

Differential Up-Regulation of Cytosolic and Membrane-Bound Heat Shock Protein 70 in Tumor Cells by Anti-Inflammatory Drugs

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ABSTRACT

Purpose: Modulation of the heat shock protein (HSP) response affects sensitivity to therapeutic agents in cancer. Here, drugs with anti-inflammatory potential (cyclooxygenase 1/2 inhibitors) and peroxidase proliferator-activated receptor- γ agonists were analyzed for their capacity to affect Hsp70 expression in human cancer cells with a divergent Hsp70 membrane expression pattern.

Experimental Design: In dose kinetics, the nonlethal concentration of acetyl-salicylic acid, celecoxib, rofecoxib, and the insulin-sensitizer pioglitazone was identified for the human adenocarcinoma cell line CX-. With the exception of CLX, which was diluted in DMSO, all reagents were dissolved in water. After treatment with the different compounds at nontoxic concentrations for 6 h, followed by a 1-h recovery period, the cytosolic Hsp70 levels were measured in CX-2 and CX- tumor cells by Western blot analysis. Fold increase was calculated in relation to the housekeeping protein tubulin. Membrane-bound Hsp70 was analyzed by flow cytometry using a FITC-labeled Hsp70-specific monoclonal antibody. Untreated cells and cells incubated with equivalent amounts of the diluting agents served as controls. The immunological function was tested in granzyme B apoptosis assays, standard ⁵¹Cr release assays, and antibody blocking studies.

Results: Compared with aqua dest, the cytoplasmic amount of Hsp70 was equally enhanced in CX-2 and CX- cells by all compounds. An increase in membrane-bound Hsp70, detected selectively in CX- cells, corresponded to an enhanced sensitivity to granzyme B- and natural killer cell-mediated kill that was blockable by using a Hsp70-specific antibody.

Conclusions: Although increase in cytosolic Hsp70 levels conferred resistance to further stress, membrane-bound Hsp70 rendered tumor cells more sensitive to the immunological attack mediated by granzyme B and natural killer cells. Our data provide a biological rationale for combining anti-inflammatory drugs with immunotherapy in cancer therapy.

INTRODUCTION

Highly conserved heat shock proteins (HSPs) inhabit nearly all subcellular compartments including the cytoplasm, mitochondria, and nucleus. Our group detected cell surface localization of Hsp70, the major stress-inducible member of the Hsp70 group, on tumor cells, but not on normal cell types (1). In the cytoplasm, Hsp70 supports *de novo* protein synthesis and chaperone transport processes across membranes during cellular development and differentiation. After environmental stress, synthesis of this molecular chaperone is strongly induced. Elevated cytoplasmic Hsp70 levels prevent cells from lethal damage induced by unfolded, aggregated, and denatured proteins (2, 3). In contrast, membrane-bound Hsp70 provides a target structure for the cytolytic attack mediated by natural killer (NK) cells (4).

By a fluorescence-activated, negative cell sorting procedure, using a Hsp70-specific antibody, a tumor subline (CX-) with low Hsp70 membrane expression (about 20%) was derived from the human adenocarcinoma cell line CX-2 that showed positive Hsp70 staining on about 45% of the cells (4). With respect to the cytoplasmic Hsp70 levels, the original cell line CX-2 and its subline, CX-, were identical. After mild heat stress at 41.8°C, both cell lines reacted equally with an induction of the Hsp70 synthesis (4). Interestingly, the divergent Hsp70 membrane phenotype remained stable at physiological and elevated temperatures. Additional studies of our group revealed that apart from heat, the membrane-interactive alkyl-lysophospholipid derivative ET-18-OCH₃ (5) and tubulin-interacting cytostatic drugs (6) were able to affect cytoplasmic and membrane-bound Hsp70 expression. Members of the propionic acid family of nonsteroidal anti-inflammatory drugs have been shown to induce heat shock factor 1 DNA binding, hyperphosphorylation of heat shock factor 1, and Hsp70 expression on the mRNA and protein level in a similar manner as heat shock.⁴ These data indicate that modulations in cytosolic and membrane-bound Hsp70 levels provide a sensitive marker for a variety of exogenous stress stimuli. In the present study, the

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⁴ S. Westerheide, L. Tai, S. Schwabenbauer, G. Matsumoto, S. Kim, R. Silverman, and R. Morimoto. Small molecule modulators of the heat shock response [abstract]. Presented at the First International Congress on Stress Responses in Biology and Medicine, Quebec, Canada, September 2003.

