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The anti-apoptotic MAP kinase pathway is inhibited in NIH3T3 fibroblasts with increased expression of phosphatidylinositol transfer protein β

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Abstract

Mouse NIH3T3 fibroblast cells overexpressing phosphatidylinositol transfer protein β (PI-TPβ, SPIβ cells) demonstrate a low rate of proliferation and a high sensitivity towards UV-induced apoptosis when compared with wtNIH3T3 cells. In contrast, SPIβS262A cells overexpressing a mutant PI-TPβ that lacks the protein kinase C-dependent phosphorylation site Ser-262, demonstrate a phenotype comparable with wtNIH3T3 cells. This suggests that the phosphorylation of Ser-262 in PI-TPβ is involved in the regulation of apoptosis. Conditioned medium (CM) from wtNIH3T3 cells contains bioactive factors, presumably arachidonic acid metabolites [H. Bunte, et al., 2006; M. Schenning, et al., 2004] that are able to protect SPIβ cells against UV-induced apoptosis. CM from SPIβ cells lacks this protective activity. However, after heat denaturation CM from SPIβ cells regains a protective activity comparable with that of wtNIH3T3 cells. This indicates that CM from SPIβ cells contains an antagonistic factor interfering with the anti-apoptotic activity present. SPIβS262A cells do not produce the antagonist suggesting that phosphorylation of Ser-262 is required. Moreover, in line with the apparent lack of anti-apoptotic activity, CM from SPIβ cells does not induce the expression of COX-2 or the activation of p42/p44 MAP kinase in SPIβ cells. In contrast, CM from wtNIH3T3 and SPIβS262A cells or heat-treated CM from SPIβ cells does induce these anti-apoptotic markers. Since we have previously shown that some of the arachidonic acid metabolites present in CM from wtNIH3T3 cells are prostaglandin (PG) E_2 and $PGF_{2\alpha}$, we investigated the effect of these PGs on cell survival. Although PGE_2 and $PGF_{2\alpha}$ were found to protect wtNIH3T3 and SPIβS262A cells against UV-induced apoptosis, these PGs failed to rescue SPIβ cells. The fact that the concentrations of PGE_2 and $PGF_{2\alpha}$ in the CM from SPIβ cells and wtNIH3T3 cells were found to be comparable suggests that the failure of these PGs to protect SPIβ cells could render these cells more apoptosis sensitive. Concomitantly, upon incubation with PGE_2 and $PGF_{2\alpha}$, an increased expression of COX-2 and activation of p42/p44 MAP kinase were observed in wtNIH3T3 and SPIβS262A cells but not in SPIβ cells. Hence, it appears that specific mechanisms of cell survival are impaired in SPIβ cells.

Keywords: Phospholipid transfer protein; Sphingomyelin metabolism; Eicosanoids; Phosphatidylinositol metabolism

1. Introduction

The mammalian phosphatidylinositol transfer protein β (PI-TPβ) is a highly conserved protein with up to 99% sequence identity between species [1,2]. It shares 77% sequence identity with its isoform PI-TPα. PI-TPβ is mainly associated with the Golgi system and may be the functional analogue of Sec14p, the major yeast PI-TP [3,4]. Although its physiological function has not yet been established, its importance follows from the observation that gene ablation of PI-TPβ in murine embryonic stem cells prevents embryonic development [5]. Murine embryos lacking PI-TPα develop normally but die within 2 weeks after birth [6]. This clearly shows that these two isoforms serve different functions in the cell.

Abbreviations: PI-TP, phosphatidylinositol transfer protein; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; PKC, protein kinase C; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; CM, conditioned medium; NCS, newborn calf serum; PBS, phosphate buffered saline; DBB, DMEM containing 0.1% bovine serum albumin; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; MAPK, mitogen-activated protein kinase; MKK, MAP kinase kinase; GPCR, G protein-coupled receptor

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In previous studies on wtNIH3T3 cells that have a 10-fold increase in PI-TPβ levels (SPIβ cells) we observed that these cells display a decreased growth rate relative to the wtNIH3T3 cells [7]. In addition, under conditions where sphingomyelin (SM) in the plasma membrane was hydrolyzed to ceramide by exogenous sphingomyelinase, SPIβ cells in contrast to wtNIH3T3 cells maintained the steady-state levels of SM in the plasma membrane suggesting that PI-TPβ was involved in this process [7]. This is in agreement with the finding that in vitro PI-TPβ binds and transfers SM in addition to PI and PC while PI-TPα lacks the ability to transfer SM [8]. Unlike SPIβ cells, mutant SPIβS262A cells were unable to instantaneously replenish SM after its degradation by exogenous sphingomyelinase, strongly suggesting that the phosphorylation of Ser-262 was required [9,10]. If SM replenishment is linked to membrane vesicle flow it could be that PI-TPβ plays a role in the budding process, an activity analogous to Sec14p function in yeast [11].

In contrast to SPIβ cells, cells with an increased expression of PI-TPα (SPIα cells) demonstrate a highly increased rate of proliferation as well as an increased survival upon induction of apoptosis [12,13]. Furthermore, it was shown that SPIα cells produce a PI-TPα/COX-2-dependent mitogenic and anti-apoptotic factor. Upon secretion, this factor is able to stimulate growth and to promote survival in wtNIH3T3 and SPIβ cells [13]. In agreement with the increased resistance of these cells towards UV-induced apoptosis, the PI-TPα-dependent survival factor induced COX-2 expression in both cell lines. In addition it had been reported that this survival factor is able to prevent apoptosis in rat motor neurons, suggesting a vital role in the central nervous system [14].

