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The action of the mast cell product tryptase on cyclooxygenase-2 (COX2) and subsequent fibroblast proliferation involves activation of the extracellular signal-regulated kinase isoforms 1 and 2 (erk1/2)

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Abstract

The mast cell product tryptase, via protease-activated receptor 2 (PAR2), induces cyclooxygenase-2 (COX2) and 15-deoxy-prostaglandin J2 (15d-PGJ2) synthesis. 15d-PGJ2, through the nuclear peroxisome proliferator activated receptor gamma (PPARγ), subsequently causes fibroblast proliferation. In this study we attempted to determine initial events of the tryptase/PAR2 signaling pathway leading to COX2 induction and fibroblast proliferation.

In human fibroblasts (HFFF2), cDNA array, RT-PCR and Western blotting studies demonstrated that tryptase, but not 15d-PGJ2, up-regulates *c-jun*, *c-fos* and COX2 expression, and phosphorylates the extracellular signal-regulated kinase isoforms 1 and 2 (erk1/2). Furthermore, tryptase effects on erk1/2, *c-jun*, *c-fos*, COX2 and cell proliferation were prevented by PD98059, an inhibitor of the mitogen-activated protein kinase kinase (MEK). Other kinases [P38, stress-activated protein kinase/*c-jun* N-terminal kinase (SAPK/JUNK), erk5], intracellular Ca^{2+} or cAMP were not affected by tryptase/PAR2. Our study identifies crucial intracellular events leading to induction of COX2 and fibroblast proliferation, i.e. a cornerstone of fibrosis.

Keywords: Tryptase; Cyclooxygenase-2; MAP kinases; Transcription factors; cAMP; Ca^{2+}

1. Introduction

Fibrosis, which involves increased numbers of fibroblasts-myofibroblasts and excessive deposition of extracellular matrix proteins, is the hallmark of numerous diseases including disorders of the lung [1–5], liver [6–8], kidney [9–11], heart [12,13] and skin [14]. Mast cells may be involved in the pathogenesis of fibrosis, since they are usually present in these cases in an increased number and show signs of activation and degranulation [15,16]. Mast cells secrete a plethora of potent mediators [17,18], which directly and/or indirectly could participate in fibrogenesis. Coculture studies of a human leukemic mast cell 1 line (HMC-1) with human orbital fibroblasts indicated up-regulation of cyclooxygenase-2 (COX2), the inducible form of the key enzyme in the synthesis of prostaglandins (PGs), and production of PGE2 in fibroblasts [19]. Since it appears

Abbreviations: 15d-PGJ2, 15-deoxy-prostaglandin J2; AP-1, activator protein-1; BMK1/ERK5, big mitogen-activated kinase 1/extracellular signal-regulated kinase isoform 5; CO_2, carbon dioxide; COX2, cyclooxygenase-2; erk1, extracellular signal-regulated kinase isoform 1; erk2, extracellular signal-regulated kinase isoform 2; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFFF2, human fetal foreskin fibroblast cell line 2; HMC-1, human leukemic mast cell 1; IU, international units; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; p38 MAPK, p38 mitogen-activated protein kinase; PAR2, protease-activated receptor 2; PCR, polymerase chain reaction; PG, prostaglandin; PI3K, phosphatidyl-inositol 3-kinase; PK, protein kinase; PPARγ, peroxisome proliferator activated receptor gamma; PPIA, peptidylprolyl isomerase A; RPL 13A, ribosomal protein L13a; RT, reverse transcription; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

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that PGs can regulate fibroblast proliferation and type I collagen production [20,21], PG and COX2 are thought to be involved in fibrotic events [22–24]. In this context, the major mast cell product, the serine protease tryptase, stimulates fibroblast proliferation via activation of the protease-activated receptor 2 (PAR2) [1,25]. We recently demonstrated that this action of tryptase through PAR2 induces expression of COX2 and subsequent PG synthesis in a human fetal foreskin fibroblast cell line (HFFF2). Among the PGs produced, 15-deoxy-prostaglandin J2 (15d-PGJ2), the natural ligand for the nuclear peroxisome proliferator activated receptor gamma (PPARγ), was pinpointed to be responsible for the tryptase-induced fibroblast proliferation [26].

In the present report we characterized the initial events associated with the tryptase/PAR2 signaling transduction pathway in human fibroblasts. We found that tryptase induces COX2 and subsequent fibroblast proliferation by a Ca^{2+}- and cAMP-independent mechanism that involves activation of the extracellular signal-regulated kinase isoforms 1 and 2 (erk1/2). We propose that our results, by elucidating details of how tryptase can stimulate COX2 expression and subsequent fibroblast proliferation, are of relevance for a better understanding of pathogenic events in fibroproliferative disorders.

2. Experimental procedures

2.1. Cell culture and reagents

HFFF2 (European Collection of Cell Cultures, Salisbury, UK) were maintained in DMEM supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich Chemie, Schnelldorf, Germany). Cells were incubated in FCS-free medium for 24 h to synchronize the cell cycle. To drive G0 cells into cell cycle, we treated serum-starved HFFF2 with medium containing 2.5% FCS. After 20 h, when cells entered into S phase [26,27], they were incubated in the presence or absence of several chemicals (see below).