effects of several nonsteroidal anti-inflammatory drugs and pioglitazone (PIO), an insulin sensitizer with anti-inflammatory capacity, were studied in CX-2 and CX- tumor cells with an initially different Hsp70 membrane phenotype. The key enzymes in the conversion of arachidonic acid to prostanoids are cyclooxygenases (COXs). Presently, two isoforms, the constitutive form (COX-1) and the inducible form (COX-2), have been identified, both of which catalyze the initial step of prostaglandin synthesis. In response to inflammatory stimuli predominantly controlled by macrophages, including interleukin-1, tumor necrosis factor α , and lipopolysaccharides, the synthesis of COX-2 was significantly up-regulated. The constitutive form COX-1 was found to interfere with housekeeping functions in the gastrointestinal tract. This property may provide an explanation for the negative side effects of COX-1 inhibitors on cells of the gastrointestinal tract. Overexpression of COX-2 has been reported for a variety of human malignancies including pancreatitis (7), pancreatic carcinoma (8), colorectal cancer (9, 10), and esophageal squamous cell carcinomas (11). COX inhibitors have been found to have beneficial effects when administered at the peak of the inflammatory response (12). Peroxidase proliferator-activated receptor (PPAR)- γ ligands inhibit dendritic cell maturation, and promote apoptosis of dendritic cells, thus representing a potential pathway for the down-regulation of immune responses initiated by inflammatory signals (13, 14). Furthermore, the synthesis of stress proteins was determined to play an important role in the resolution of acute phase reactions. Due to the presence of reactive oxygen species heme oxygenase 1 (also termed Hsp32), the inducible enzyme in the heme catabolism and members of the Hsp70 family were found to be strongly up-regulated. Depending on the subcellular localization, Hsp70 mediates different immunological effects. On one hand, high cytoplasmic Hsp70 levels have been reported to contribute to antiapoptotic mechanisms (15–17); on the other hand, membrane-bound Hsp70 has been found to stimulate the cytolytic activity mediated by NK cells (1, 18) by forcing them to secrete high amounts of the proapoptotic enzyme granzyme B (19). Because of this dual activity, it was important to study not only the cytosolic Hsp70 levels but also the amount of membrane-bound Hsp70. Evidence has accumulated that nonsteroidal anti-inflammatory drugs exert antineoplastic as well as anti-inflammatory effects. *In vitro* assays showed that acetylsalicylic acid (ASA) inhibits the growth of colon, ovarian carcinomas (20, 21), and leukemic cells (22). However, the mechanisms underlying this phenomenon remained unclear. In the present study, the effects of several anti-inflammatory reagents were tested for their capacity to modulate Hsp70 expression in tumor cells with an initially high and low Hsp70 membrane phenotype. Furthermore, functional consequences were studied with respect to their sensitivity toward granzyme B and NK cells secreting granzyme B.

MATERIALS AND METHODS

Cells and Cell Culture. The human colon carcinoma cell line CX2 [TZB 61005; Tumorbank Deutsches Krebsforschungszentrum, Heidelberg, Germany (4)] and partner cell line CX-, which has low membrane expression of Hsp70, were grown in RPMI 1640 (GibcoBRL, Eggenstein, Germany) supplemented

with 10% heat-inactivated FCS (BioWhittaker, Walkersville, MD), 6 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin; GibcoBRL). To obtain exponential cell growth, cells were trypsinized, counted, and seeded at a cell density of 0.5×10^6 cells/ml regularly, every 3 days. All experiments were performed on day 2 after cell passage.

Viability Assays. Cell viability was evaluated by trypan blue (GibcoBRL) and propidium iodide (Sigma, Munich, Germany) incorporation. Untreated and pretreated cells were incubated for 1 min with trypan blue or propidium iodide and analyzed by either light microscopy or flow cytometry. After incubation with propidium iodide, 5000 cells were analyzed on a FACSCalibur instrument (BD Biosciences, Heidelberg, Germany).

Treatment with COX Inhibitors and Insulin Sensitizer. CX-2 or CX- tumor cells were incubated with different doses of the following compounds: COX-1/COX-2 inhibitor ASA (Aspisol; Bayer Vital, Leverkusen, Germany); COX-2 inhibitor celecoxib [CLX (Celebrex; Pharmacia, Pfizer, Karlsruhe, Germany)]; COX-2 inhibitor rofecoxib (RFX; Vioxx, MSD Sharp & Dohme, Haar, Germany); and insulin sensitizer PIO (Actos, Takeda Pharma, Aachen, Germany), with anti-inflammatory capacity. All agents were freshly prepared as described for medical applications. With the exception of CLX, which was dissolved in DMSO (D-889; Sigma), all other reagents were diluted in aqua dest. Cells were incubated with different concentrations, ranging from 0.5 to 10 μ M for ASA, 100 to 750 μ M for CLX, 1 to 400 μ M for RFX, and 1 to 600 μ M for PIO, for 6 h at 37°C and 5% CO₂. The drug incubation period was chosen to study nonlethal effects. Furthermore, previously published data from our group indicate that in aqueous solution, most clinically applied solutions become inactivated within a few hours (23). After treatment, the cells were washed twice in ice-cold PBS and resuspended in fresh culture medium for a recovery period of 1 h at 37°C.