In earlier studies we have shown that SPIβ cells, when compared with wtNIH3T3 cells, are very susceptible towards UV-induced apoptosis, whereas the SPIβS262A cells behave similar to wtNIH3T3 cells [15]. Here we report that two abundant COX-1,-2 dependent arachidonic acid metabolites, PGE_2 and $PGF_{2\alpha}$ do not protect SPIβ cells against apoptosis whereas wtNIH3T3 and SPIβS262A cells are protected. In addition, we report that conditioned medium (CM) from SPIβ cells contains a heat-labile antagonist masking the survival factor present. This antagonist prevents the activation of the anti-apoptotic p42/p44 MAP kinase pathway and the upregulation of COX-2.

2. Materials and methods

2.1. Materials

Prostaglandins PGE_2 and $PGF_{2\alpha}$ were obtained from Sigma, polyclonal antibodies against COX-1/COX-2 from Cayman; anti-p42/p44 MAP kinase antibodies where obtained from Cell Signaling technology, DMEM and NCS from Invitrogen; PGE_2 ELISA kit was obtained from Cayman.

2.2. Cell culture

All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with 44 mM $NaHCO_3$. Cells were maintained at 7.5% CO_2 at 37 °C in a humidified atmosphere. Wild type cells used were designated ATCC CRL 1658 NIH3T3 cells. The SPIα cells were obtained as described in Snoek et al. [12]. The SPIβ cells and SPIβS262A cells were obtained as described in van Tiel et al. [7] and van Tiel et al. [9].

2.3. Preparation of conditioned medium

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the medium was replaced with 13 ml of DMEM containing 0.1% bovine serum albumin (DBB medium). After 24 h the medium was collected and centrifuged (10 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Neutral lipids were extracted from CM with two 39 ml portions of ethyl acetate (after adjusting to pH 2.0 with formic acid) [16]. CM was heat-denatured by incubation for 20 min at 80 °C, then centrifuged for 10 min at 17,500×*g* and the supernatant used for experiments. Under standard conditions 90% confluent cells were incubated with CM derived from an identical surface of cells (i.e. 9.5 cm^2 of cells per well of a six-well dish was incubated with the amount of CM or neutral lipid extract derived from 9.5 cm^2 of cells).

2.4. Induction of apoptosis

Induction of apoptosis by serum starvation: Cells were seeded in 6-well plates and grown under standard conditions for 48 h until ca 85% confluency. Growth medium was removed and the cells were washed with PBS. The cells were incubated with DMEM containing 0.1% bovine serum albumin (DBB medium) for 16 h at 37 °C and the percentage of apoptotic cells was morphologically determined as the percentage of cells that was in the process of blebbing.

Induction of apoptosis by tumor necrosis factor α (TNFα): Cells were seeded in 6-well plates and grown under standard conditions for 48 h until ca 85% confluency. Growth medium was removed and cells were washed with PBS. The cells were incubated with DBB medium containing cycloheximide (2.5 μg/ml) and TNFα (10 ng/ml) and the cells were incubated for 7 h at 37 °C and the percentage of apoptotic cells was morphologically determined as the percentage of cells that was in the process of blebbing.

Induction of apoptosis by UV irradiation: Cells were seeded in 6-well plates and grown for 48 h until ca 85% confluency. Growth medium was removed and cells were washed with PBS. Before UV treatment, the cells were incubated for 4 h in DBB medium. To investigate the effects on the sensitivity to apoptosis, CM or prostaglandins were added to this DBB during the incubation prior to UV irradiation. The medium was removed and the cells were given a UV dose (200 J/m^2) using a Stratalinker (Stratagene). After UV irradiation, the cells were incubated with DBB at 37 °C. At the indicated time points cell death was morphologically determined as described above.

2.5. Determination of COX-1 and COX-2 levels

Cells were grown in 21 cm^2 dishes to 80–90% confluency. Cells were washed twice with PBS and lysed in 20 mM Tris–HCl pH 7.5 containing 0.1% (v/v) NP_{40}. The cell lysate was centrifuged at 17,500×*g* for 10 min at 4 °C and the protein content of the supernatant fraction determined using the Bradford assay [17]. Equal amounts of supernatant proteins (50 μg) were subjected to SDS-PAGE on a 12% gel and Western blot analysis was performed using an antibody specific for COX-1 or COX-2. To determine whether equal amounts of protein were analyzed, blots were stained with ponceau S and scanned. The levels of COX-1 or -2 on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program. In some experiments prior to harvesting the cells were incubated with CM for 5 h.

2.6. Determination of p42/44 MAP kinase levels

Cells were grown in 21 cm^2 dishes to 80–90% confluency. Cells were washed twice with PBS and lysed in 20 mM Tris–HCl pH 7.5 containing 0.1% (v/v) NP_{40}, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM aprotinin and 1 mM PMSF. Sample preparation was performed as described above. Western blot analysis was performed using an antibody specific for p42/44 MAP kinase and immunoreactive bands were quantified. To determine whether equal amounts of protein were analyzed, blots were stained with ponceau S and scanned. In some experiments prior to harvesting the cells were incubated with CM for 10 min.

