T3 cells.

**Effects of simultaneous treatment with quinolone and UVA on the release of PGE_2_, 6-keto-PGF_1α_, LTB_4_, and lactate dehydrogenase (LDH) from 3T3 cells.** After 1 ml of phenol red-free RPMI medium containing SPFX or LVFX at 1, 10, or 100 μM was added to the well, the culture plates were irradiated with UVA for 5 min. For the vehicle control, quinolone-free medium without phenol red was added to the well. For the non-UVA control, the culture plates were kept for 5 min under the ambient conditions of an experimental room in which UVA intensity was detected at less than 0.01 mW/cm^2_. The quinolone solution was then replaced with the medium containing 10% FBS, and the culture plates were placed in the incubator for 24 hr. The incubation medium was then collected and centrifuged at 800 rpm for 5 min, and the supernatant was stored at -20°C until use for eicosanoid or LDH assay. PGE_2_ concentration was measured for all samples, eicosanoid or LDH assay. PGE_2_ and lactate dehydrogenase (LDH) activity were determined by ELISA methods using commercial kits (Biotrack, Amersham International Plc., UK). The concentrations of PGE_2_, 6-keto-PGF_1α_, and LTB_4_, 6-keto-PGF_1α_, and LTB_4_ were measured by enzyme immunoassay (EIA) methods using commercial kits (Biotrack, Amersham International Plc., UK).

**LDH assay.** LDH activity was measured using a commercial kit (LDH test Wako, Wako Pure Chemical Co., Japan). Briefly, 50 μl of the sample was mixed with 50 μl of nitroblue tetrazolium (0.74 mg/ml), and the mixture was left at room temperature for 45 min. After addition of 100 μl of di-lithium lactate (25 mg/ml) to the mixture, the absorbance was measured at 560 nm within 90 min.

**Statistical analysis.** All data were analyzed using Dunnett’s multiple range test.

**RESULTS**

**Effect of simultaneous treatment with quinolone and UVA on the release of PGE_2_, 6-keto-PGF_1α_, and LTB_4_ from 3T3 cells.** Figure 2 shows the concentration of PGE_2_ in the incubation medium of 3T3 cells. Without UVA, there is no difference in PGE_2_ concentration between the vehicle control, SPFX, and LVFX groups at any concentration (Fig. 2a). Treatment of the cells with SPFX at 100 μM and UVA markedly increased the PGE_2_ concentration in the medium, while that with SPFX at 1 or 10 μM and UVA or LVFX at any concentration and UVA showed no effect on the level (Fig. 2b). In contrast, UVA-preirradiated SPFX and LVFX had no effect on the PGE_2_ concentration at a concentration up to 100 μM (Fig. 2c). 6-Keto-PGF_1α_ level in the incubation medium is shown in Fig. 3: the pattern of change of this prostaglandin was similar to that of PGE_2_. While SPFX or LVFX at 100 μM without UVA and LVFX at 100 μM with
UVA had no effect, SPFX at 100 μm with UVA markedly increased 6-keto-PGF\textsubscript{1α} concentration. However, LTB\textsubscript{4} concentration in the incubation medium after treatment with 100 μm SPFX or 100 μm LVFX with or without UVA was comparable to that of the control (Fig. 4).

Effect of indomethacin on PGE\textsubscript{2} level. Figure 5 shows the effect of indomethacin on the elevation of PGE\textsubscript{2} level induced by SPFX and UVA. The treatment with 100 μm SPFX and UVA markedly increased PGE\textsubscript{2} concentration in the incubation medium to 55 pg/50 μl, compared to 8 pg/50 μl in the control. Pre- and posttreatment with indomethacin at 0.1, 1, and 10 μm significantly decreased the elevated PGE\textsubscript{2} concentration to 2, 5, and 3 pg/50 μl, respectively. The concentrations of PGE\textsubscript{2} in the indomethacin treatment groups lower than those in the control may have been caused by the inhibitory effect of indomethacin on other factors influencing PGE\textsubscript{2} production in 3T3 cells such as stimulation due to experimental manipulations.

Effect of treatment with quinolone plus UVA on LDH level. Figure 6 shows the leakage of LDH from 3T3 cells into the medium, which is a marker of cell membrane damage or cell lysis. Conversely, 100 μm SPFX or 100 μm LVFX without UVA and 100 μm SPFX with UVA showed lower LDH levels than the control, whereas 100 μm LVFX with UVA showed the enzyme level similar to the control. These results suggest that none of the treatments induced severe membrane damage or death in 3T3 cells under the present experimental conditions.

DISCUSSION

We examined the production of arachidonic acid metabolites induced by quinolone phototoxicity in Balb/c 3T3 mouse fibroblast cells in vitro. Treatment with SPFX at 100 μm plus UVA irradiation for 5 min markedly increased levels of PGE\textsubscript{2} and 6-keto-PGF\textsubscript{1α}, but not that of LTB\textsubscript{4}. SPFX or LVFX alone up to 100 μm, 10 μm SPFX, or 100 μm or less LVFX plus UVA irradiation, or UVA-preirradiated quinolone up to 100 μm had no effect. The cyclooxygenase inhibitor indomethacin even at 0.1 μm completely reversed the PGE\textsubscript{2} elevation induced by 100 μm SPFX with UVA.