Preparation of Cell Lysates. After treatment and a recovery period, cytoplasmic fractions were prepared from 5×10^6 cells by incubation of PBS-washed cell pellets in 10 mM Tris-buffered saline (pH 7.5) containing 1% NP40 (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma), as described previously (5).

SDS-PAGE and Immunoblotting. Equal protein amounts (5 μ g) derived from freshly prepared cell lysates were separated electrophoretically on a 10% SDS-PAGE (24). After SDS-PAGE, the proteins were transferred onto nitrocellulose membrane (PALL Corp.) according to a standard protocol of Towbin *et al.* (25). Nonspecific binding to nitrocellulose was blocked with 5% skim milk in PBS with 0.5% Tween at room temperature for 30 min. Membranes were incubated with an anti-Hsp70-specific monoclonal antibody (clone 7F4; 1:1000 dilution) and a secondary antibody (goat antirat IgG peroxidase-conjugated antibody; 1:2000 dilution; Dako, Hamburg, Germany), each for 1 h. Immune complexes were detected using the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Braunschweig, Germany). Protein bands were scanned and quantified using ImageMaster 1D Elite Version 3.00 (Amersham Pharmacia Biotech). To ensure that equal protein amounts have been subjected to the gel and to calculate the relative increase in cytoplasmic Hsp70, the blots

were stained with the anti-tubulin-specific antibody AB-1 (clone DM1A; Oncogene, San Diego, CA).

Flow Cytometry. Immunofluorescence studies were performed using FITC-labeled Hsp70-specific monoclonal antibody (cmHsp70.1; clone C92F3B1; IgG1; multimmune GmbH, Regensburg, Germany), phycoerythrin-labeled MHC class I-specific monoclonal antibody (W6/32; CBL139F; Cymbus, Chandler's Ford, UK), and FITC/phycoerythrin-labeled, isotype-matched control antibodies (IgG1 and IgG2a; Immuntotech, Marseilles, France). Briefly, 0.1×10^6 cells were incubated with the fluorescence-labeled antibodies at 4°C for 30 min. Immediately after staining and two washing steps in ice-cold PBS/2% FCS, quantitative flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). The percentage of positively stained cells was determined as the number of positively stained cells minus the number of cells stained with an isotype-matched negative control antibody. Only viable, propidium iodide-negative cells were gated and analyzed.

Apoptosis Assay. CX- cells were kept untreated, treated with γ -irradiation (1×20 Gy), or treated with ASA (5 μ M) or RFX (100 μ M) at 37°C for 6 h. After washing and a 1-h recovery period, cells were incubated with enzymatically active, freshly prepared granzyme B [10 ng/ml; Hölzel Diagnostica (Cologne, Germany) or kindly provided by Chris Froelich (Northwestern University)] at 37°C for 12 h. As controls, CX- cells were treated with granzyme B (10 ng/ml) alone or with the topoisomerase inhibitor camptothecin, as a positive control for apoptosis, at a final concentration of 4 μ g/ml. Apoptotic cell death was measured by annexin V-FITC staining on a FACSCalibur instrument (BD Biosciences), as described previously (19).

Cytotoxicity Assay. NK cell-mediated cytotoxicity was measured in a 4-h standard ^{51}Cr release assay. NK cells were derived from monocyte-depleted peripheral lymphocytes by CD3 depletion, according to a standard protocol of Miltenyi Biotech (Dreieich, Germany). The purity of NK cells used in the assays was always >90%. After incubation with Hsp70 peptide TKD (2 μ g/ml) plus low-dose interleukin-2 (100 IU/ml) for 4 days, activated NK cells were used as effector cells in the cytotoxicity assay. Untreated CX- colon carcinoma cells or CX- cells pretreated with nonlethal doses of RFX (100 μ M) and PIO (150 μ M) were used as target cells. For blocking studies, the mouse monoclonal antibody cmHsp70.1 (multimmune GmbH) was used at a final concentration of 5 μ g/ml for 1×10^6 cells. After labeling, the target cells were incubated for 20 min with antibody at room temperature. Then the cells were counted, diluted in fresh medium, and used as target cells. The percentage of specific lysis was calculated as follows: [(experimental release - spontaneous release)/(maximal release - spontaneous release)] $\times 100$.