2.2. Gene array

Gene expression was evaluated in HFFF2 incubated for 1 h in the presence or absence of 120 mIU/ml recombinant human β-tryptase (Promega, Mannheim, Germany) or 1 μM 15d-PGJ2 (Cayman Chemical, Ann Arbor, MI, USA) using commercial chemiluminescent array kits (SuperArray, Biomol, Hamburg, Germany). Background intensity was subtracted and the integrated density corresponding to each gene was quantified only for genes with expression levels higher than 10% of those detected for the reference/housekeeping gene peptidylprolyl isomerase A (PPIA). Data were standardized for non-specific variation expressing the results relative to the intensity the mean of the reference/housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PPIA, ribosomal protein L13a (RPL 13A) and β-actin. Gene expression ratios were calculated by dividing the standardized integrated density of the treated samples by the standardized integrated density of the non-treated samples.

2.3. Reverse transcription (RT) and PCR analysis

Gene expression was evaluated in HFFF2 incubated for 5, 10, 20 and/or 30 min in the presence or absence of 120 mIU/ml tryptase alone, 120 mIU/ml tryptase plus 10 μM and 30 μM PD98059 (Alexis Deutschland, Grünberg, Germany) or 1 μM 15d-PGJ2. Chemicals were added directly to the cell culture medium in order to avoid possible alterations in the gene expression resulting from medium change. RNA extraction was performed using Rneasy mini kit (Qiagen, Hilden, Germany). Followed, RT using oligo-dT_{15} primers and PCR amplification were performed [26]. Information about the oligonucleotide primers used and cDNAs isolated are given in Table 1. The identity of PCR products was verified by sequencing with a fluorescence-based dideoxy sequencing reaction and an automated sequence analysis on an ABI model 373A DNA sequencer. PCR bands were quantified by densitometry and normalized to cyclophilin and α-tubulin reference/housekeeping genes.

2.4. Western blot

HFFF2 were plated on 60-mm dishes (Nunc, Wiesbaden, Germany) and incubated for 5, 10, 20 and/or 30 min in the presence or absence of 120 mIU/ml tryptase alone, 120 mIU/ml tryptase plus 10 and 30 μM PD98059 or 1 μM 15d-

Table 1
Oligonucleotide primers for polymerase chain reaction (PCR) amplification of cDNAs obtained by reverse transcription (RT) from human fetal foreskin fibroblasts (HFFF2)

Target	Primers		Bp	Annealing temperature (°C)
	Sense	Antisense		
COX2	5′ -GCAAATCCTTGCTGTTCC-3′	5′ -GGAGGAAGGGCTCTAGTA-3′	368	55
c-fos	5′ -GCCTAACCGCCACGATGATGT-3′	5′ -GCCCCTCCTGCCAATGCTCTG-3′	395	60
c-jun	5′ -GGAAACGACCTTCTATGACGATGCCCTCAA-3′	5′ -CCCTCCTGCTCATCTGTCACGTTCTT-3′	312	60
Cyclophilin	5′ -CTCCTTTGAGCTGTTTGCAG-3′	5′ -CACCACATGCTTGCCATCC-3′	325	55
α-Tubulin	5′ -CACCCGTCTTCAGGGCTTCTTGGTTT-3′	5′ -CATTTCACCATCTGGTTGGCTGGCTC-3′	476	54

GenBank accession numbers: COX2, U04636; *c-fos*, K00650; *c-jun*, J04111; cyclophilin, Y00052; α-tubulin, X01703.

PGJ2. Immunoblots were performed as described [28] by using a monoclonal mouse anti-human phospho-extracellular signal-regulated kinase isoforms 1 and 2 (phospho-erk 1/2) antibody (1:500; Cell Signaling Technology, New England Biolabs, Frankfurt am Main, Germany), a polyclonal rabbit anti-human erk1/2 antiserum (1:1000; Cell Signaling Technology, New England Biolabs), a polyclonal rabbit anti-human phospho-p38 mitogen-activated protein kinase (phospho-p38 MAPK) antiserum (1:1000; Cell Signaling Technology, New England Biolabs), a polyclonal sheep anti-phospho big mitogen-activated kinase 1/extracellular signal-regulated kinase isoform 5 (phospho-BMK1/erk5) antiserum (1:1000; Upstate Biotechnology, Biomol Feinchemikalien, Hamburg, Germany), a polyclonal rabbit anti-phospho stress-activated protein kinase/*c-jun* N-terminal kinase (phospho-SAPK/JNK) antiserum (1:500; Calbiochem, Merck KGaA, Darmstadt, Germany) and a monoclonal mouse anti-human β-cytoplasmic actin antibody (1:5000; Sigma-Aldrich). Western blot bands were quantified by densitometry and normalized to β-actin reference/housekeeping gene.

2.5. Proliferation assays

Approximately 7×10^3 HFFF2 were seeded in 96-well plates (Nunc) and incubated for 24 h in the presence or absence of 120 mIU/ml tryptase alone, 10 and 30 μM PD98059 alone, 120 mIU/ml tryptase plus 10 μM and 30 μM PD98059 or 1 μM 15d-PGJ2. Cell proliferation was determined by using the CellTiter 96 Aqueous One Solution cell-proliferation assay (Promega). The specificity and sensitivity of this method was evaluated previously in our lab by comparison with a [3H]thymidine incorporation assay [29].