2.7. Measurement of ceramide and sphingomyelin levels

Cells were grown to 80% confluency and total lipids were extracted by the method of Bligh and Dyer [18]. Ceramide levels were determined using the *Escherichia coli* diacylglycerol kinase assay as described [19]. Briefly, the lipids were incubated at room temperature for 30 min in the presence of β-octylglucoside/dioleoyl-phosphatidyl glycerol micelles, 2 mM dithiothreitol, 5 μg of proteins from the diacylglycerol kinase membranes, and 2 mM ATP (mixed with [γ-^{32}P]ATP) in a final volume of 100 μl. After Bligh and Dyer extraction the lipids were separated by thin layer chromatography (TLC) in chloroform:acetone:methanol:acetic acid:H_2O (50:20:15:10:5, by vol.) and the radioactivity associated with ceramide-phosphate was measured. Ceramide levels were quantified using external standards and were normalized to phosphate. SM levels were determined as described previously [7].

3. Results

3.1. Apoptosis sensitivity of wtNIH3T3, SPIβ and SPIβS262A cells

Previously we have shown that SPIβ cells are sensitive towards UV-induced apoptosis [15]. Exposure of the wtNIH3T3, SPIβ and SPIβS262A cells to other apoptotic stimuli (i.e. 10 ng/ml TNFα, 2.5 μg/ml; serum starvation) showed that the increased apoptotic response of the SPIβ cells was not restricted to UV irradiation. Under all conditions tested, SPIβ cells showed a significantly higher extent of apoptosis than wtNIH3T3 and SPIβS262A cells (Fig. 1A). This is illustrated by Fig. 1B, which shows the extent of blebbing 90 min after UV irradiation. These images, at all time points shown, have been visually analyzed to determine the extent of apoptosis.

Because SM metabolism may play a role in apoptosis [20–23] , we analyzed SM and ceramide in SPIβ, SPIβS262A and wtNIH3T3 cells. In agreement with previous studies [7], SM levels were comparable in the three cell lines, (i.e. 59 pmol/nmol of total lipid). Similar to other studies, we observed that SM separated in two bands by TLC [24,25]. The lower band represents short-chain (C16:0) and the upper band long-chain (C24:0/1) SM species. However, we noticed that the relative proportion of the two classes of SM species was different in the three cell lines (Fig. 1C). Specifically,