RESULTS

Definition of the Nontoxic Concentration of Different COX Inhibitors in Human Adenocarcinoma Cell Line CX- with Initial Low Hsp70 Membrane Expression. The sensitivity of CX- and CX-2 tumor cells toward exogenous stress factors was tested after γ -irradiation with a single dose of 1×20 Gy, followed by a recovery period of 24 h. Previous kinetic studies revealed that a dose of 1×10 Gy was nonlethal for

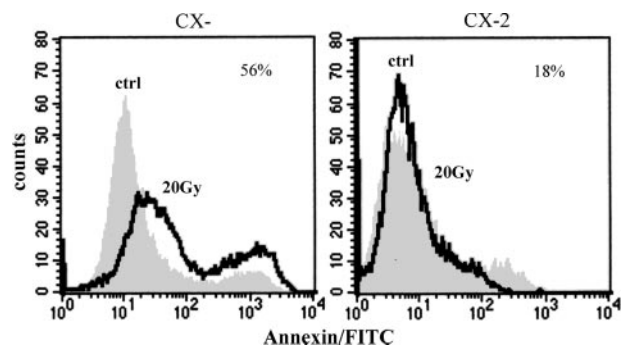


Fig. 1 Apoptotic cell death was measured by annexin V-FITC staining in CX-2 and in CX- tumor cells that were untreated (gray-shaded area) or treated with γ -irradiation (a single dose of 20 Gy) followed by a recovery period of 24 h (solid black line). The percentage apoptotic cells after treatment is indicated in the top right corner of each graph. The data represent one experiment of three identical experiments.

CX- cells.⁵ In Fig. 1, the percentage of annexin V-FITC-positive cells, as a parameter of apoptosis, is shown in the top right corner of each graph. After irradiation (1×20 Gy), 56% of the CX- tumor cells but only 18% of the CX-2 tumor cells underwent apoptotic cell death. These data clearly indicate that CX- tumor cells are more sensitive to irradiation-induced stress as compared with CX-2 tumor cells. Therefore, cell viability after treatment with different COX inhibitors was predominantly tested in CX- tumor cells. The chemical structure of the COX-1/COX-2 inhibitor ASA (180.15 Da) and the COX-2 inhibitors CLX (381.38 Da) and RFX (314.47 Da) is illustrated in Fig. 2, together with that of the insulin sensitizer PIO (392.90 Da). PIO has been shown to exhibit anti-inflammatory capacity *in vitro*. To identify the nontoxic dose of ASA, exponentially growing CX-2 and CX- tumor cells were kept untreated (control) or incubated with 0.5, 1, 5, or 10 μ M ASA for 6 h, followed by a recovery period of 24 h. As illustrated in Fig. 3, A and B, ASA was nonlethal for both CX2 and CX- tumor cells, up to a concentration of 5 μ M. At 10 μ M, cell viability of CX-2 and CX- tumor cells significantly decreased ($P < 0.05$). The effects of the reagents CLX, RFX, and PIO were tested only on CX- tumor cells at the following concentrations: CLX, 100, 200, 500, and 750 μ M; RFX, 1, 100, 200, and 400 μ M; and PIO, 1, 150, 300, and 600 μ M.

After a washing step and a 24-h recovery period in fresh medium, cells were harvested, and the absolute number of viable and dead cells was determined. Under physiological conditions, the doubling time of untreated CX- cells was 24 h. A concentration of 5 μ M ASA (Fig. 3B), 500 μ M CLX (Fig. 3C), 100 μ M RFX (Fig. 3D), and 150 μ M PIO (Fig. 3E) was determined as nonlethal for CX- tumor cells. Concentrations above these levels resulted in a decreased cell viability after a 6-h incubation period. All additional experiments were performed

⁵ M. Gehrmann, J. Marienhagen, H. Eicholtz-Wirth, E. Fritz, J. Ellwart, M. Jäättelä, T. Zilch, G. Multhoff. Dual function of membrane-bound Hsp70 and Bag 4: protection against radiation-induced effects and target structures for NK cells, submitted for publication.

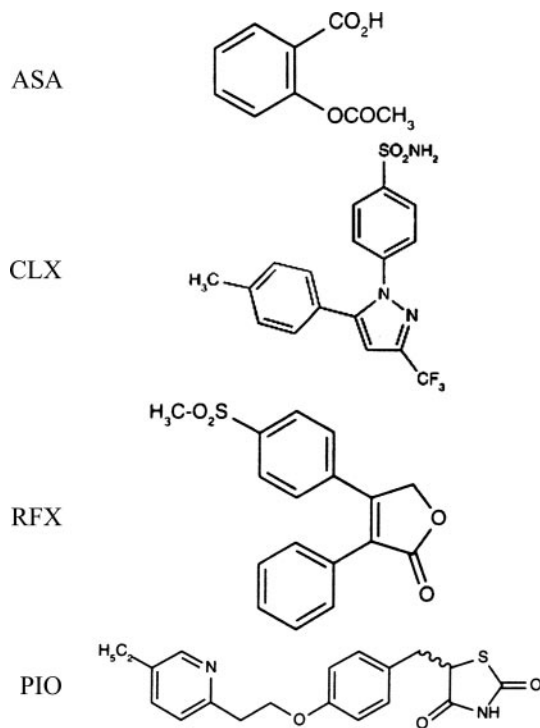


Fig. 2 Chemical structure of the nonsteroidal anti-inflammatory drugs cyclooxygenase (COX)-1/2 inhibitor acetyl-salicylic acid (ASA; 180.15 Da), the COX-2 inhibitors celecoxib (CLX; 381.38 Da) and rofecoxib (RFX; 314.47 Da), and the insulin sensitizer pioglitazone (PIO; 392.90 Da) with anti-inflammatory and COX-2 inhibitory capacity.

with the indicated (see the *underlined* values in Fig. 3, A–E) nontoxic concentrations of each compound.