2.6. Measurements of intracellular Ca^{2+}

Ca^{2+} measurements were performed on HFFF2 treated with tryptase alone (120 mIU/ml), heparin alone (10 μM, Serva, Heidelberg, Germany), tryptase (120 mIU/ml) plus heparin (10 μM), PAR2 agonist peptide SLIGKV (10 μM, Genzentrum, University of Munich, Germany), thrombin (1 nM, Sigma-Aldrich) or trypsin (22 μM, PAA, Cölbe, Germany) as previously described [30]. Briefly, cells were grown in DMEM supplemented with 10% FCS on coverslips for 24 h. Medium was replaced by fresh DMEM supplemented with 10% FCS containing 5 μM fluo-4-AM (Molecular Probes, Eugene, OR, USA) and cells were loaded for 30 min at 37 °C and 5% carbon dioxide (CO_2). Finally, cells were washed in DMEM without FCS and fluorescence measurements were performed every 2 s for a total of 1000 s.

2.7. Determinations of cAMP

Measurements were performed on HFFF2 treated with tryptase (120 mIU/ml) using a commercially available kit

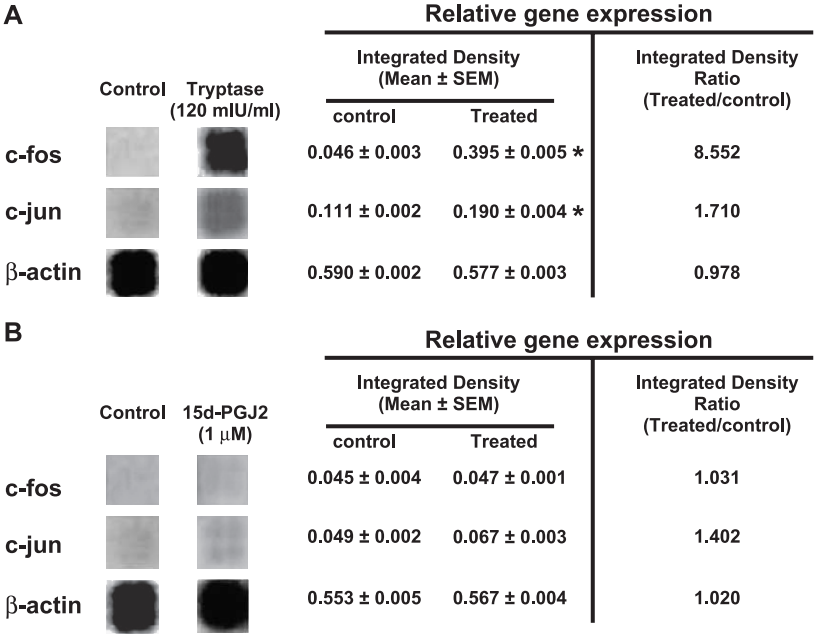

Fig. 1. Gene expression profile in human fetal foreskin fibroblasts (HFFF2) treated with tryptase and 15d-PGJ2. Gene expression was evaluated in HFFF2 cells incubated for 1 h in the presence or absence of 120 mIU/ml human recombinant tryptase (A) or 1 μM 15d-PGJ2 (B) using commercial gene array kits, as described under "Experimental procedures". Chemiluminescence signals were digitized and the integrated density corresponding to each gene was quantified. Results were normalized to the expression levels of the mean of reference/housekeeping genes (i.e. β-actin). The figure shows results obtained from one of three representative gene arrays that yielded comparable results. Numbers given are the mean ± SEM of these three experiments. Gene expression ratios corresponding to *c-fos*, *c-jun* and β-actin were calculated by dividing the normalized integrated density of the treated samples by the normalized integrated density of the non-treated samples. *$P<0.05$ vs. the corresponding control group (untreated).

(Cayman Chemical). After different incubation times (20 min, 1 h, 3 h) samples (supernatants and cell pellets) were frozen until analysis. In order to increase the sensitivity of detection samples and standards were acetylated before the assay. Under these conditions the standard curve was linear between 0.03 and 3.00 pmol/ml.

2.8. Data analysis

Data acquisition, analysis, statistics and presentation were performed using PRISM 3.0 (GraphPad Software, San Diego, CA, USA), and SigmaPlot (Wavemetrics, Lake Oswego, OR, USA). For cDNA array, PCR and Western blot assays, the integrated density corresponding to each signal was quantified using SCION IMAGE (SCION Corporation, Frederick, MD, USA). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Student's *t*-test for two comparisons or Student-Newman-Keuls test for multiple comparisons. Data reported represent mean±SEM.