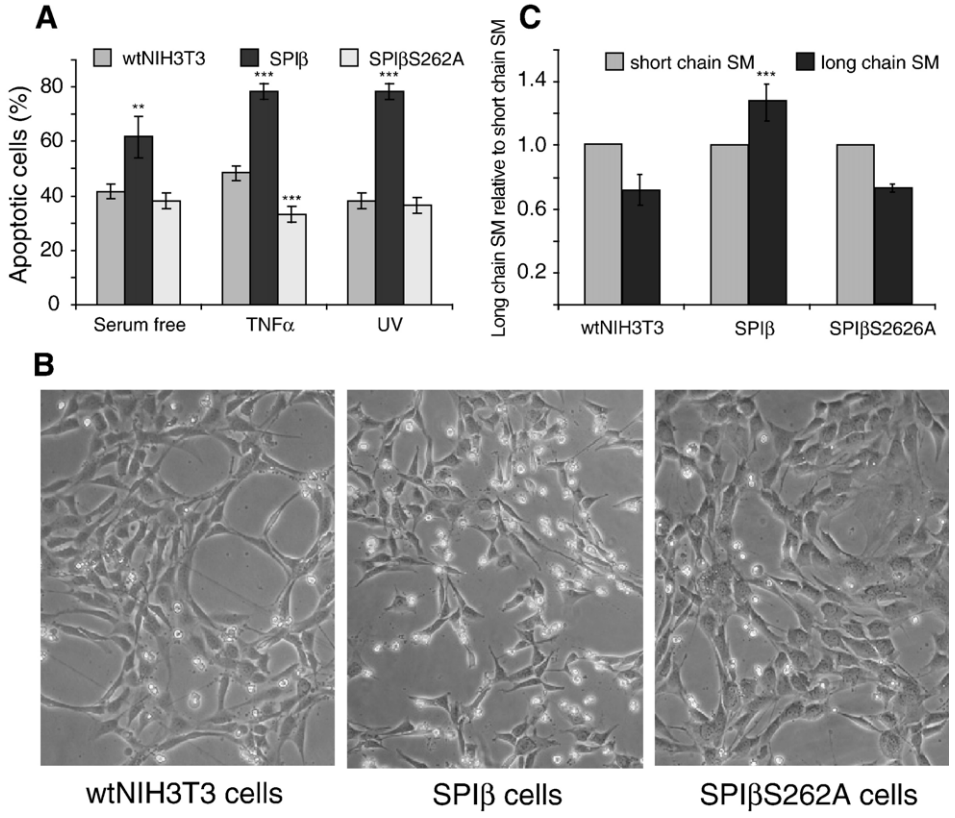

Fig. 1. (A) Survival of wtNIH3T3, SPIβ and SPIβS262A cells upon induction of apoptosis by UV irradiation, TNFα or serum deprivation. Cells were grown to 90% confluency. Serum starvation: growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB) and the cells were incubated for 16 h at 37 °C. TNFα induced apoptosis: growth medium was replaced by DBB containing cycloheximide (2.5 μg/ml) and TNFα (10 ng/ml) and the cells were incubated for 7 h at 37 °C. UV induced apoptosis: growth medium was replaced by DBB and cells were incubated for 4 h at 37 °C. After removal of DBB the cells were irradiated with 200 J/m^2, fresh DBB was added to the cells and incubated for 3 h at 37 °C. The number of apoptotic cells (blebbing) was determined by visual analysis. Results ± SD represent the mean values of at least three independent experiments. *p*-values were calculated relative to the survival of wtNIH3T3 cells (**$p<0.01$; ***$p<0.001$). (B) Photographs of blebbing wtNIH3T3, SPIβ and SPIβS262A cells. Cells were grown to 85% confluency. Cells were incubated for 4 h at 37 °C with DBB. After removal of DBB the cells were irradiated with 200 J/m^2 and fresh DBB was added. Images were taken 90 min after UV irradiation on a Nikon TE2000 U inverted microscope. Left panel, wtNIH3T3 cells; middle panel, SPIβ cells; right panel, SPIβS262A cells. (C) Long and short chain SM levels in wtNIH3T3, SPIβ and SPIβS262A cells. Cells were grown to 80% confluency and total lipids were extracted by the method of Bligh and Dyer [18]. Results ± SD represent the mean values of at least three independent experiments. *p*-values were calculated relative to the level of long chain SM in wtNIH3T3 cells (***$p<0.001$).

wtNIH3T3 and SPIβS262A cells have relatively more short chain than long chain SM species (ratio of long chain over short chain of 0.7), whereas SPIβ cells have relatively less short chain than long chain SM species (ratio of 1.25). Ceramide analysis of wtNIH3T3 and SPIβ cells showed that the species composition was similar to that of SM (data not shown).

In agreement with previous observations PGE_2 and $PGF_{2\alpha}$ are able to protect wtNIH3T3 mouse fibroblast cells against apoptosis (Fig. 2A) [26,27]. Incubation of SPIβ cells with these prostaglandins did not protect these cells against UV-induced apoptosis, whereas CM from wtNIH3T3 cells did protect (Fig. 2B). The concentration of PGE_2 and $PGF_{2\alpha}$ used were 0.5 and 0.1 ng/ml respectively. These concentrations are comparable to the levels detected in CM from SPIα cells and are approximately 5-fold higher compared to the levels detected in CM from wtNIH3T3 [13] and SPIβ cells. A similar protection was observed when neutral lipid extracts from this CM were used (data not shown). Similar to wtNIH3T3, SPIβS262A cells were protected by both $PGE_2/PGF_{2\alpha}$ and CM from wtNIH3T3 cells (Fig. 2A–C).

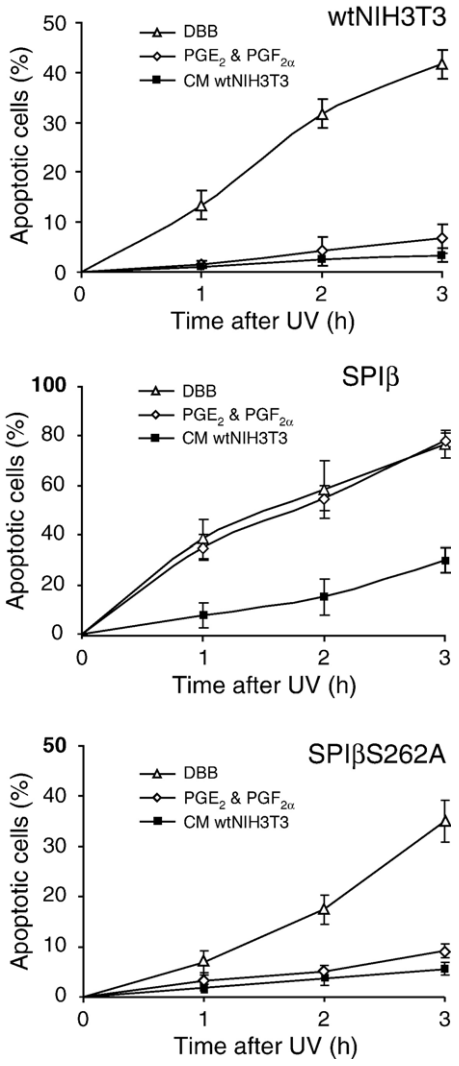

Fig. 2. Survival of wtNIH3T3, SPIβ and SPIβS262A cells upon induction of apoptosis by UV irradiation. Cells were grown to 90% confluency. The growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from wtNIH3T3 cells and the cells were incubated for 4 h at 37 °C. After removal of DBB or CM, the cells were irradiated with UV light (200 J/m^2). Fresh DBB was added to the cells and the number of apoptotic cells (blebbing) was determined by visual analysis at the indicated times. Results±SD represent the mean values of at least three independent experiments performed in duplicate.

