Phosphatidylinositol transfer protein α regulates growth and apoptosis of NIH3T3 cells: involvement of a cannabinoid 1-like receptor

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Abstract **Mouse fibroblast cells overexpressing phosphatidylinositol transfer protein α [PI-TPα; sense PI-TPα (SPIα) cells] show a significantly increased rate of proliferation and an extreme resistance toward ultraviolet- or tumor necrosis factor-α-induced apoptosis. The conditioned medium (CM) from SPIα cells or the neutral lipid extract from CM stimulated the proliferation of quiescent wild-type NIH3T3 cells. CM was also highly effective in increasing resistance toward induced apoptosis in both wild-type cells and the highly apoptosis-sensitive SPIβ cells (i.e., wild-type cells overexpressing PI-TPβ). CM from SPIα cells grown in the presence of NS398, a specific cyclooxygenase-2 (COX-2) inhibitor, expressed a diminished mitogenic and antiapoptotic activity. This strongly suggests that at least one of the bioactive factor(s) is an eicosanoid. In accordance, SPIα cells express enhanced levels of COX-1 and COX-2. The antiapoptotic activity of CM from SPIα cells tested on SPIβ cells was inhibited by ~50% by pertussis toxin and suramin as well as by SR141716A, a specific antagonist of the cannabinoid 1 receptor. These inhibitors had virtually no effect on the COX-2-independent antiapoptotic activity of CM from SPIα cells. The latter results imply that PI-TPα mediates the production of a COX-2-dependent eicosanoid that activates a G-protein-coupled receptor, most probably a cannabinoid 1-like receptor.**—Schenning, M., C. M. van Tiel, D. van Manen, J. C. Stam, B. M. Gadella, K. W. A. Wirtz, and G. T. Snoek. **Phosphatidylinositol transfer protein α regulates growth and apoptosis of NIH3T3 cells: involvement of a cannabinoid 1-like receptor.** ***J. Lipid Res.*** **2004.** 45: **1555–1564.**

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Phosphatidylinositol transfer proteins (PI-TPs) belong to a family of highly conserved proteins that in vitro can catalyze the transfer of phosphatidylinositol, phosphatidylcholine, and sphingomyelin between membranes (1, 2). In mammalian tissues, at least two isoforms are identified: PI-TPα, which is localized in the nucleus and cytosol, and PI-TPβ, which is associated with the Golgi system (3). Possible cellular functions of these proteins have been obtained from experiments with permeabilized cells (4), reconstituted Golgi membrane systems (5), and cells in which the expression of the proteins has been altered (6–8). In permeabilized, cytosol-depleted cells, both isoforms restored guanosine 5′-[γ−thio]triphosphate-stimulated protein secretion as well as phospholipase C-mediated inositol lipid signaling (9–11). On the other hand, intact mouse fibroblast cells with increased expression of PI-TPα [sense PI-TPα (SPIα) cells] showed an enhanced phospholipase A_2 (PLA_2)-mediated degradation of PI that was not observed in cells with an increased expression of PI-TPβ (SPIβ cells) (7, 8). Convincing evidence for distinct cellular functions was obtained by genetic approaches. Murine embryonic stem cells deficient in PI-TPβ fail to develop, whereas the embryonic development of cells deficient in PI-TPα proceeds normally (12, 13). In the latter case, the mice die within 2 weeks of birth.

The rate of proliferation of SPIα cells is significantly increased, with a cell cycle duration of 13 h compared with 21 h for wild-type cells, indicating that PI-TPα may be involved in the production of a mitogenic factor (7). The observation that in SPIα cells a PI-specific PLA_2 is activated implies that, in addition to lysoPI, a significant amount of arachidonic acid is produced because PI is

Abbreviations: CB1, cannabinoid 1; CM, conditioned medium; COX, cyclooxygenase; DBB, DMEM/bicarbonate containing 0.1% BSA; GPCR, G-protein-coupled receptor; NCS, newborn calf serum; PGE_2, prostaglandin E_2; PI-TP, phosphatidylinositol transfer protein; PLA_2, phospholipase A_2; PVA, polyvinyl alcohol; SPIα, sense PI-TPα; UV, ultraviolet; wtNIH3T3, wild-type NIH3T3 cells.

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highly enriched in this fatty acid (14). Arachidonic acid is the main precursor in the synthesis of eicosanoids, including prostaglandins, leukotrienes, thromboxanes, and prostacyclins. These arachidonic acid metabolites play important roles in many cellular processes, such as thrombosis (15), inflammation (16, 17), cell growth (18), and apoptosis (19). Eicosanoids are synthesized in response to external stimuli in which the release of arachidonic acid from phospholipids by PLA_2 is the rate-limiting step (20). In particular, the prostaglandins, the synthesis of which depends on cyclooxygenase-1 (COX-1) and COX-2, play key roles in cell growth and cell survival as well as in processes such as carcinogenesis and inflammation (21). In general, COX-1 is expressed constitutively, whereas COX-2 can be induced by various physiological stimuli (22–24). Furthermore, it has been suggested that an increase in arachidonic acid metabolism as mediated by COX-1 or COX-2 depends on proteins that coordinate the release of arachidonic acid from phospholipids (25).

In the present study, we show that PI-TPα is involved in the regulation of proliferation and of apoptosis sensitivity. We present evidence that this regulation occurs through the COX-2-dependent production and secretion of eicosanoid factor(s). One of these factors most likely acts on the G-protein-coupled cannabinoid 1 receptor, thereby displaying both autocrine and paracrine activity.

MATERIALS AND METHODS

Materials

Indomethacin (Sigma), NS398 (Cayman Chemical, Ann Arbor, MI), ELISA kit (Cayman), COX-1/COX-2 antibody (Cayman), [^{3}H]thymidine (Amersham), [^{14}C]arachidonic acid (Amersham), 4′,6-diamidino-2-phenyindole (DAPI) (Sigma), Silica Gel 60 TLC plates (Merck), prostaglandin E_2 (PGE_2), PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, PGA_2 (Sigma), suramin (Sigma), and pertussis toxin (Sigma) were obtained from the suppliers indicated. The cannabinoid receptor antagonists SR141716A and SR144538 were a kind gift of Dr. G. van Zadelhoff (Section Bioorganic Chemistry, Bijvoet Institute, Utrecht University).