Effects of Anti-Inflammatory Drugs on Cytoplasmic Hsp70 Levels in CX– and CX-2 Cells. Exponentially growing colon adenocarcinoma CX– and CX-2 cells with initially different Hsp70 membrane expression were incubated with either aqua dest or ASA (5 μ M) diluted in aqua dest for 6 h, followed by a recovery period of 1 h. After Western blot analysis using a Hsp70-specific monoclonal antibody, the cytoplasmic amount of Hsp70 was measured by comparative laser scan densitometry. Staining of the blots with an antibody directed against tubulin revealed that equal protein amounts had been subjected to SDS-PAGE. As shown in Fig. 4A, ASA significantly increased the cytoplasmic Hsp70 levels in CX– and CX-2 cells. One representative Western blot analysis of the Hsp70 and tubulin staining before and after nonlethal ASA treatment is illustrated in the *top panels* of Fig. 4A; the mean values of six independent experiments are shown as *graphs* in the *bottom panel*. This result is in line with previous data of other laboratories showing a synergistic up-regulation of Hsp70 after a combined treatment with heat and ASA in K562 and endothelial cells (26, 27). Furthermore, we examined whether COX-2 inhibitors CLX (Fig. 4B) and RFX (Fig. 4C) exhibit similar effects on cytoplasmic Hsp70 levels. We showed that exposure of CX– and CX-2 tumor cells to nonlethal concentrations of CLX (500 μ M) and RFX (100 μ M) both resulted in an increase in the amount of cytoplasmic Hsp70 comparable with

that shown for ASA. However, it is important to note that ASA and RFX were diluted in aqua dest and did not affect Hsp70 synthesis, whereas CLX was diluted in DMSO. Incubation with the membrane-interactive compound DMSO already increased cytoplasmic Hsp70 levels in both tumor sublines. With respect to these findings, it was impossible to separate the Hsp70-inducing effects of CLX and DMSO.

In addition to CLX and RFX, the insulin-sensitizing drug PIO was tested in a similar manner. After uptake into the cytosol, PIO is known to bind PPAR- γ , a nuclear hormone receptor. In a complex with the retinoid X receptor, DNA binding is enabled. PPAR- γ inhibits the expression of pro-inflammatory cytokines including tumor necrosis factor α , IFN- γ , and interleukin-2 (28). After incubation of CX– and CX-2 tumor cells with nonlethal concentrations of PIO (150 μ M), the increase in cytoplasmic Hsp70 levels (Fig. 4D) was comparable with those shown for ASA, CLX, DMSO, and RFX.

Effects of Anti-Inflammatory Drugs on Membrane-Bound Hsp70 in CX– and CX-2 Cells. Besides its intracellular chaperoning function, membrane-bound Hsp70 is known to stimulate NK cell activity. Cell membrane-bound Hsp70 acts as a tumor-selective target recognition structure (1, 4, 29). To evaluate the immunostimulatory function of ASA, CLX, RFX, and PIO treatment on tumor cells, we investigated membrane expression of Hsp70. We were interested in determining whether enhanced cytoplasmic Hsp70 levels correlate with an increase in the amount of membrane-bound Hsp70. Membrane localization of Hsp70 in untreated or COX inhibitor-treated CX– and CX-2 tumor cells was studied by flow cytometry using a Hsp70-FITC-labeled monoclonal antibody.

Under physiological conditions, 23% of the CX– tumor cells and 45% of the CX-2 tumor cells appeared to be Hsp70 membrane positive (Fig. 5). Incubation with aqua dest at a volume equivalent to that used for the compounds did not affect Hsp70 membrane expression significantly. As shown in Fig. 5A (*top six panels*), preincubation of CX– cells with ASA resulted in a significant increase in the percentage of viable Hsp70-positive cells, whereas the percentage of Hsp70-positive cells in CX-2 tumor cells remained unaffected. By comparison with untreated control cells, the amount of Hsp70-positive cells increased significantly from 23% to 42% in CX– cells. The fold increase derived from independent experiments using CX– cells was 1.7-fold (Fig. 5A, *bottom panels*). No significant increase was observed with CX-2 tumor cells (45% versus 50%). As shown in Fig. 5B, similar results were obtained with DMSO and the COX-2 inhibitor CLX at nonlethal concentrations. DMSO alone increases the amount of Hsp70-positive CX– cells from 19% to 32%. After treatment with CLX, the amount of membrane-bound Hsp70-positive cells showed a 12% increase, to 26% (Fig. 5B, *top panels*). Neither DMSO nor CLX affected Hsp70 membrane expression of CX-2 tumor cells. Similar results could be obtained with RFX (Fig. 5C) and PIO (Fig. 5D). Again, Hsp70 membrane expression increased in CX– cells from 19% to 31% (1.5-fold) for RFX and from 19% to 27% (1.3-fold) for PIO, whereas that of CX-2 cells remained unaltered and high. The MHC class I expression, used as a positive control, remained stable and high (>95%) before and after treatment with any of the tested reagents (data not shown).