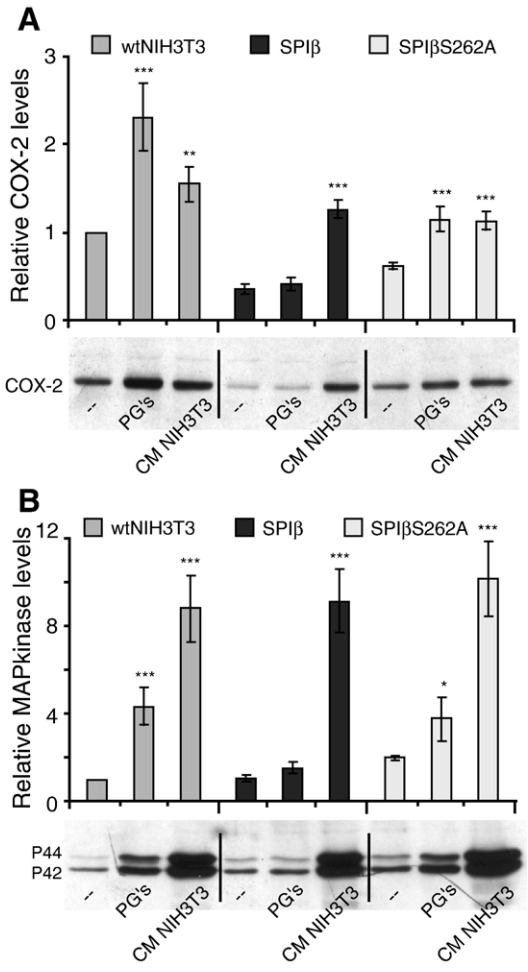

Fig. 3. (A) Expression of COX-2 in wtNIH3T3, SPIβ and SPIβS262A upon incubation with $PGE_2/PGF2_\alpha$ or CM from wtNIH3T3 cells. Cells were grown to 90% confluency and incubated with DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from wtNIH3T3 cells for 5 h at 37 °C. Equal amounts of cell lysate protein (30 μg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a COX-2 specific antibody. Representative experiment performed in triplicate. *p*-values were calculated relative to the COX-2-immunoband obtained from the cells incubated with DBB (**$p<0.01$; ***$p<0.001$). (B) Phosphorylation of p42/p44 MAP kinase in wtNIH3T3, SPIβ and SPIβS262A upon incubation with $PGE_2/PGF_{2\alpha}$ or CM from wtNIH3T3 cells. Cells were grown to 90% confluency and incubated with DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from NIH3T3 cells for 10 min at 37 °C. Equal amounts of cell lysate protein (25 μg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS-PAGE followed by Western blot analysis using a p42/p44 MAP kinase specific antibody. Representative experiment performed in triplicate. *p*-values were calculated relative to the p42/p44 MAPK-immunobands obtained from the cells incubated with DBB (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

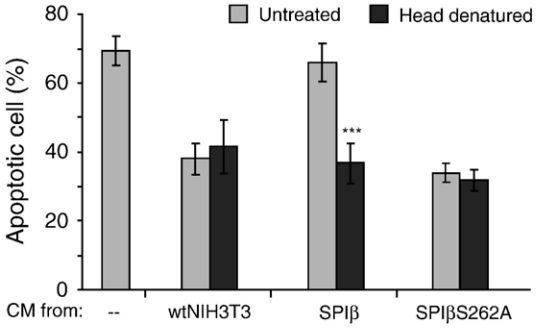

Fig. 4. Survival of SPIβ cells upon induction of apoptosis by UV irradiation after preincubation with CM from wtNIH3T3, SPIβ and SPIβS262A cells with or without heat denaturation. SPIβ cells were grown to 90% confluency. The growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB) or by CM and the cells were incubated for 4 h at 37 °C. After removal of DBB the cells were irradiated with UV light (200 J/m^2), fresh DBB was added to the cells and incubated for 3 h at 37 °C. The number of apoptotic cells (blebbing) was determined by visual analysis. Results ± SD represent the mean values of at least three experiments performed in duplicate. *p*-values were calculated relative to the survival of SPIβ cells incubated with the untreated conditioned medium (***$p<0.001$).

3.2. Expression of cyclooxygenase-1 and -2

The anti-apoptotic activity of CM from wtNIH3T3 and SPIα cells is caused by the presence of eicosanoids, the synthesis of which is (partially) dependent on COX-2 activity [13]. As shown by Western blot analysis, the level of COX-2 in SPIβ cells is reduced when compared to wtNIH3T3 cells (Fig. 3A; lanes 1 and 4). Again the SPIβS262A cells resembled the wild type cells as the levels of COX-2 were comparable in both cell lines (Fig. 3A; lanes 1 and 7). Arachidonic acid metabolites produced by COX-2 can stimulate the expression of this enzyme via an autocrine pathway [28,29]. By using an ELISA kit we showed that the amount of PGE_2 secreted by the SPIβ cells (0.16 ng/ml) is similar to that of wtNIH3T3 cells (0.1 ng/ml). Therefore the reduced level of COX-2 in SPIβ cells is not linked to a decreased level of PGE_2. When SPIβ cells were incubated for 5 h with PGE_2 in combination with $PGF_{2\alpha}$, COX-2 levels remained the same (Fig. 3A; lanes 4 and 5), whereas the COX-2 levels of wtNIH3T3 and SPIβS262A cells were increased (Fig. 3A; lanes 2 and 8). This strongly suggests that the upregulation of COX-2 by PGE_2/$PGF_{2\alpha}$ is inhibited in SPIβ cells. On the other hand, incubation of SPIβ cells with CM from wtNIH3T3 cells did increase the COX-2 levels (Fig. 3A; cf. lanes 4 and 6). A similar upregulation of COX-2 was observed for wtNIH3T3 cells (Fig. 3A; cf. lanes 1 and 3) and SPIβS262A cells (Fig. 3A; cf. lanes 7 and 9). This suggests that the upregulation of COX-2 in SPIβ cells by CM from wtNIH3T3 cells may be linked to the increased survival upon UV irradiation under the same conditions. Lack of COX-2 induction by PGE_2 and $PGF_{2\alpha}$ in SPIβ cells agrees with the failure to protect these cells (Fig. 2B). In contrast to COX-2, expression levels of COX-1 were similar in all three cell lines and did not change upon incubation with PGs or CM from wtNIH3T3 cells (data not shown).