Preparation of conditioned medium

Cell cultures (75 or 150 cm^2 dishes) were grown to 80–90% confluency. The medium was replaced by 5 or 10 ml of DMEM/bicarbonate (Bic) containing 0.1% BSA (DBB). This medium was left on the cells for 24 h. After removal, the medium was centrifuged (5 min at 1,000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Under standard conditions, quiescent cells were incubated with CM that was derived from an identical surface of cells (i.e., each 9.5 cm^2 well of a six-well dish was incubated with the amount of CM that was conditioned for 24 h by 9.6 cm^2 of cells).

Cell culture and growth assays

All cells were cultured in DMEM containing 10% newborn calf serum (NCS) and buffered with $NaHCO_3$ (44 mM) in a 7.5% CO_2-humidified atmosphere at 37°C. NIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) and PI-TPβ (SPIβ cells) were made as described previously (7, 8). In this study, we have used two different SPIα cell lines (SPI2 and SPI8), giving essentially identical results.

To determine growth rate, cells (1×10^4 per well) were seeded on 24-well plates. After 24 h, the medium was replaced by 1 ml of DMEM/Bic containing 0.1% NCS. After 24–48 h, the medium was replaced by 250 μl of CM or 250 μl of DMEM/Bic/0.1% NCS supplied with extracts of the medium. The volume of medium that had been conditioned by 2 cm^2 of cells was added to each well of a 24-well (2 cm^2 each) plate. After 8 h, [^{3}H]thymidine (0.5 μCi/well in 50 μl of DMEM/Bic/0.1% NCS) was added to the cells. After 16 h (overnight), the medium was removed, the cells were washed four times with phosphate-buffered saline, and 1 ml of methanol per well was added. The methanol was removed after 20 min, and the cells were left to dry on air. A total of 0.5 ml of 0.1 N NaOH was added, and the cells were incubated for 30 min at 37°C and scraped of. The cells were mixed with 4.5 ml of scintillation liquid and counted. Controls were cells incubated with 250 μl of DMEM/Bic/0.1% NCS. Maximal stimulation was obtained by the addition of DMEM/Bic containing 10% NCS.

Ultraviolet radiation of cell cultures

For ultraviolet (UV) treatment, cells were grown on a 6- or 12-well dish. Before UV treatment, the cells were incubated for 4 h in DBB. All of the compounds that were tested for activity were added to this DBB during the incubation. The medium was removed and UV treatment was performed in a Stratalinker (Stratagene) with the indicated dose (standard, 200 J/m^2). Cells were incubated overnight (19 h) with pertussis toxin (300 ng/ml) in DMEM containing 10% NCS followed by incubation of pertussis toxin in CM from SPIα cells for 4 h; suramin and the cannabinoid 1 and 2 receptor antagonists SR141716A (1 μM) and SR144538 (1 μM) were incubated for 4 h in CM from SPIα cells.

After treatment with UV light, 1.5 ml of DBB was added and cells were incubated at 37°C. At the indicated times, cell death was morphologically scored as the percentage of cells that are in the process of blebbing, which have a condensed nucleus. Also, for visualization of condensed nuclei, the cells were grown on glass cover slips and similarly treated. The cells were fixed in cold methanol (−20°C) and subsequently incubated with 1 μg/ml DAPI in methanol for 5 min at room temperature. Cells were washed once with methanol and once with PBS and mounted in Mowiol (Hoechst, Frankfurt, Germany) supplemented with 0.1% paraphenylene diamine. Fluorescent DNA-DAPI complexes were visualized with a Leica inverted microscope.

Flow cytometric analysis of DNA fragmentation

Cells were grown in 9 cm^2 dishes to 80% density. Cells were treated as described above (preincubation with CM for 4 h), UV treatment was performed, and 1.5 ml of DBB was added to the cultures. Cells were kept at 37°C. After 2 h, the medium was collected and cultures were washed with 1 ml of PBS. This PBS was combined with the medium and centrifuged to collect floating cells. Cell cultures were incubated with 1 ml of 8 mM EGTA in PBS for 5 min, resuspended, and centrifuged for 5 min at 800 rpm at 4°C. Both cell pellets were combined and resuspended in 100 μl of PBS supplemented with 0.5% polyvinyl alcohol (PVA), vortexed, and kept on ice for 30 min. A total of 900 μl of 70% ethanol in PBS (0°C) was layered carefully on top of the PBS-PVA. The sample was mixed and stored at −20°C.

Before flow cytometric analysis, nuclei were extracted and stained to detect nuclear fragmentation as a sign of apoptosis [the methodology with slight modifications is described by Darzynkiewicz and Li (26)]. Briefly, the cell suspension was centrifuged for 3 min at 2,200 rpm at 4°C. The supernatant was removed, and the pellet was resuspended in 1 ml of PBS and again centrifuged for 3 min at 2,200 rpm. The pellet was resuspended in 1 ml of a mixture of 50% PBS and 50% extraction buffer (0.2 M

Na_2HPO_4, 4 mM citric acid, pH 7.8). The suspension was kept for 10 min at room temperature. Nuclei were collected by centrifugation (3 min at 2,200 rpm), the pellet was resuspended in 1 ml of PBS containing propidium iodide (50 μg/ml) and RNase (50 μg/ml), and the suspension was incubated in the dark for at least 30 min. Before flow cytometric analysis, the mixture was filtered through a 70 μm cell strainer (Falcon) to remove clustered nuclei and other debris. Flow cytometric analysis of the extracted and stained nuclei (and fragments thereof) was performed on a FACScan flow cytometer equipped with a 100 mW argon laser exciting at 488 nm (Becton Dickinson, San Mateo, CA). The propidium iodide fluorescence of nonaggregated nuclear events was detected in fluorescence detector FL-3 (630 nm long-pass emission detector) in linear mode. Because it is likely that an apoptizing nucleus will fall apart into more than one event in the subdiploid area, the term apoptotic events will be used rather than apoptotic nuclei. This technique renders a relatively low scoring of apoptotic cells compared with visual scoring, as nuclear apoptotic events are still at an early stage at 2 h after UV irradiation.