The results of the present study suggest that the simultaneous presence of 100 μm SPFX and UVA light stimulated the production of PGs, but not LT, via cyclooxygenase activation in 3T3 cells. These findings correspond to those of our previous in vivo study, in which indomethacin, dexmethasone (a phospholipase A\textsubscript{2} inhibitor) and phenindone (a dual inhibitor of cyclooxygenase and 5-lipoxygenase) inhibited auricular inflammation caused by SPFX phototoxicity in Balb/c mice, but that AA-861 (a 5-lipoxygenase inhibitor) had no effect (Shimoda et al., 1996). These in vivo and in vitro studies indicate that PGE\textsubscript{2} and probably 6-keto-PGF\textsubscript{1α} play a possible role in the mechanism of skin inflammation induced by quinolone phototoxicity, but that 5-lipoxygenase products such as LTs do not. In contrast, treatment with LVFX up to 100 μm and UVA had no effect on PG concentration in the 3T3 cell culture medium. This lesser effect of LVFX in contrast to that of SPFX appears to be consistent with our previous in vivo study, in which oral administration of 50 mg/kg SPFX or 800 mg/kg LVFX followed by UVA irradiation induced auricular inflammation, while that of 400 mg/kg LVFX with UVA did not (Shimoda et al., 1993). Quinolones substituted with a fluorine at the 8 position of the quinolone ring have been reported to possess stronger phototoxic potential than quinolones with other substituents at this position (Domagala, 1994). SPFX is substituted with fluorine at the 8 position, but LVFX is not; the lack of effect of LVFX on PG production in the present study is therefore thought to be due to the lower phototoxic potential of the drug, resulting from its structural characteristics. To the best of our knowledge, no reports have appeared describing toxicokinetic studies of SPFX or LVFX in mice in terms of their phototoxicity. The phototoxicity-inducing concentration of quinolones should be determined and compared between that in the incubation medium in vitro and that in the auricular tissue in vivo. However, it is impossible to do that because a validated method for SPFX is not established in our laboratory.

It has been reported that LFLX and ENX undergo greater degradation under UVA irradiation than NFLX, CPFX, and OFLX, and that the former quinolones had stronger phototoxicity than the latter in mice (Marutani et al., 1993). Moreover, the spectral changes of quinolones under UVA irradiation were related to the inhibition rate of lymphocyte mitogenic activity under stimulation by concanavalin A (Shimizu et al., 1995). In the present study, however, UVA-preirradiated SPFX did not increase PGE\textsubscript{2} concentration in the culture medium of 3T3 cells, corresponding to the result of the previous report that intraauricular injection of UVA-irradiated quinolone did not induce skin inflammation (Wagai and Tawara, 1991). These findings suggest that photoproducts of SPFX and LVFX are not involved as causative factors in quinolone phototoxicity.

In general, photodynamic reactions occurring in the simultaneous presence of photosensitizer and exciting light are implicated in the mechanisms of drug phototoxicity. Reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxy radical, and singlet oxygen generated from type I and II reactions have been reported to induce lipid peroxidation in cell membrane components, leading to cell damage (Giorotti, 1990). Under UVA irradiation, naldixic acid (NA) and PD117596 may generate hydroxy radicals and/or singlet oxygen (Fernández and Cárdenas, 1990; Robertson et al., 1991); further, Y-2611, SPFX, LFLX, NA, CPFX, and ENX induced lipid peroxidation of human erythrocyte membrane or squalene (Wada et al., 1994; Fujita and...
FIG. 2. Prostaglandin E₂ concentration in culture medium of Balb/c 3T3 mouse fibroblast cells. The cells were treated with sparfloxacin (SPFX) or levofloxacin (LVFX) (a), SPFX or LVFX and UVA irradiation for 5 min (0.5 J/cm²) (b), or UVA-preirradiated SPFX or LVFX (c), and incubated in quinolone-free medium for 24 hr. Vertical bars represent standard deviations, n = 4. *Statistically different from the control group (p < 0.05).

Matsuo, 1994). In addition, antioxidants such as catalase and dimethyl sulfoxide inhibited the early auricular thickening of SPFX phototoxicity in Balb/c mice (Shimoda et al., 1996). These previous investigations suggest that the initiation of skin inflammation by quinolone phototoxicity derives from reactive oxygen species generated from photodynamic reactions and subsequent lipid peroxidation in some cells. In the present study, however, LDH leakage from 3T3 cells was not detected, suggesting that the cells could produce and release PGs without severe membrane damage or death. Recently, reactive oxygen species have been reported to exert signaling functions and regulate gene expression (Toledano and Leonard, 1991; Schreck et al., 1991), and pyrrolidine dithiocarbamate, an antioxidant, was reported to inhibit inducible nitric oxide synthase and cyclooxygenase–protein expression induced by interleukin-1 (Tetsuka et al., 1996). Therefore, it is possible that reactive oxygen species generated from SPFX under UVA irradiation modulate the cyclooxygenase gene or protein expression without cell membrane damage in the present study.