3. Results

3.1. Defining the signaling mechanism by which tryptase induces COX2

As recently demonstrated in HFFF2, the stimulatory action of tryptase on fibroblast proliferation is a two-step process. Tryptase via PAR2 induces rapid expression of COX2, resulting in PG synthesis. Then, 15d-PGJ2, acting via PPARγ, initiates fibroblast proliferation [26]. In order to distinguish between the signaling events associated to tryptase/PAR2 and 15d-PGJ2/PPARγ, we compared the results obtained from cDNA array studies. Whereas numerous genes were regulated by both treatments, tryptase and 15d-PGJ2, only tryptase increased the levels of the transcription factors *c-fos* and *c-jun* (Fig. 1A and B). Employing RT-PCR we confirmed that tryptase induces *c-fos* and *c-jun* expression (Fig. 2A), while 1 μM 15d-PGJ2, i.e. a concentration clearly inducing cell proliferation [26], had no effect (Fig. 2B), Thus, induction of *c-fos* and *c-jun* in HFFF2 results from tryptase/PAR2 and occurs prior to the binding of 15d-PGJ2 to PPARγ.

Western blot experiments were performed to determine whether the effect of tryptase on COX2 in HFFF2 is mediated via protein kinases (PKs). Levels of phospho-SAPK/JNK, phospho-p38 MAPK and phospho-BMK1/erk5, were not affected in the presence of tryptase or 15d-PGJ2 (data not shown). Nevertheless, addition of 120 mIU/ml tryptase to cells, i.e. a concentration stimulating proliferation in HFFF2 (Fig. 3C) and increasing COX2 expression at mRNA level (Fig. 3A) and also at protein level after 30 min [26], induced phosphorylation of erk1 and erk2, but did not change total protein levels of erk1 and erk2 (Fig. 4A). In contrast, whereas treatment of HFFF2 with

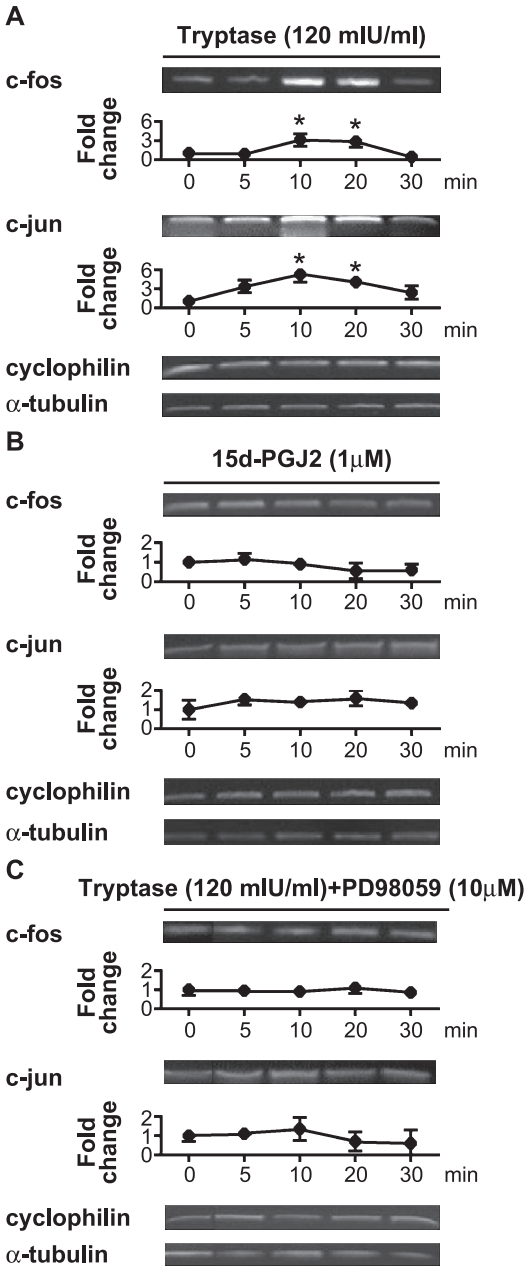

Fig. 2. Tryptase-stimulated *c-fos* and *c-jun* expression in human fetal foreskin fibroblasts (HFFF2) is associated with the erk pathway. HFFF2 were incubated in the absence or presence of 120 mIU/ml tryptase (A), 1 μM 15d-PGJ2 (B) or 120 mIU/ml tryptase plus 10 μM PD98059 (C) for 5, 10, 20 and 30 minutes. Expression of *c-fos* and *c-jun* was monitored by RT-PCR using specific primers as described under "Experimental procedures". Representative ethidium bromide-stained agarose gels show results obtained from one of three experiments that yielded comparable results. In all experiments, PCR bands were densitometrically evaluated. Results are expressed as "fold change" relative to the control (0 min), which was assigned a value of 1. Data shown were normalized to cyclophilin and α-tubulin genes. In line plot graphics, the mean ± SEM from three independent experiments (four replicates per experiment) performed on different cell preparations is shown. *$P<0.05$ vs. the corresponding control group (0 min).