Determination of COX-1 and COX-2

Cells were grown in 21 cm^2 dishes to 80–90% density. Cells were washed twice with PBS and CM was added. Cells were harvested after 5 h. To harvest the cells, the medium was removed, cells were washed twice with PBS, and the dishes were frozen. After thawing, the cells were incubated with 150 μl of buffer containing 0.1% Nonidet P40 in 20 mM Tris (pH 7.2) for 5 min at room temperature, scraped off, and put on ice. The cell lysate was centrifuged for 10 min at 14,000 rpm at 4°C, and the supernatant was used to determine protein content using the Bradford assay (27). Equal amounts of protein of all samples were prepared for gel electrophoresis. A total of 20 μg of supernatant protein was subjected to SDS-PAGE on a 12.5% gel and analyzed by Western blotting using antibodies against COX-1 and COX-2 (Cayman). Quantification of bands on film was performed by scanning with a Bio-Rad GS 700 imaging densitometer equipped with an integrating program.

Extraction of [^{14}C]arachidonic acid-labeled metabolites from CM

Cells were grown to 60% confluency. The cells were labeled for 24 h with [^{14}C]arachidonic acid (0.1 μCi per well on a six-well plate in 1 ml of DMEM/Bic containing 10% NCS). To analyze labeled arachidonic acid metabolites in CM, the label medium was removed after 24 h and replaced by DBB. After 24 h, this medium was collected and centrifuged to remove floating cells. Arachidonic acid metabolites in the CM were extracted and separated as described by Tai, Tai, and Hollander (28). Shortly, 0.03 ml of 12 M formic acid was added per milliliter of CM. The mixture was extracted with two 3 ml portions of ethyl acetate. The combined extracts were evaporated under N_2. The residue was taken up in acetone and quantitatively spotted on a Silica Gel 60 TLC plate. The TLC plate was developed in the organic phase of ethyl acetate-acetic acid-isooctane-water (11:2:5:10, v/v) as the solvent system. Radioactivity was monitored by scanning the plate with a Berthold Tracemaster 20 Automatic TLC-Linear analyzer.

RESULTS

Mitogenic activity in the CM from SPIα and wild-type NIH3T3 cells

Previously, we had shown that SPIα cells have a cell cycle duration of 13 h compared with 21 h for wild-type NIH3T3 (wtNIH3T3) cells (7). In view of this highly increased rate of proliferation, CM from SPIα cells were tested for the production of a mitogenic factor by measuring the incorporation of [^{3}H]thymidine into DNA of serum-starved (24–48 h) wtNIH3T3 cells. As shown in **Fig. 1A**, the CM from SPIα cells increased DNA synthesis by a factor of 1.6. For comparison, CM from wtNIH3T3 cells increased the DNA synthesis by a factor of 1.1. This indicates that SPIα cells produce a mitogenic factor that is secreted into the medium.

Given the enhanced degradation of PI in SPIα cells as reflected in the relatively high levels of lysoPI and inositol phosphates (7), it is most likely that arachidonic acid also is released, which subsequently can be converted by COXs and lipoxygenases into metabolites to be secreted into the medium (29). To isolate prostaglandins, leukotrienes, and

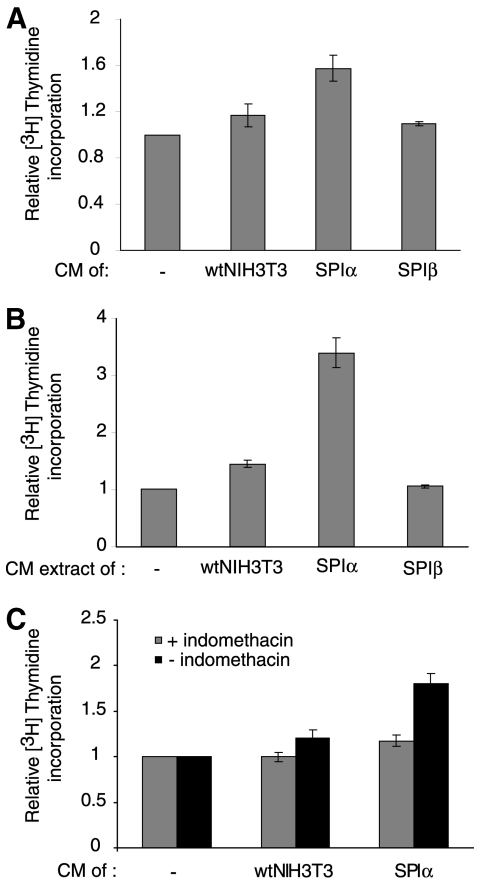

Fig. 1. Mitogenic activity of conditioned medium (CM) from wild-type NIH3T3 (wtNIH3T3), sense phosphatidylinositol transfer protein (PI-TP) α (SPIα), and SPIβ cells tested on quiescent wtNIH3T3 cells. CM was prepared and the neutral lipid extract obtained as described in Materials and Methods. Mitogenic activity was determined by measuring the incorporation of [^{3}H]thymidine into DNA as described in Materials and Methods and is presented relative to the control consisting of DMEM/bicarbonate containing 0.1% BSA (DBB). The activities of CM (A), of the neutral lipid extract (B), and of CM from wtNIH3T3 and SPIα cells prepared in the presence or absence of 10 μM indomethacin (C) are shown. CM prepared in the presence of 50 μM NS398 gave results identical to indomethacin. Results ± SD represent mean values of at least three experiments.

other metabolites from CM, we used a neutral extraction procedure (28). As shown in Fig. 1B, the lipid extract of CM from SPIα cells increased DNA synthesis in quiescent wtNIH3T3 cells by 3.5-fold, whereas the extracts of CM from wtNIH3T3 had only a slight effect. By comparing Fig. 1A, B, it appears that the mitogenic activity in the neutral lipid extracted is enhanced relative to that of CM. When the medium was conditioned in the continuous presence of 10 μM indomethacin, a nonselective inhibitor of COX-1 and COX-2, the CM was no longer mitogenic for quiescent wtNIH3T3 cells (Fig. 1C). In the presence of 50 μM NS398, a selective inhibitor of COX-2, the ensuing CM displayed a similar lack of mitogenic activity, indicating that the mitogenic factor(s) is dependent on COX-2 activity (data not shown).