3.3. p42/p44-MAP kinase activation

Activation of p42/p44 MAP kinase is commonly observed after hormone or polypeptide growth factor induced proliferation or cell survival [30–33]. Incubation of wtNIH3T3, SPIβ and SPIβS262A cells with CM from wtNIH3T3 cells induced a rapid activation of p42/p44 MAP kinase (Fig. 3B; cf. lanes 3, 6, 9). Upon incubation with PGE_2 and $PGF_{2\alpha}$, p42/p44 MAP kinase was activated in wtNIH3T3 and SPIβS262A cells but not in SPIβ cells (Fig. 3B; cf. lanes 2, 5, 8). These data

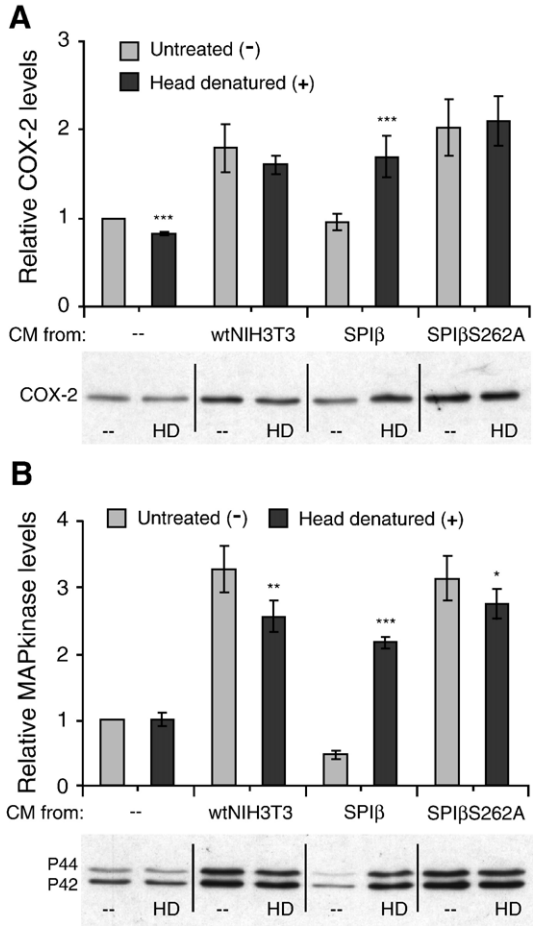

Fig. 5. (A) Expression of COX-2 in SPIβ cells upon incubation with CM from wtNIH3T3, SPIβ and SPIβS262A with or without heat denaturation of the CM. Cells were grown to 90% confluency and incubated for 5 h at 37 °C with CM from NIH3T3, SPIβ or SPIβS262A cells, either heat denatured or not. Equal amounts of cell lysate protein (30 μg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a COX-2 specific antibody. Representative experiment performed in triplicate. *p*-values were calculated relative to the COX-2-immunoband obtained from the SPIβ incubated with the untreated conditioned medium (***$p<0.001$). (B) Phosphorylation of p42/p44 MAP kinase in SPIβ cells upon incubation with CM from wtNIH3T3, SPIβ and SPIβS262A with or without heat denaturation of the CM. Cells were grown to 90% confluency and incubated for 10 min at 37 °C with CM from wtNIH3T3, SPIβ or SPIβS262A cells, either heat denatured or not. Equal amounts of cell lysate protein (25 μg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a p42/p44 MAP kinase specific antibody. Representative experiment performed in triplicate. *p*-values were calculated relative to the p42/p44 MAPK-immunobands obtained from the SPIβ incubated with the untreated conditioned medium (*$p<.05$; **$p<0.01$; ***$p<0.001$).