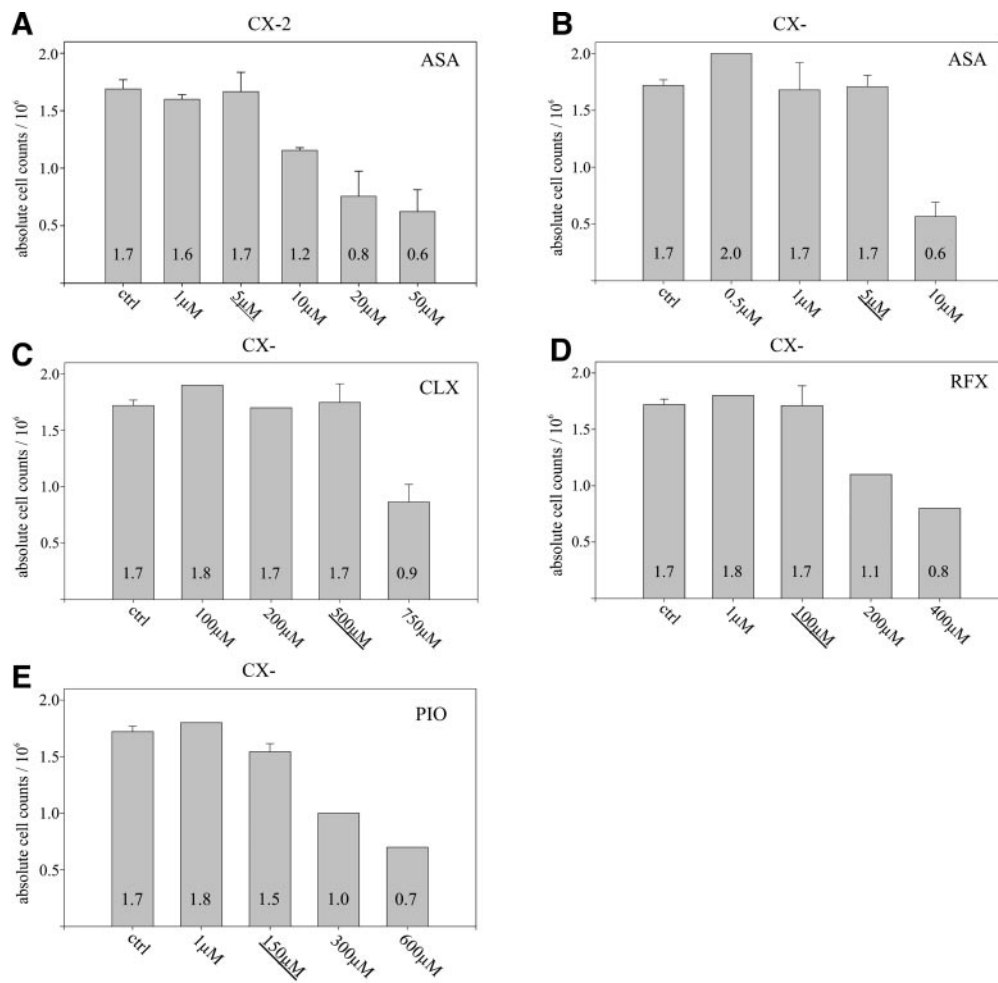


Fig. 3 Influence of different anti-inflammatory drugs on cell viability of human adenocarcinoma cell lines CX-2 and CX- with initially intermediate (45%) and low (20%) heat shock protein 70 membrane expression. The absolute number of viable cells was determined by flow cytometry after propidium iodide staining. **A**, cell death was determined in CX-2 tumor cells after a 6-h incubation period with 0, 1, 5, 10, 20, and 50 μM acetyl-salicylic acid (ASA) and a recovery period of 24 h ($n = 3$). **B**, cell death was determined in CX- tumor cells after a 6-h incubation period with 0, 0.5, 1, 5, and 10 μM acetyl-salicylic acid and a recovery period of 24 h ($n = 4$). **C**, cell death was determined in CX- tumor cells after a 6-h incubation period with 0, 100, 200, 500, and 750 μM celecoxib (CLX) and a recovery period of 24 h ($n = 4$). **D**, cell death was determined in CX- tumor cells after a 6-h incubation period with 0, 1, 100, 200, and 400 μM rofecoxib (RFX) and a recovery period of 24 h ($n = 3$). **E**, cell death was determined in CX- tumor cells after a 6 h incubation period with 0, 1, 150, 300, and 600 μM pioglitazone (PIO) and a recovery period of 24 h ($n = 4$). Bar charts represent the number of PI-negative viable cells \pm SE derived from three to four independent experiments. The nonlethal concentration of each drug is underlined.