Both arachidonic acid metabolites and reactive oxygen species are known to be involved in the mechanisms of UV light-induced skin inflammation (Hawk et al., 1983; Soter, 1990; Black, 1987). Moreover, UVA irradiation has been reported to stimulate cultured human fibroblasts and mouse C3H10T1/2 cells to release arachidonic acid via phospholipase C and the diacylglycerol lipase enzyme system, followed by an increase in cyclooxygenase products depending on oxygen and calcium ion concentration rather than lipoxygenase products (Hanson and DeLeo, 1989). A low dose of UVA also increased protein kinase C (PKC) activity in cultured mouse fibroblast C3H10T1/2 cells (Matsui and DeLeo, 1990), and PKC activation is known to trigger arachidonic acid release and PG synthesis (Parker et al., 1987). Oxidant-mediated metabolism of phospholipids produces several metabolites, including phosphatidic acid, diacylglycerol, lyso phosphatidylcholine, and arachidonic acid, which can modulate PKC activity (Natarajan, 1995). Although it is unclear which pathway was involved in the mechanism of PG production by quinolone plus UVA irradiation in the present
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Recent studies have shown that PG production can be enhanced by reactive oxygen species. In particular, cyclic nucleotide binding to PKC may stimulate PG synthesis in fibroblasts. Recently, cyclooxygenase was shown to have at least two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-2 is predominant in inflammatory reactions, and the parallel induction of COX-2 and phospholipase A2 exaggersates PG release. It has been identified in fibroblasts (Kujubu et al., 1991; Fletcher et al., 1992; O’Banion et al., 1992), macrophages (Mitchell et al., 1993; Lee et al., 1992) and endothelial cells (Hla and Neilson, 1992; Akarasereenont et al., 1994); it is therefore possible that the increased PG release from fibroblasts in the present study was mediated by COX-2 activation in the cells. The participation of macrophages and endothelial cells in the development of quinolone phototoxicity should be considered in vivo. Keratinocytes are also thought to have a possible role in skin inflammation induced by quinolone phototoxicity. It has been reported that keratinocytes release various cytokines and arachidonic acid metabolites under UV irradiation (Soter, 1990), and that UV irradiation enhances bradykinin-induced PG synthesis in keratinocytes and fibroblasts (Pentland and Jacobs, 1991). Mast cells, however, can be ruled out from the mechanisms of quinolone phototoxicity because SPFX phototoxicity was induced in mast cell-deficient WBB6F1-W/Wv mice (Shimoda et al., 1996).

FIG. 3. 6-Ketoprostaglandin F₁₀ concentration in culture medium of Balb/c 3T3 mouse fibroblast cells. The cells were treated with sparfloxacin (SPFX) or levofloxacin (LVFX) and ultraviolet-A (UVA) irradiation for 5 min (0.5 J/cm²), and incubated in quinolone-free medium for 24 hr. Vertical bars represent standard deviations, n = 4. *Statistically different from the control group (p < 0.05).

FIG. 4. Leukotriene B₄ concentration in culture medium of Balb/c 3T3 mouse fibroblast cells. The cells were treated with sparfloxacin (SPFX) or levofloxacin (LVFX) and ultraviolet-A (UVA) irradiation for 5 min (0.5 J/cm²), and incubated in quinolone-free medium for 24 hr. Vertical bars represent standard deviations, n = 4.

FIG. 5. Effect of indomethacin on prostaglandin E₂ concentration in culture medium of Balb/c 3T3 mouse fibroblast cells. Following pretreatment with indomethacin for 5 min, the cells were treated with 100 μM sparfloxacin (SPFX) and ultraviolet-A (UVA) irradiation for 5 min (0.5 J/cm²). The cells were then incubated in the medium containing indomethacin for 24 hr. Vertical bars represent standard deviations, n = 4. *Statistically different from the control group. *Statistically different from the 100 μM SPFX group (p < 0.05).

FIG. 6. Lactate dehydrogenase leakage into culture medium from fibroblast Balb/c 3T3 cells. The cells were treated with sparfloxacin (SPFX) or levofloxacin (LVFX) and ultraviolet-A (UVA) irradiation for 5 min (0.5 J/cm²), following incubation in quinolone-free medium for 24 hr. Lactate dehydrogenase activity was determined by absorbance at 560 nm (OD₅₆₀). Vertical bars represent standard deviations, n = 4. *Statistically different from the control group (p < 0.05).
In conclusion, the present study showed that concurrent treatment with 100 µm SPFX and UVA irradiation stimulated Balb/c 3T3 mouse fibroblast cells to produce PGs via cyclooxygenase activation, that photoproducts of SPFX and LVFX did not induce PG production, and that this production was not associated with cell damage. These results suggest that PGs released from dermal fibroblasts by quinolones with UVA irradiation may contribute to the development of skin inflammation in vivo.

REFERENCES