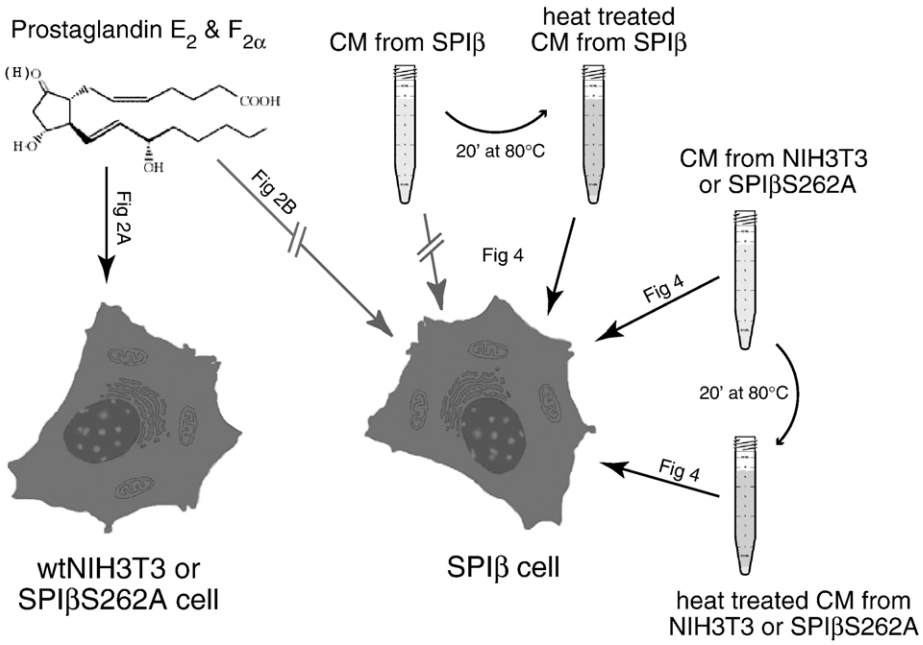

Fig. 6. Effect of prostaglandins and conditioned medium on the survival of wtNIH3T3 and SPIβ cells. Prostaglandin E_2 and prostaglandin $F_{2\alpha}$ protect wtNIH3T3 and SPIβS262A cells against UV-induced apoptosis but fail to do so with SPIβ cells. The anti-apoptotic activity present in CM from SPIβ cells is masked by the presence of an antagonist, which is inactivated upon heat treatment. Heat treatment of CM from wtNIH3T3 and SPIβS262A cells has no effect on the anti-apoptotic activity. This suggests that the expression and phosphorylation of PI-TPβ is responsible for the production of the antagonist.

support the finding that PGE_2 and $PGF_{2\alpha}$ do protect wtNIH3T3 and SPIβS262A cells against apoptosis but fail to protect SPIβ cells, indicating that in SPIβ cells this signal pathway for stimulation of proliferation and cell survival is inhibited.

3.4. Secretion of an antagonist of anti-apoptotic activity

SPIα cells secrete a highly potent anti-apoptotic and mitogenic factor(s) [12,13]. Although to a lesser extent, wtNIH3T3 cells also produce bioactive factors. Indeed, when prior to UV irradiation SPIβ cells were incubated with CM from wtNIH3T3 cells instead of DBB, the extent of apoptosis was decreased by 45% (Fig. 4). Incubation of SPIβ cells with CM from SPIβ cells did not prevent apoptosis. However, upon heat treatment (20 min at 80 °C) CM from SPIβ cells expressed an anti-apoptotic activity comparable to that from wtNIH3T3 cells (Fig. 4). As a control, heat treatment of CM from wtNIH3T3 and SPIβS262A cells had little effect on the anti-apoptotic activity indicating that this activity was heat stable. This strongly suggests that CM from SPIβ cells contains a heat-labile factor (antagonist) that interferes with the anti-apoptotic activity present. The addition of CM from SPIβ cells containing the antagonist did not increase the rate of apoptosis in the wild type cells and apoptosis resistant SPIα cells, which suggests that the antagonist has no pro-apoptotic activity. CM from SPIβS262A cells resembled that from wtNIH3T3 cells indicating that phosphorylation of PI-TPβ is required for the production of the antagonist.

In agreement with the observations for cell survival, CM from SPIβ cells after heat-treatment (20 min 80 °C) induced COX-2 in SPIβ cells (Fig. 5A; cf. lanes 2 and 6) to the same extent as CM from wtNIH3T3 cells (Fig. 5A; cf. lanes 4 and 6) and SPIβS262A cells (Fig. 5A; lanes 6 and 8) whereas the untreated CM from SPIβ cells had no effect on COX-2 expression (Fig. 5A; lane 5). Similarly, the untreated CM from SPIβ has no effect on the level of p42/p44 MAP kinase, whereas the heat-treated CM from SPIβ cells is able to activate MAPK (Fig. 5B; cf. lanes 5 and 6). For comparison, the untreated CM from wtNIH3T3 and SPIβS262A cells are able to activate MAPK in SPIβ cells (Fig. 5B; cf. lanes 3, 4, 7 and 8), resulting in protection of these cells against UV induced apoptosis (Fig. 4).

The effect of $PGE_2/PGF_{2\alpha}$ and conditioned media on the survival of wtNIH3T3 and SPIβ cells are summarized in Fig. 6.

4. Discussion

Previously we have shown that a ten-fold increase of PI-TPβ in wtNIH3T3 mouse fibroblast cells (SPIβ cells) significantly increases the sensitivity towards apoptosis induced by UV-radiation [15]. Here we show that SPIβ cells incubated with TNFα and serum starvation are much more prone to apoptosis indicating that the increased apoptotic response was not restricted to UV irradiation. A role of PI-TPβ in cell survival was also indicated by the finding that initially, using another expression vector (pSG5) than the one currently used (pBK-CMV) we failed to obtain stable SPIβ cell lines. Due to high levels of PI-TPβ we routinely observed that these cells died after 4–5 passages. Previously we have shown that PI-TPβ is mainly associated with the Golgi system and that this association requires the PKC-dependent phosphorylation of Ser-262 as the mutant PI-TPβ(S262A) is present throughout the cell

[3,9,10]. By overexpressing PI-TPβ(S262A) to a level comparable to that of PI-TPβ (9.0 and 10.6 ng per 100 μg cytosolic protein, respectively) the ensuing SPIβS262A cells have a sensitivity towards apoptosis comparable to that of wtNIH3T3 cells [15]. This strongly suggests that there is a relationship between the phosphorylation of PI-TPβ and the sensitivity towards apoptosis.