The effects of COX inhibitors on cytoplasmic and membrane-bound Hsp70 levels in CX- and CX-2 tumor cells are summarized in Table 1. At nonlethal concentrations, all compounds including DMSO were able to induce the synthesis of Hsp70, regardless of their initial membrane expression pattern. An equivalent volume of aqua dest, the dissolvent of all compounds except CLX, did not affect Hsp70 expression. In contrast, the amount of membrane-bound Hsp70 was only enhanced in tumor cells that initially had low membrane Hsp70 expression.

Immunological Relevance of a Drug-Induced Increase in Hsp70 Membrane Expression. In an effort to determine whether an increase in membrane-bound Hsp70 correlated with an enhanced sensitivity to granzyme B-mediated apoptosis, control cells and ASA-, RFX-, and PIO-pretreated (nonlethal dose

for 6 h) CX- cells were incubated with granzyme B (10 ng/ml) for 12 h. In line with previous results (19), the percentage of apoptotic CX- cells, which initially exhibit low Hsp70 membrane expression (19%), was not elevated after contact with granzyme B (17% *versus* 16%). However, after treatment with a nontoxic dose of ASA (5 μM), Hsp70 membrane expression was up-regulated from 19% to 47%. As shown in Table 2, this increase in the amount of membrane-bound Hsp70 corresponded to a 1.9-fold elevation in annexin V-FITC positivity (17% to 32%), indicating that membrane-bound Hsp70 might facilitate uptake of granzyme B and thus cause apoptotic cell death. Similar results were obtained for RFX- and PIO-treated CX- cells with an increased Hsp70 membrane expression (31% and 27% Hsp70 membrane positive, respectively). The percentage of apoptotic cells increased from 14% to 34% for RFX