The aqueous phase of the lipid extract was not active (data not shown), indicating that all mitogenic activity was present in the organic phase. Similarly, the neutral lipid extract from the postnuclear cell lysates was inactive, indicating that all mitogenic activity formed was released in the medium.

Cell survival upon UV irradiation

Because the rate of proliferation is often correlated with apoptosis sensitivity (30, 31), we compared the apoptotic sensitivity of the wtNIH3T3 and SPIα cells upon UV irradiation. First, apoptosis was analyzed by quantifying the number of blebbing cells at 2 h after exposing the cells to variable doses of UV radiation (**Fig. 2A**). Over a range from 20 to 400 J/m^2, the SPIα cells were almost completely resistant toward apoptosis, compared with wtNIH3T3 cells being up to 40% apoptotic. Apoptosis as a function of time upon a UV dose of 200 J/m^2 confirmed that SPIα cells are highly resistant toward UV-induced apoptosis (Fig. 2B). Under the same conditions, the apoptosis sensitivity of cells overexpressing PI-TPβ (SPIβ) was also tested. Compared with the wtNIH3T3 cells, the SPIβ cells were found to be even more sensitive toward UV-induced apoptosis. This indicates that SPIα cells have acquired a high resistance against apoptosis.

Factors that stimulate cell growth often also demonstrate antiapoptotic activity (32, 33). To investigate the relationship between the mitogenic factor and apoptosis sensitivity, we investigated whether CM from SPIα cells is able to protect wtNIH3T3 cells against UV-induced apoptosis. Upon incubation for 4 h with CM from SPIα cells, the wtNIH3T3 cells were fully protected (Fig. 2C). Similar protection was observed when the wild-type cells were incubated with the neutral lipid extract of CM from SPIα cells. The CM from SPIα cells is also very effective in protecting the highly sensitive SPIβ cells (34) against UV-induced apoptosis (Fig. 2D). Given that indomethacin inhibited the mitogenic activity of CM from SPIα cells (Fig. 1C), we also determined the effect of indomethacin on the antiapoptotic activity. As shown in Fig. 2D, CM collected in the presence of indomethacin (10 μM) was less effective in protecting SPIβ cells against UV-induced apoptosis. However, it is clear that the inhibitory effect of

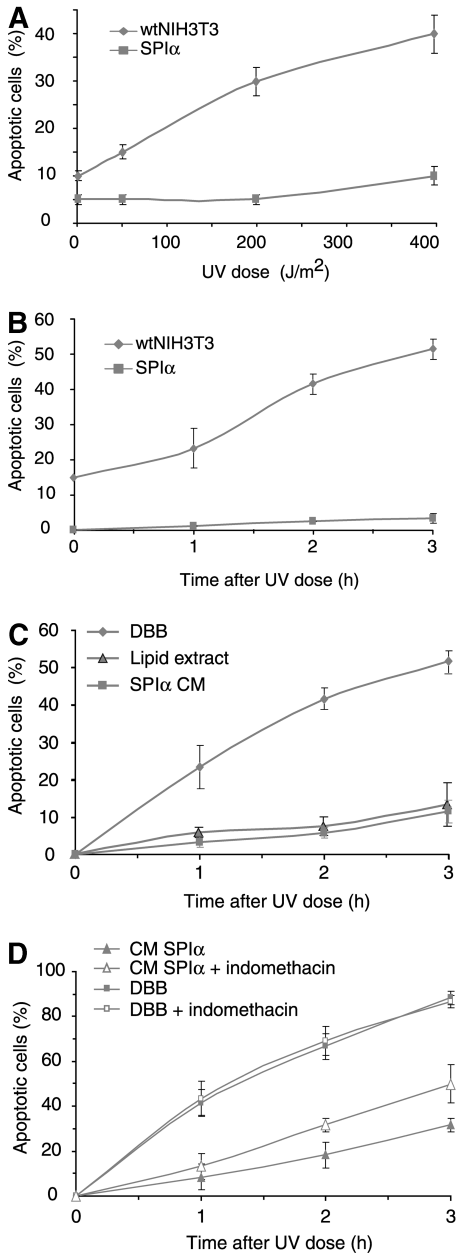

Fig. 2. Survival of wtNIH3T3 and SPIα cells upon induction of apoptosis by ultraviolet (UV) radiation. Cells were grown to 90% confluency. The growth medium was replaced by DBB and the cells were incubated for 4 h at 37°C. After removal of DBB, the cells were irradiated as indicated, fresh DBB was added to the cells, and the number of apoptotic cells (blebbing) was counted at the indicated times. A: Percentage of apoptotic cells in wtNIH3T3 and SPIα cell cultures at 2 h after increasing doses of UV radiation. B: Time course of apoptosis in wtNIH3T3 and SPIα cell cultures after radiation with 200 J/m^2. C: wtNIH3T3 cells were incubated with DBB, with CM from SPIα cells, and with the neutral lipid extract thereof. At time 0, DBB and CM from SPIα cells were removed, cell cultures were radiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined at 0, 1, 2, and 3 h. D: SPIβ cells were incubated with DBB, with DBB containing indomethacin (50 μM), with CM from SPIα cells, or with CM from SPIα cells prepared in the presence of indomethacin (50 μM). At time 0, the media were removed, cell cultures were radiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined at 0, 1, 2, and 3 h. Results ± SD represent mean values of at least three experiments.

indomethacin on the mitogenic activity of CM from SPIα cells is more pronounced than its effect on the antiapoptotic activity (cf. Figs. 1C and 2D). This suggests that either the factor affects cell survival at a much lower concentration than cell growth or that the SPIα cells produce an additional antiapoptotic factor independent of COX-1/COX-2.