On the other hand, recent studies concluded that the Golgi localization of PI-TPβ is not dependent on the phosphorylation of Ser-262 [34,35]. We do not have an explanation for this discrepancy. However, we do find that increased levels of PI-TPβS262A have no effect on the apoptosis sensitivity and thus, regardless of Golgi localization, it is clear that phosphorylation of PI-TPβ is required to affect apoptosis.

Two important parameters linked to apoptosis are activation of p42/p44 MAP kinase and expression of COX-2 [36–38]. In general, inhibition of COX-2 expression enhances apoptosis and more specifically reduces the incidence and progression of tumors in animal models [39–42]. Phosphorylation of p42/p44 MAP kinase through the Ras > Raf > MAP kinase kinase (MKK) cascade is associated with proliferation, protection against apoptosis and angiogenesis [30–33]. Here we show that the prostaglandins PGE_2/$PGF_{2\alpha}$, although present in CM from SPIβ cells, are unable to upregulate COX-2 in SPIβ cells, but are able to do so in wtNIH3T3 and SPIβS262A cells, showing that the pathway of upregulation is blocked in SPIβ cells (Fig. 3A). This results in a lower basal level of COX-2 in SPIβ cells and prevents prostaglandins (PGs) from protecting SPIβ cells against apoptosis. In addition we show that these PGs cannot activate p42/p44 MAP kinase in SPIβ cells whereas this pathway is activated in wtNIH3T3 and SPIβS262A cells (Fig. 3B). These observations may explain why SPIβ cells are more prone to apoptosis and why PGE_2 and $PGF_{2\alpha}$ are unable to induce survival in SPIβ cells. CM from SPIβ cells appeared to lack the survival activity present in the CM from NIH3T3 cell. Interestingly, CM from SPIβ cells acquired survival activity upon heating, indicating that the expression of PI-TPβ is responsible for the production and secretion of a component that interferes with the action of the intrinsic survival factors present. Attempts to gain insight into the nature of the PI-TPβ-dependent 'antagonist' were inconclusive. Experiments using protein synthesis inhibitors and analysis of [^{14}C]serine-labeled SM metabolites secreted by SPIβ cells revealed no significant differences compared with control cells.

The relationship between SM metabolism and apoptosis has been investigated extensively. B-cell receptor-triggered apoptosis is associated with an early rise of C16 ceramide leading to the subsequent formation of long chain C24 ceramide via activation of effector caspases [43]. Apoptotic stimuli including TNFα [44], ionizing radiation [45] and B-cell receptor cross-linking [43] can generate ceramide by the induction of SM hydrolysis through the action of sphingomyelinases or the *de novo* pathway. In contrast to SPIβS262A and wtNIH3T3 cells, SPIβ cells maintain the total level of SM under conditions where SM is degraded in the plasma membrane to ceramide by exogenous sphingomyelinase [7,9]. Although the mechanism by which PI-TPβ regulates the rapid conversion of ceramide to SM is not known, the data again strongly suggest that PI-TPβ must be phosphorylated at Ser-262 in order to maintain the cellular SM levels. Although increased levels of PI-TPβ appear to be required for SPIβ cells to rapidly convert ceramide to SM [7], we found that steady-state levels of ceramide in SPIβ and wtNIH3T3 cells are similar. However, we did observe that the molecular species of the fatty acids of SM (Fig. 1B) and ceramide are different with SPIβ cells having relatively less short chain (C16) and more long chain species (C24:1/0) compared with wtNIH3T3 cells. To date it is not known whether the sensitivity towards apoptosis of SPIβ cells is related to this shift from short chain to long chain ceramide/SM. However, it could be that the relative enrichment of long chain SM species in SPIβ cells has an effect on the plasma membrane and, thereby, affect the properties of membrane proteins [46].

We propose that as a consequence of an antagonist blocking of the autocrine action of the survival factor present in CM from SPIβ cells, the SPIβ cells are more sensitive towards induced apoptosis (summarized in Fig. 6). In addition, the failure of PGE_2/$PGF_{2\alpha}$ to both activate p42/p44 MAP kinase and to upregulate COX-2 levels may also explain why SPIβ cells are more prone to apoptosis (summarized in Fig. 6). At this point our data suggest that since an increased expression of PI-TPβ promotes apoptosis, the deletion of PI-TPβ may have a prohibitive effect on apoptosis. Since apoptosis is an essential event during early embryonic development, the proposed role of PI-TPβ in apoptosis may explain why the generation of a PI-TPβ knockout mouse has failed [6]. Understanding why and how expression of a single protein decreases the rate of proliferation as well as survival of cells might be of interest for research on methods to decrease the growth of rapidly proliferating tumor cells that have gained resistance against induction of apoptosis.

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