1 μM 15d-PGJ2 significantly increased cell proliferation [26], erk1/2 phosphorylation remained unchanged (data not shown). This implies that phosphorylation and activation of

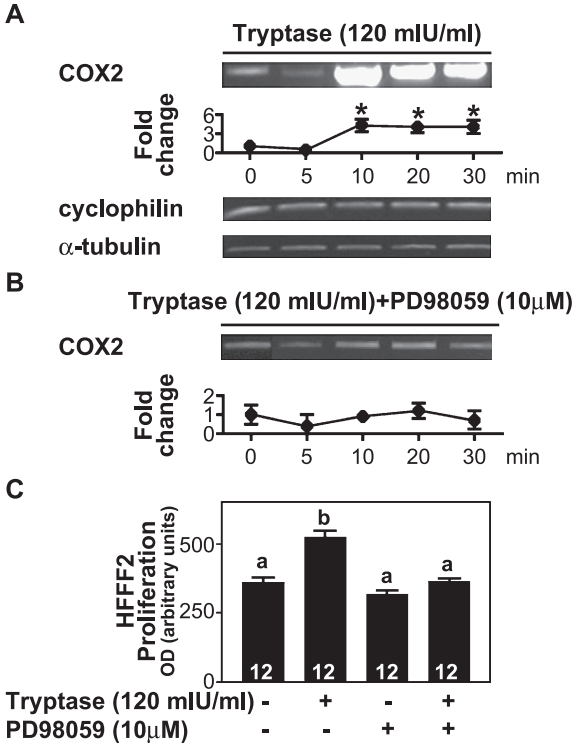

Fig. 3. Tryptase-stimulated COX2 expression and cell proliferation in human fetal foreskin fibroblasts (HFFF2) is associated with the erk pathway. HFFF2 were incubated in the absence or presence of 120 mIU/ml tryptase (A) or 120 mIU/ml tryptase plus 10 μM PD98059 (B) for 5, 10, 20 and 30 min. Expression of COX2 was monitored by RT-PCR using specific primers as described under "Experimental procedures". Representative ethidium bromide-stained agarose gels show results obtained from one of three experiments that yielded comparable results. In all experiments, PCR bands were densitometrically evaluated. Results are expressed as "fold change" relative to the control (0 min), which was assigned a value of 1. Data shown were normalized to cyclophilin and α-tubulin genes. In line plot graphics, the mean ± SEM from three independent experiments (four replicates per experiment) performed on different cell preparations is shown. *P<0.05 vs. the corresponding control group (0 min). (C) Cell cycle synchronized HFFF2 cells, as described under "Experimental procedures", were incubated in the presence or absence of 120 mIU/ml tryptase alone, 10 μM PD98059 alone or 120 mIU/ml tryptase plus 10 μM PD98059. Cell proliferation was determined by using the CellTiter 96 Aqueous One Solution cell-proliferation assay (Promega, Mannheim, Germany). Data represent the mean±SEM of n=12 replicate wells per treatment. All groups were compared; different top letters indicate statistically significant differences between the groups (P<0.05). This figure shows results obtained from one of three experiments performed on different cell preparations that yielded comparable results.

erk1/2 are related to tryptase activating via PAR2 and occur prior to the effect of 15d-PGJ2/PPARγ, which then leads to fibroblast proliferation.

When HFFF2 were treated with tryptase plus 10 μM PD98059, a specific inhibitor of MEK known to prevent activation of erk1 and erk2 [31], the stimulatory action of tryptase on the expression of *c-fos*, *c-jun* (Fig. 2C) and COX2 (Fig. 3B) was abolished. Moreover, treatment of HFFF2 with 10 μM PD98059 (as well as 30 μM PD98059; data not shown), blocked tryptase-induced erk1 and erk2 phosphorylation (Fig. 4B) and prevented tryptase-induced cell proliferation (Fig. 3C). Thus, increased erk1 and erk2 phosphorylation is required for