In addition to counting blebbing cells, we quantified the percentage of apoptotic cells by analyzing DNA fragmentation by flow cytometric analysis of nuclei that were labeled with propidium iodide (**Fig. 3**). Flow cytometric analysis carried out at 2 h after UV irradiation confirmed that the extent of apoptosis increased in the order SPIα < wtNIH3T3 < SPIβ. When SPIβ cells were incubated for 4 h with CM from SPIα cells before UV treatment, the percentage of apoptotic events was significantly decreased, confirming the antiapoptotic activity of the SPIα medium. This protective effect was reduced using SPIα medium prepared in the presence of indomethacin.

The different sensitivity of NIH3T3 and SPIα cells toward UV-induced apoptosis was confirmed by analysis of nuclear condensation using DAPI staining. Before serum starvation, the nuclear DNA of all wtNIH3T3 and SPIα cells was intact (**Fig. 4A, D**). After sensitization by incubation for 4 h with DBB, the nuclei of some wtNIH3T3 cells showed DNA condensation, whereas the nuclei of SPIα cells were completely unaffected (Fig. 4B, E). This shows that in the case of wtNIH3T3 cells, serum deprivation by DBB is already an apoptotic signal. Two hours after UV radiation, the nuclear DNA of the wtNIH3T3 cells showed a significant increase in condensation, whereas the effect on the nuclear DNA of SPIα cells was still minimal (Fig. 4C, F).

The mitogenic factor is an arachidonic acid metabolite

Indomethacin and NS398 block the production of the mitogenic activity, indicating that the bioactive factor in CM from SPIα cells is an arachidonic acid metabolite (Fig. 1C). To obtain additional evidence, we prepared CM from SPIα cells labeled for 24 h with [^{14}C]arachidonic acid, extracted the medium with ethyl acetate, and separated the lipid extract by TLC. As shown in **Fig. 5**, four major [^{14}C]arachidonic acid metabolite peaks were present in CM from SPIα cells (right panel), whereas the bulk of ^{14}C label in CM from wtNIH3T3 cells ran with arachidonic acid (left panel). Similar analyses of CM from SPIα cells prepared in the presence of NS398 (50 μM), a specific COX-2 inhibitor, or of 10 μM indomethacin (data not shown) showed that three of the four metabolite peaks were absent. Using standards, it could be shown that PGE_2

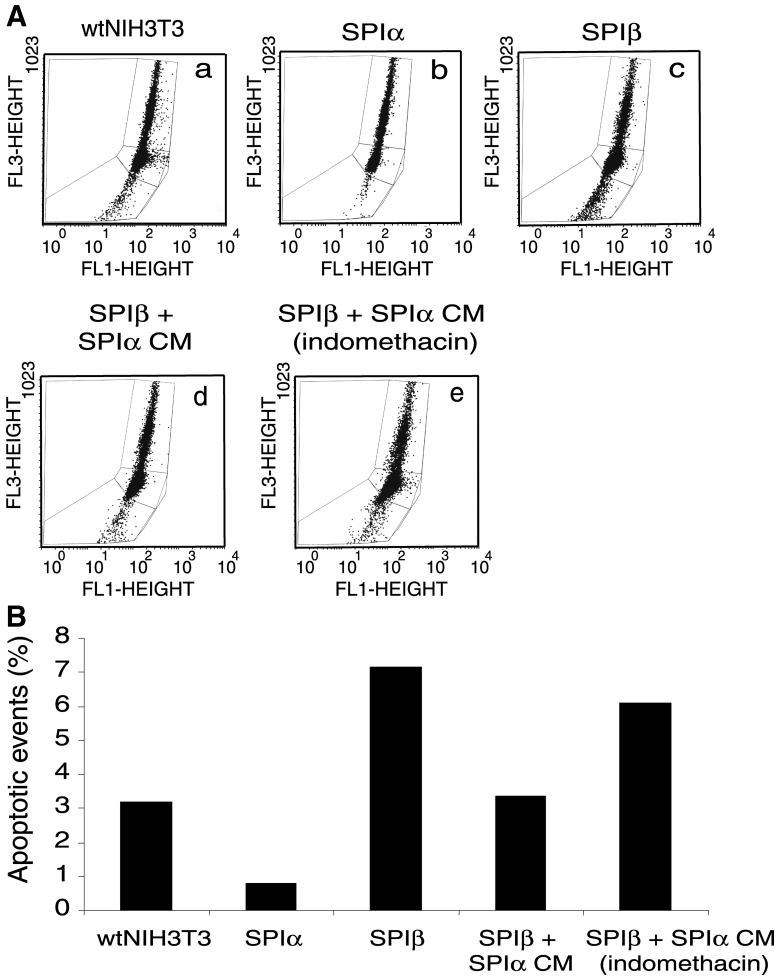

Fig. 3. Survival of wtNIH3T3, SPIα, and SPIβ cells after UV radiation as determined by flow cytometric analysis of the nuclei labeled by propidium iodide. Cells were collected at 2 h after UV radiation (200 J/m^2) and prepared for flow cytometric analysis as described in Materials and Methods. A: Size-intensity diagram of the nuclei labeled with propidium iodide from wtNIH3T3 (a), SPIα (b), and SPIβ (c) cells incubated for 4 h with DBB before UV radiation and from SPIβ cells incubated for 4 h with CM from SPIα cells prepared in the absence (d) or presence of 50 μM indomethacin (e). B: Percentage of apoptotic events representing the nuclei with reduced DNA (see area of A, bottom left). FL1, fluorescent signal from a photomultiplier tube with an excitation wavelength of 488 nm and an emission wavelength of 495–525 nm; FL2, fluorescent signal with an excitation wavelength of 488 nm and an emission wavelength of 630 nm.

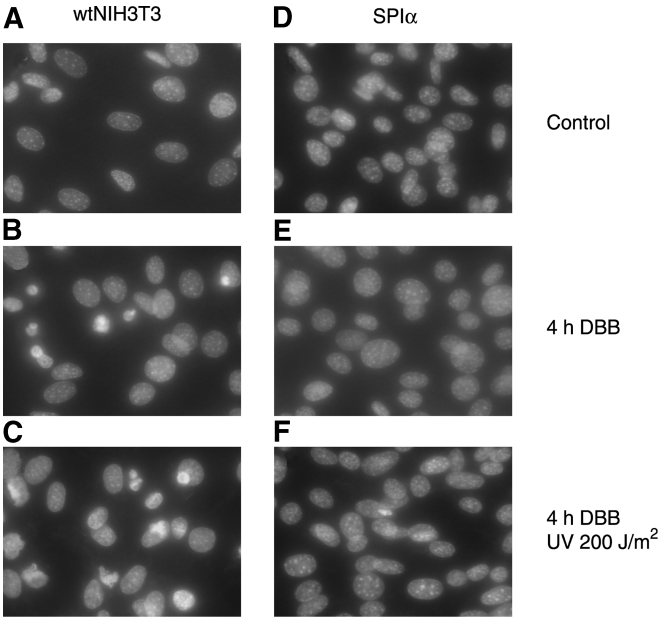

Fig. 4. Survival of wtNIH3T3 and SPIα cells upon serum starvation and UV radiation determined by 4′,6-diamidino-2-phenyindole (DAPI) staining of condensed DNA. wtNIH3T3 cells (A–C) and SPIα cells (D–F) grown on glass cover slips were fixed and stained with DAPI as described in Materials and Methods. A and D: Control cells. B and E: Cells incubated with DBB for 4 h (serum starvation). C and F: Cells incubated for 4 h with DBB, radiated with 200 J/m^2 UV, and fixed and stained after 1 h.

and $PGF_{2\alpha}$ coeluted with two of the arachidonic metabolite peaks. By using an ELISA kit (Cayman) it was determined that SPIα cells produced five times more PGE_2 compared with wtNIH3T3 (0.5 and 0.1 μg/ml, respectively). However, it was shown that both prostaglandins, either separate or combined, were unable to stimulate the growth of quiescent wtNIH3T3 cells in the concentration range present in CM from SPIα cells. Similar results were obtained by using PGE_1, PGD_2, PGA_2, and $PGF_{1\alpha}$ (data not shown), indicating that the COX-2-dependent mitogenic factor must be an as yet unidentified eicasonoid.

COX-1 and COX-2 expression

Given the high production of a mitogenic factor(s) by SPIα cells, we investigated the expression of COX-1 and COX-2 in these cells by Western blotting. In SPIα cells, COX-1 and COX-2 are increased 4- and 7.5-fold, respectively, compared with wtNIH3T3 cells (**Fig. 6**). In addition, incubation with CM from SPIα cells for 5 h gives rise to a 3-fold increase of COX-2 in wtNIH3T3 cells and a 2-fold increase in SPIβ cells, in agreement with the increased resistance of these cells toward UV-induced apoptosis (Fig. 2C, D). Under these conditions, CM from SPIα cells has little effect on COX-1.

Involvement of a G-protein-coupled receptor

To identify the mode of action of the antiapoptotic activity in CM from SPIα cells, inhibitors of G-protein-coupled receptors (GPCRs) were used. Incubation of SPIβ cells with CM from SPIα cells in the presence of suramin (300 μM) or pertussis toxin (300 ng/ml) inhibited the antiapoptotic effect by ∼40–50% (**Fig. 7**). A specific group of eicosanoids, the endocannabinoids, has been shown to inhibit neurodegeneration in rat brain by activation of a GPCR, the cannabinoid 1 (CB1) receptor (35, 36). Because PI-TPα appears to play an important role in maintaining neural integrity (37), we investigated whether there was a relationship between the paracrine stimulation of SPIβ cells by CM from SPIα cells and the CB1 and CB2 receptors. Incubation with the CB1 receptor antagonist SR141716A (1 μM) for 4 h reduced the antiapoptotic activity of CM from SPIα cells by 40% (Fig. 7). Under these conditions, the CB2 receptor antagonist SR144538 (1 μM) appeared to have no effect. On the other hand, in contrast to pertussis toxin, suramin, and SR141716A, which had no effect on the apoptosis sensitivity of the SPIβ cells under control conditions (i.e., DBB), the CB2 receptor antagonist SR144538 itself showed a distinct antiapoptotic activity (Fig. 7). Hence, we cannot exclude the possibility that the antiapoptotic factor also acts through the activation of the CB2 receptor. Moreover, addition of pertussis toxin, suramin, or SR141716A to CM from SPIα cells prepared in the presence of NS398, a specific COX-2 inhibitor, failed to result in a significant further decrease of antiapoptotic activity (data not shown). These results strongly suggest that at least one of the antiapoptotic factors produced by SPIα cells is a COX-2-dependent endocannabinoid, acting through the activation of a CB1-like receptor.

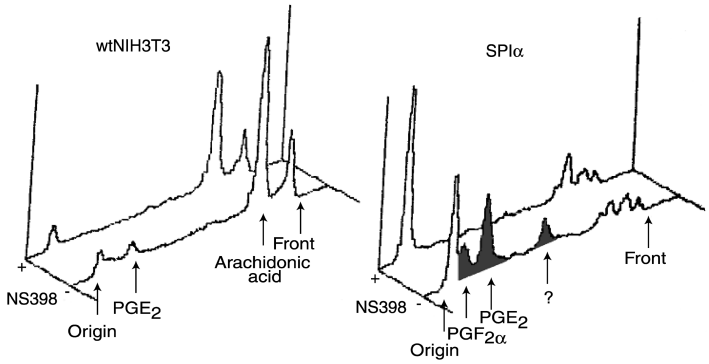

Fig. 5. Thin layer chromatography scans of radioactive compounds in CM from wtNIH3T3 and SPIα cells prepared in the absence or presence of NS398 (50 μM). Cells were labeled to equilibrium with [^{14}C]arachidonic acid, CM was prepared and extracted by ethyl acetate, and the neutral lipid extract was separated and analyzed as described in Materials and Methods. The prostaglandins PGE_2 and $PGF_{2\alpha}$ and arachidonic acid were used as referents.