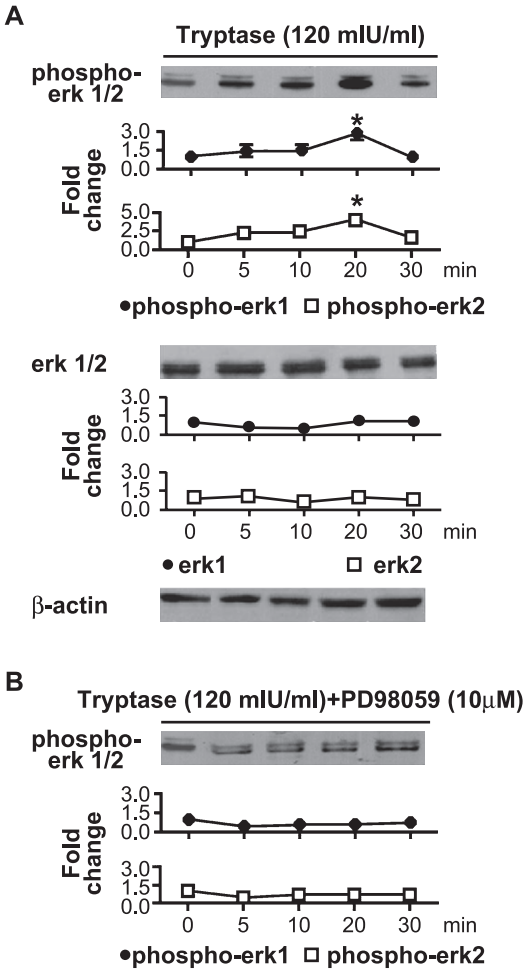

Fig. 4. Tryptase-induced extracellular signal-regulated kinase isoforms 1 and 2 (erk1/2) phosphorylation in human fetal foreskin fibroblasts (HFFF2). (A) HFFF2 were incubated in the absence or presence of 120 mIU/ml tryptase for 5, 10, 20 and 30 min. Expression of erk 1/2 protein as well as phosphorylation of erk 1/2 was monitored by Western blot using specific anti-phospho erk 1/2 and anti-erk 1/2 antibodies as described under "Experimental procedures". Equal amounts of proteins were loaded on all lanes as confirmed by β-actin blotting. Representative immunoblots show results obtained from one of three experiments that yielded comparable results. In all experiments, Western blot bands were densitometrically evaluated. Results are expressed as "fold change" relative to the control (0 min), which was assigned a value of 1. Data shown were normalized to β-actin. In line plot graphics, the mean ± SEM from three independent experiments (four replicates per experiment) performed on different cell preparations is shown. *P<0.05 vs. the corresponding control group (0 min). (B) HFFF2 were incubated in the absence or presence of 120 mIU/ml tryptase plus 10 μM PD98059 for 5, 10, 20 and 30 min, and phosphorylation of erk 1/2 was studied by Western blot. Representative immunoblots show results obtained from one of three experiments that yielded comparable results. Western blot bands were densitometrically evaluated. Results are expressed as "fold change" relative to the control (0 min), which was assigned a value of 1. Data shown were normalized to β-actin. In line plot graphics, the mean ± SEM from three independent experiments (four replicates per experiment) performed on different cell preparations is shown.

induction of *c-fos*, *c-jun* and COX2 expression, and subsequent fibroblast proliferation.

3.2. Tryptase does not induce changes of intracellular Ca^{2+} and cAMP levels in human fibroblasts (HFFF2)

Treatment of HFFF2 with 1 nM thrombin (a PAR1, PAR3 and PAR4 agonist) and 22 μM trypsin (a PAR1, PAR2, PAR3 and PAR4 agonist) rapidly increased intracellular Ca^{2+} levels (Fig. 5). Nevertheless, 120 mIU/ml tryptase and 10 μM PAR2 activating peptide (SLIGKV), concentrations effectively inducing COX2 and cell proliferation, failed to induce changes of intracellular Ca^{2+} levels in all experiments performed (Fig. 5). Moreover, combination of tryptase with 10 μM heparin, which is known to be essential for maintaining tryptase integrity and activity [32,33], had no effect on intracellular Ca^{2+} concentration (Fig. 5).

Furthermore, we observed that after 1 h of treatment of HFFF2 with tryptase (120 mIU/ml) intracellular cAMP levels as well as cAMP released into the incubation media were not altered (Fig. 6). Additional experiments performed with HFFF2 incubated for 20 min or 3 h in the absence or presence of 120 mIU/ml tryptase yielded comparable results (data not shown). Thus, the tryptase activation of PAR2 in