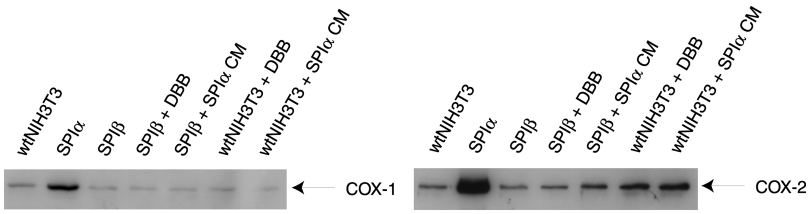

Fig. 6. Western blot analysis of cyclooxygenase-1 (COX-1; left) and COX-2 (right) in wtNIH3T3, SPIα, and SPIβ cells and in wtNIH3T3 and SPIβ cells incubated for 4 h with DBB or CM from SPIα cells (SPIα CM). Cells were lysed, and the cytosolic fractions (aliquots of 25 μg of protein) were analyzed by SDS-PAGE and Western blotting using specific anti-COX-1 and anti-COX-2 antibodies as described in Materials and Methods. To ensure that identical amounts of protein were analyzed, gels and blots were routinely checked by Ponceau S/Coomassie Brilliant Blue staining.

DISCUSSION

In this paper, we show that NIH3T3 cells overexpressing PI-TPα (SPIα cells) are almost completely resistant toward UV-induced apoptosis compared with wtNIH3T3 cells. When CM from SPIα cells was added to wtNIH3T3 or SPIβ cells, which are extremely sensitive to UV radiation, these cells also became apoptosis resistant. In addition, CM from SPIα cells as well as the neutral lipid extract derived from CM stimulates the growth of quiescent wtNIH3T3 cells. Antiapoptotic and mitogenic activity in the CM was significantly diminished when the SPIα cells were incubated with COX-2 inhibitors during preparation of the CM. In agreement with previous observations on SPIα cells being enriched in lysoPI (7), CM from SPIα cells is highly enriched in arachidonic acid metabolites (e.g., 0.5 μg/ml PGE_2 compared with 0.1 μg/ml for wtNIH3T3 cells). This enrichment reflects the constitutive activation of PLA_2 by PI-TPα, resulting in the degradation of PI into lysoPI and arachidonic acid, which is subsequently converted into eicosanoids, among them PGE_2 and $PGF_{2\alpha}$. When prepared in the presence of the COX-2 inhibitor NS398, some major arachidonic acid metabolites were absent from CM of SPIα cells, suggesting that these compounds constituted a major part of the mitogenic and antiapoptotic activity (Fig. 4).

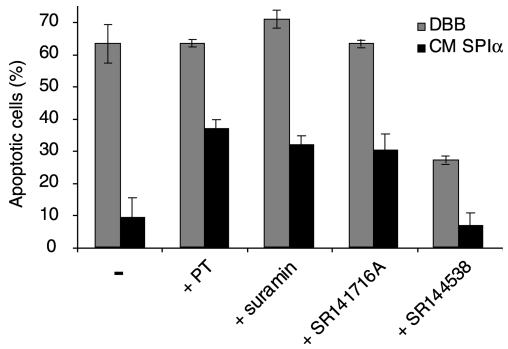

Fig. 7. Survival of SPIβ cells by CM from SPIα cells in the presence of inhibitors of G-protein-coupled receptors (GPCRs) and antagonists of cannabinoid 1 (CB1) or CB2 receptor. Cells were grown to 90% confluency. The growth medium was removed and the SPIβ cells were incubated for 4 h with DBB, with CM from SPIα cells, with CM from SPIα cells containing inhibitors of GPCRs [pertussis toxin (PT; 300 ng/ml) and suramin (300 μM)], and with CM from SPIα cells containing an antagonist of the CB1 receptor (SR141716A; 1 μM) or the CB2 receptor (SR144538; 1 μM). Gray bars represent data with DBB; black bars represent data with CM from SPIα cells. At time 0, the media were removed, cell cultures were radiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined by visual scoring (blebbing cells) at 3 h. Results ± SD represent mean values of four experiments.

PI-TPα is localized in the cytosol and in the nucleus (3, 38). The relevance of the nuclear localization is not clear yet. However, the presence of an active PI metabolism in the nucleus is well documented (39). Furthermore, nuclei of several mammalian cells have been shown to contain an active acylation-deacylation cycle involving PLA_2 activity [reviewed in ref. (40)]. Additionally, because mammalian nuclei also contain a significant level of arachidonoylPI (41), it remains to be established whether the PI-TPα-mediated production of lysoPI and arachidonic acid occurs partially in the nucleus.

In many cell types, PGE_2 is the major COX-2-dependent product known to promote cell growth in an autocrine and paracrine manner (42). The mitogenic effects of PGE_2 are mediated by the activation of E-prostanoid receptors (43). When PGE_2 alone or together with $PGF_{2\alpha}$ was tested on quiescent wtNIH3T3 cells, we failed to observe the growth-promoting effect. However, PGE_2 and $PGF_{2\alpha}$ enhanced to some extent the survival of these cells after UV radiation. On the other hand, incubation of SPIβ cells with these prostaglandins had no effect on the survival of the SPIβ cells, which remained extremely sensitive (data not shown). This strongly suggests that SPIα cells produce another COX-2-dependent mitogenic eicasanoid, different from PGE_2 or $PGF_{2\alpha}$, the identity of which remains to be established. Similarly, we assume that these metabolites are also active in protecting cells against induced apoptosis.