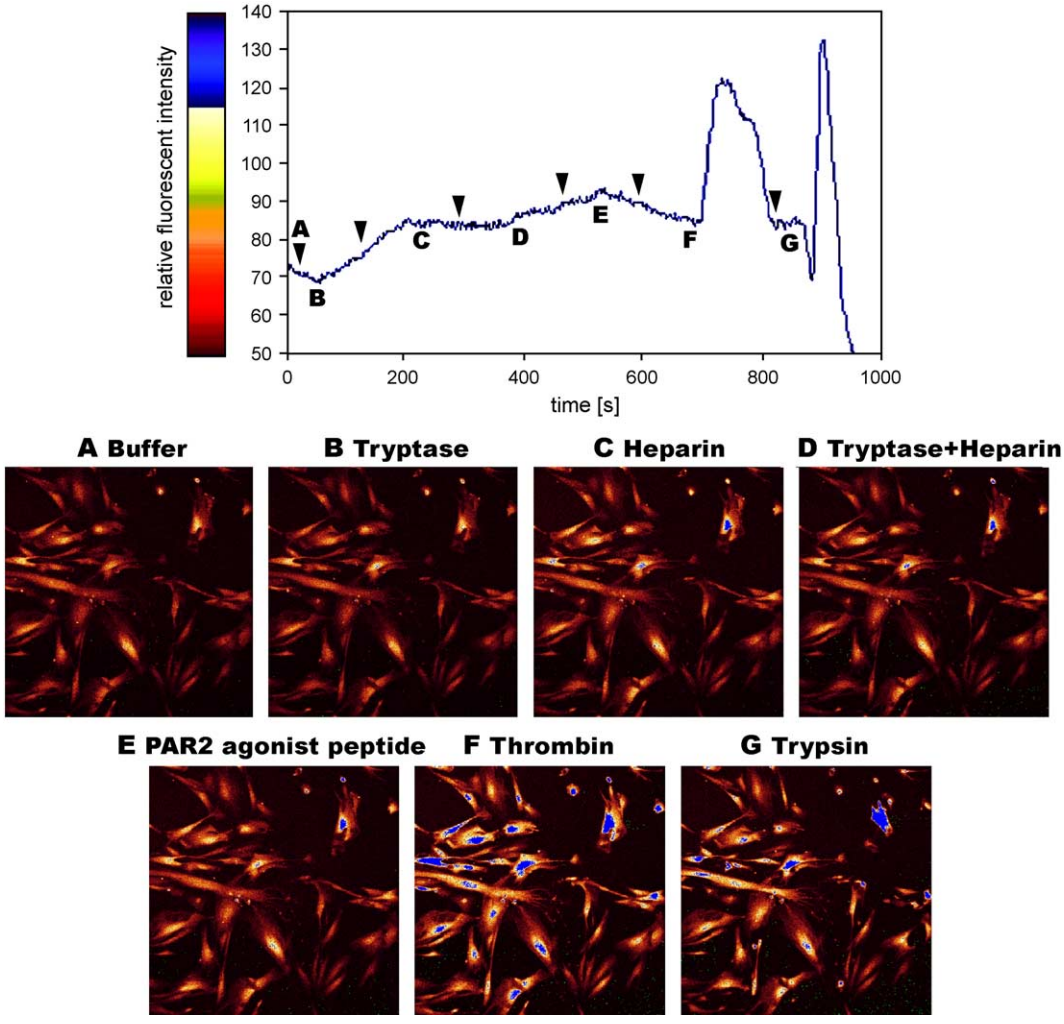

Fig. 5. Tryptase does not increase intracellular Ca^{2+} levels in human fetal foreskin fibroblasts (HFFF2). HFFF2 were loaded with the Ca^{2+} sensitive dye fluo 4-AM and fluorescent intensity was measured every 2 s for a total of 1000 s. Note that in the top part of the figure the detailed changes in Ca^{2+} levels in one single, representative cell are depicted, while the bottom part of the figure gives the corresponding pseudo-colored viewing area of the experiment. Experiments were repeated five times with a total of 50 cells examined in detail. Top panel: Line plot graphic showing the time-course of intracellular Ca^{2+} changes for one representative cell sequentially treated with buffer (A, arrowheads), 120 mIU/ml tryptase (B), 10 μM heparin (C), 120 mIU/ml tryptase plus 10 μM heparin (D), 10 μM PAR2 agonist peptide SLIGKV (E), 1 nM thrombin (F) and 22 μM trypsin (G). Data are expressed as relative fluorescent intensities corresponding to a pseudo color scale from red/yellow to yellow/blue. Modifications in the sequence of drugs addition were employed in several independent experiments. Bottom panel: color images of fluo 4-AM loaded HFFF2 cells. Color changes from red/yellow to yellow/blue represent increased intracellular Ca^{2+} levels. Note that the color images A–G in the bottom panel correspond to the capital letters A–G in the line plot graphic of the top panel indicating the time points at which the different drugs (A, buffer; B, 120 mIU/ml tryptase; C, 10 μM heparin; D, 120 mIU/ml tryptase plus 10 μM heparin; E, 10 μM PAR2 agonist peptide SLIGKV; F, 1 nM thrombin; G, 22 μM trypsin) were applied and the color image pictures were taken.

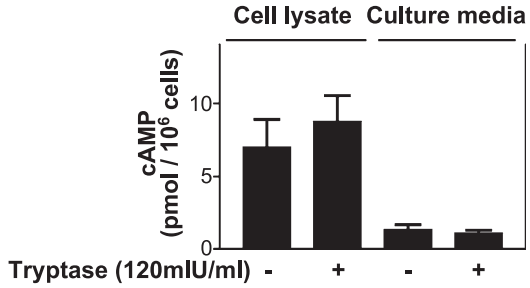

Fig. 6. Tryptase does not induce changes of cAMP levels in human fetal foreskin fibroblasts (HFFF2). HFFF2 were incubated for 1 h in the absence or presence of 120 mIU/ml tryptase. Intracellular cAMP concentrations and cAMP secreted into the incubation media, were determined by a commercially available kit (Cayman Chemical). Data represent mean ±SEM of n=12 replicate wells per treatment. This figure shows results obtained from one of two to three experiments performed on different cell preparations that yielded comparable results. Significant differences between control and tryptase-treated groups were not detected.

HFFF2 is not linked to intracellular Ca^{2+} signaling or cAMP production.

4. Discussion

We recently found that tryptase via activation of PAR2 induces expression of COX2 and subsequently stimulates PG synthesis in HFFF2. The stimulatory action of tryptase on proliferation was shown to be a two-step process and 15d-PGJ2, the natural ligand for PPARγ, is responsible for the tryptase-induced fibroblast proliferation [26]. In the present study, we focused on the characterization of the signaling transduction pathway leading to COX2 induction and subsequent cell proliferation in human fibroblasts.

To this end, and to be able to clearly distinguish between tryptase- and 15d-PGJ2-initiated signaling events, we analyzed gene expression profiles in HFFF2 treated with tryptase and 15d-PGJ2 by employing a cDNA array approach. Results revealed that both tryptase and 15d-PGJ2 regulate genes that directly or indirectly play roles in the enhancement of cell proliferation, mitosis, control of cell cycle, suppression of apoptosis and generation of the extracellular matrix and cell adhesion (results not shown). We found, however, a set of genes that were differentially expressed. Combined cDNA array studies and RT-PCR experiments indicated that only tryptase induces the expression of the transcription factors *c-fos* and *c-jun* in HFFF2, whereas their expression levels remain unchanged in the presence of 15d-PGJ2. It has been extensively demonstrated that activation of COX2 gene expression involves the activator protein-1 (AP-1) family members, *fos* and *jun,* [34–36] supporting a role of these transcription factors in the regulation of COX2. In addition, other mechanisms controlling the expression of proteins associated with rapid responses to proliferation have recently been described. In this context, p38 activation and lack of phosphatidyl-inositol 3-kinase (PI3K) activity were linked to COX2 mRNA stability [37–39].

Previous reports implied PKs as integral parts of the signaling pathways of several members of the PAR family. For example, PAR2- and PAR4-mediated action in HMC-1 involves erk1/2 [40]. Whereas PAR2 stimulates erk and p38 MAPK in astrocytoma cells without any detectable activation of SAPK/JNK [41], in human peripheral blood eosinophils expressing PAR2, erk1 and erk2, p38 MAPK and SAPK/JNK are activated [42]. Furthermore, tryptase-induced mitogenesis in airway smooth-muscle cells requires activation of erk1 and erk2 [43]. We therefore analyzed whether tryptase activates different PKs in HFFF2. We found that tryptase increased phosphorylation of erk1 and erk2, but it did not induce phosphorylation of SAPK/JNK, erk5 or p38. In contrast, 15d-PGJ2 did not regulate any of the PKs studied, suggesting that enhanced erk1 and erk2 phosphorylation in HFFF2 is linked to tryptase/PAR2 and occurs prior to the induction of COX2 and subsequent production/action of 15d-PGJ2. That erk1/2 phosphorylation and activation are also crucial for fibroblast proliferation was confirmed in our study by treatment of HFFF2 with PD98059, which is known to prevent phosphorylation of erk1 and erk2 [34]. We found that PD98059 blocked phosphorylation of erk1 and erk2, expression of *c-fos*, *c-jun* and COX2 and importantly, subsequent fibroblast proliferation. Our results are in line with studies demonstrating that the erk pathway is essential for cellular proliferation of human gingival fibroblasts [44] and WI-38 fetal human fibroblasts [45]. Furthermore, previous reports show that MAPKs play a key regulatory role in the regulation of COX2 expression in a macrophage-cell line [46] and in mammary epithelial cells transformed with a transmembrane receptor with tyrosine kinase activity [32]. Tryptase action in human peripheral blood eosinophils expressing PAR2, involves erk1/2 and the AP-1 family members *fos* and *jun*, but in contrast to our results also SAPK/JNK and p38 MAPK [42].

Several pieces of evidences indicate that activation of PAR1 [47,48], PAR2 [49,50] and PAR4 [47,48] mobilizes intracellular Ca^{2+} concentration. While the PAR2 agonist tryptase stimulates Ca^{2+} mobilization in human bronchial smooth muscle [49], keratinocytes [51], myenteric neurons [52], myocytes [53] and endothelial cells [51], tryptase activation of PAR2 is not related to changes of cytosolic Ca^{2+} in Chinese hamster lung fibroblasts [18] and human dermal fibroblasts [54]. We likewise observed that tryptase and PAR2 activating peptide did not affect intracellular Ca^{2+} in HFFF2 cells. As heparin is essential for maintenance of tryptase tetrameric structure and therefore its activity, and heparin antagonists are potent inhibitors of mast cell tryptase [35,36,55], we also treated HFFF2 with tryptase in combination with heparin, but intracellular Ca^{2+} concentration remained unaffected. Methodological problems can be ruled out, since HFFF2 responded to thrombin (a PAR1, PAR3 and PAR4 agonist) and trypsin (a PAR1, PAR2, PAR3

and PAR4 agonist), which all increased intracellular Ca^{2+} levels. Taken together, these results indicate that, in HFFF2, tryptase activation of PAR2 does not involve changes in cytosolic Ca^{2+} levels. Consequently, the effects of thrombin and trypsin on intracellular Ca^{2+} concentration are likely due to their action on other members of the PAR family, namely PAR1, PAR3 and/or PAR4.

In thoracic aorta [56] and keratinocytes [57] activation of PAR2 involves changes in cAMP levels. In our studies with HFFF2 cells, we did not observe changes in the intracellular or in the extracellular cAMP levels after treatment of HFFF2 with tryptase. Thus, tryptase activation of PAR2 in HFFF2 is not linked to induction of cAMP production.

In summary, our work reveals an as yet unknown intracellular signaling pathway initiated by tryptase in human fibroblasts. This pathway leading to induction of *c-fos*, *c-jun* and COX2, and subsequent to cell proliferation, does not involve Ca^{2+} and cAMP, but crucially depends on phosphorylation of erk1 and erk2. Several evidences support a link among early response genes (*c-jun*, *c-fos*) and fibrosis in lung [2–4], kidney [10], liver [6,8] and heart [12]. Moreover, induction of erk pathway has been related to pulmonar [3,5], renal [9,11], hepatic [7] and cardiac fibrosis [13]. Thus, our work by elucidating details of how tryptase, the major mast cell product, can stimulate COX2 expression and subsequent fibroblast proliferation may bear clinical relevance for prevention or treatment of fibroproliferative diseases.

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