It is well established that the induction of COX-2 prevents apoptosis by generating antiapoptotic prostaglandins as well as by removing the proapoptotic substrate

arachidonic acid (42, 44, 45). In the case of the SPIα cells, the arachidonic acid released by PI-TPα from PI is effectively removed by COX-2, the level of which is highly increased compared with wtNIH3T3 and SPIβ cells (Fig. 6). Incubation of the SPIβ cells with CM from SPIα cells increased the level of COX-2 but not of COX-1. This could explain why wtNIH3T3 and SPIβ cells are protected against apoptosis by CM from SPIα cells. On the other hand, CM from SPIα cells prepared in the presence of the COX-1/COX-2 inhibitor indomethacin still showed considerable antiapoptotic activity (Figs. 2D, 3). This indicates that either the factor affects cell survival at a much lower concentration than cell proliferation or that, in addition to producing COX-dependent factors, SPIα cells also produce COX-independent antiapoptotic factors.

Further insight into the nature of the factor was provided by the inhibitory effect of both GPCR inhibitors and the CB1 and CB2 receptor antagonists on the antiapoptotic activity of CM from SPIα cells (46, 47). The 40–50% reduction in antiapoptotic activity induced by pertussis toxin, suramin, and the specific CB1 receptor antagonist SR141716A strongly suggests that the antiapoptotic factor produced by SPIα cells is an endocannabinoid, which acts via activation of the CB1 receptor (Fig. 7). CM from SPIα cells prepared in the absence or presence of COX-2 inhibitors (indomethacin or NS398) in combination with the GPCR/CB1 inhibitors showed similar antiapoptotic activity (i.e., 60% of the control). This indicates that the activation of the CB1 receptor is solely attributable to the COX-2-dependent cannabinoid (48, 49). On the other hand, to date there is no direct evidence for the presence of a CB1 receptor in mouse fibroblasts. Hence, we cannot exclude the possibility that the antagonist SR141716A acts on a CB1-like receptor.

These findings demonstrate a possible link between the production of endocannabinoids and PI-TPα activity. Decreased expression of PI-TPα in rat brain is known to cause serious neurodegeneration (37), whereas the endocannabinoid anandamide has been shown to act as an endogenous protective factor of the brain against acute neuronal damage (35). Our results provide evidence that the cell survival (e.g., the prevention of neurodegeneration) by PI-TPα is linked to the PLA_2-dependent release of arachidonic acid, part of which is subsequently converted into bioactive eicosanoids by COX-2. The possible mechanism by which cell survival depends on PI-TPα is presented in a model (**Fig. 8**).

Recently, mice lacking PI-TPα were shown to die within 2 weeks after birth as a result of massive physiological defects, including spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia (13). Because *PI-TPα*$^{-/-}$ mice develop to term and are phenotypically normal, it appears that PI-TPα is not required for embryonic development. However, immediately after birth, these mutant mice failed to thrive, demonstrating among other defects increased apoptosis throughout the cerebellum. The importance of PI-TPα for normal brain function is also evident from the vibrator mouse, which has severe neurological disorders attributable to reduced PI-TPα levels in the brain (37). Given that PI-TP transfer activity is particularly

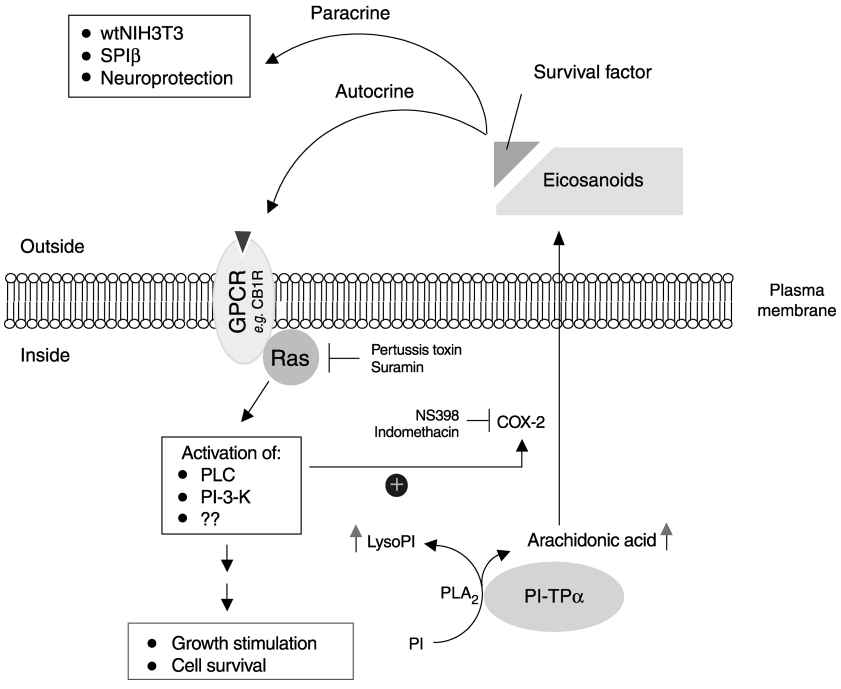

Fig. 8. The regulatory role of phosphatidylinositol transfer protein (PI-TPα) in the production of a bioactive eicosanoid. In the model presented, PI-TPα activates a PI-specific phospholipase A_2 (PLA_2) leading to an increased release of arachidonic acid, which is subsequently converted into eicosanoids by COX-2. Part of these eicosanoids constitute the survival factor(s), which has antiapoptotic activity by acting through the activation of a GPCR, possibly CB1-like receptor. PLC, phospholipase C; PI-3-K, phosphatidylinositol-3-kinase.

high in synaptosome and myelin fractions from rat brain as a result of high levels of PI-TPα (50), the neuronal cells of mice may be well protected against apoptosis by PI-TPα mediating the synthesis of the antiapoptotic factor. Along this line, it will be interesting to investigate whether the early death of *PI-TPα*$^{-/-}$ mice after birth can be prevented by the administration (either in food or intravenously) of the eicosanoid factor produced by SPIα cells.

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