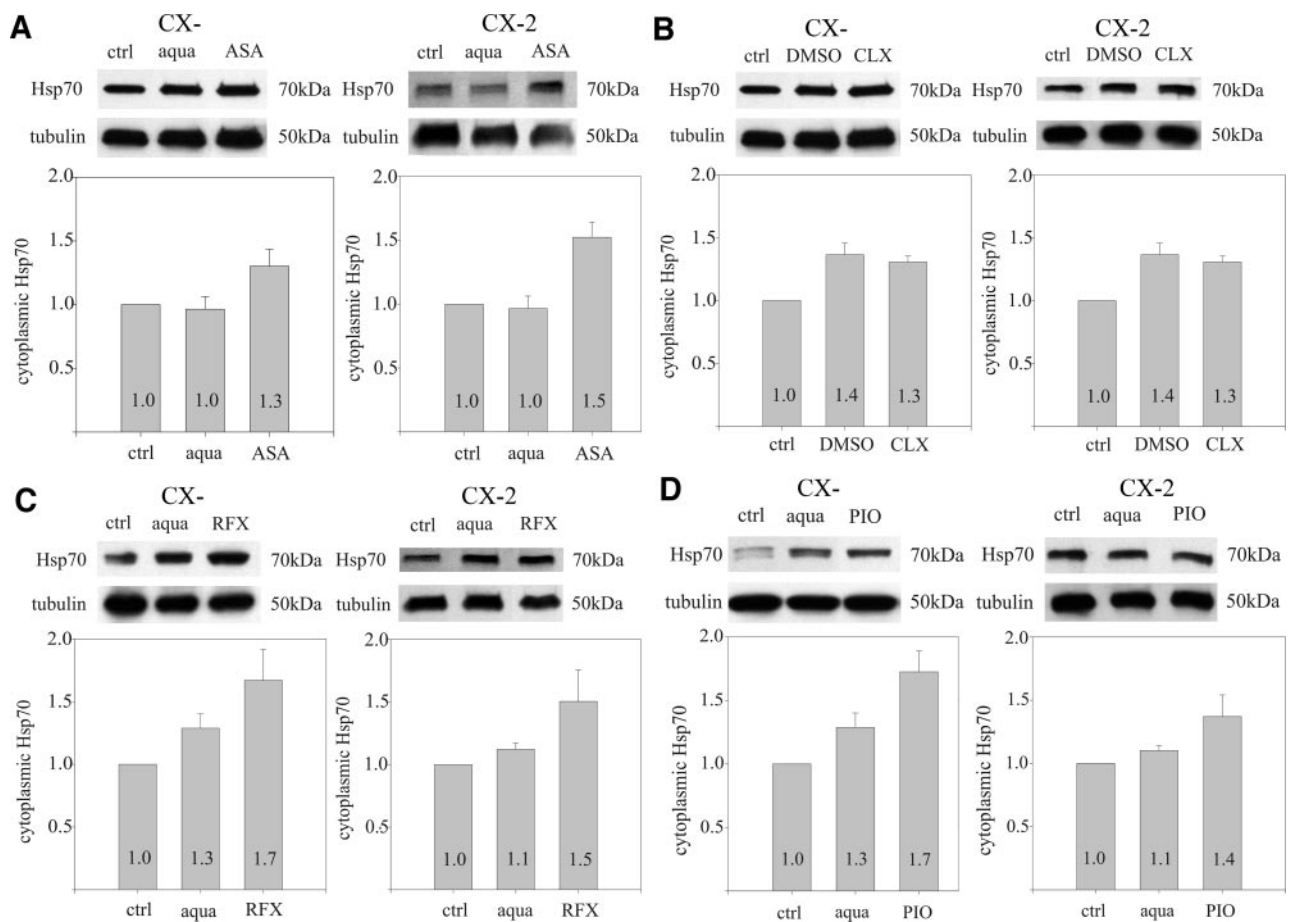


Fig. 4 Influence of nontoxic concentrations of cyclooxygenase inhibitors on the cytoplasmic amount of heat shock protein (HSP) 70 in CX- and CX-2 tumor cells. Equal amounts of protein were run on 10% SDS-PAGE and transferred to nitrocellulose membranes. After Western blotting using a Hsp70 (7F4)- and tubulin (DM1A)-specific monoclonal antibody, the immunoblots were quantified by densitometry. **A**, representative Hsp70 immunoblots of CX- and CX-2 cells that were kept untreated or incubated with aqua dest (45.5 μ l) or 5 μ M acetyl-salicyl acid (ASA; 45.5 μ l). The relative (mean of three to six independent experiments) increase is shown in the *graphs* at the *bottom*. **B**, representative Hsp70 immunoblots of CX- and CX-2 cells that were kept untreated (*ctrl*) or incubated with DMSO (47.5 μ l) or 500 μ M celecoxib (CLX; 47.5 μ l). The relative increase (mean of six independent experiments) is shown in the *graphs* at the *bottom*. **C**, representative Hsp70 immunoblots of CX- and CX-2 cells that were kept untreated or incubated with aqua dest (192.6 μ l) or 100 μ M rofecoxib (RFX; 192.6 μ l). The relative increase (mean of three to five independent experiments) is shown in the *graphs* at the *bottom*. **D**, representative Hsp70 immunoblots of CX- and CX-2 cells that were kept untreated or incubated with aqua dest (192.6 μ l) or 5 μ M pioglitazone (PIO; 192.6 μ l). The relative increase (mean of three to five independent experiments) is shown in the *graphs* at the *bottom*. The relative increase of cytoplasmic Hsp70 related to the housekeeping protein tubulin for each compound and its dissolvent is illustrated graphically as fold increase \pm SE.

(2.4-fold) and from 15% to 28% (1.9-fold) for PIO after a 12-h incubation period with granzyme B. As a control for apoptosis, CX- cells were also incubated with the topoisomerase inhibitor camptothecin. After treatment with camptothecin, the amount of apoptotic CX- cells also was elevated from 21% to 33% (1.6-fold).

To further prove the relevance of the COX inhibitor-induced elevated Hsp70 membrane expression as a target structure for NK cell-mediated cell kill, cytotoxicity assays were performed using Hsp70 peptide-activated NK cells (18) as effector cells and untreated, RFX-treated, and PIO-treated CX- cells as target cells. Concomitant with an increased Hsp70 membrane expression, lysis of CX- tumor cells was up-regulated after treatment with RFX and PIO (Fig. 6, *A* and *B*, *left panels*). Furthermore, the Hsp70-specific antibody (cmHsp70.1)

was able to reduce this elevated lysis of RFX (Fig. 6*A*, *right panel*)- and PIO (Fig. 6*B*, *left panel*)-pretreated CX- cells down to the level of untreated CX- tumor cells. These data indicate that lysis of RFX- and PIO-pretreated CX- cells is mediated through Hsp70.

DISCUSSION

Depending on the subcellular localization, Hsp70 confers different immunological functions. On one hand, elevated cytoplasmic Hsp70 levels are thought to play a role in protection against cell damage induced by exogenous stress stimuli including heat shock, tumor necrosis factor, oxidative stress, cytostatic drugs, and radiation (17, 30–32). On the other hand, plasma membrane-bound Hsp70 has been determined as a tumor-selec-

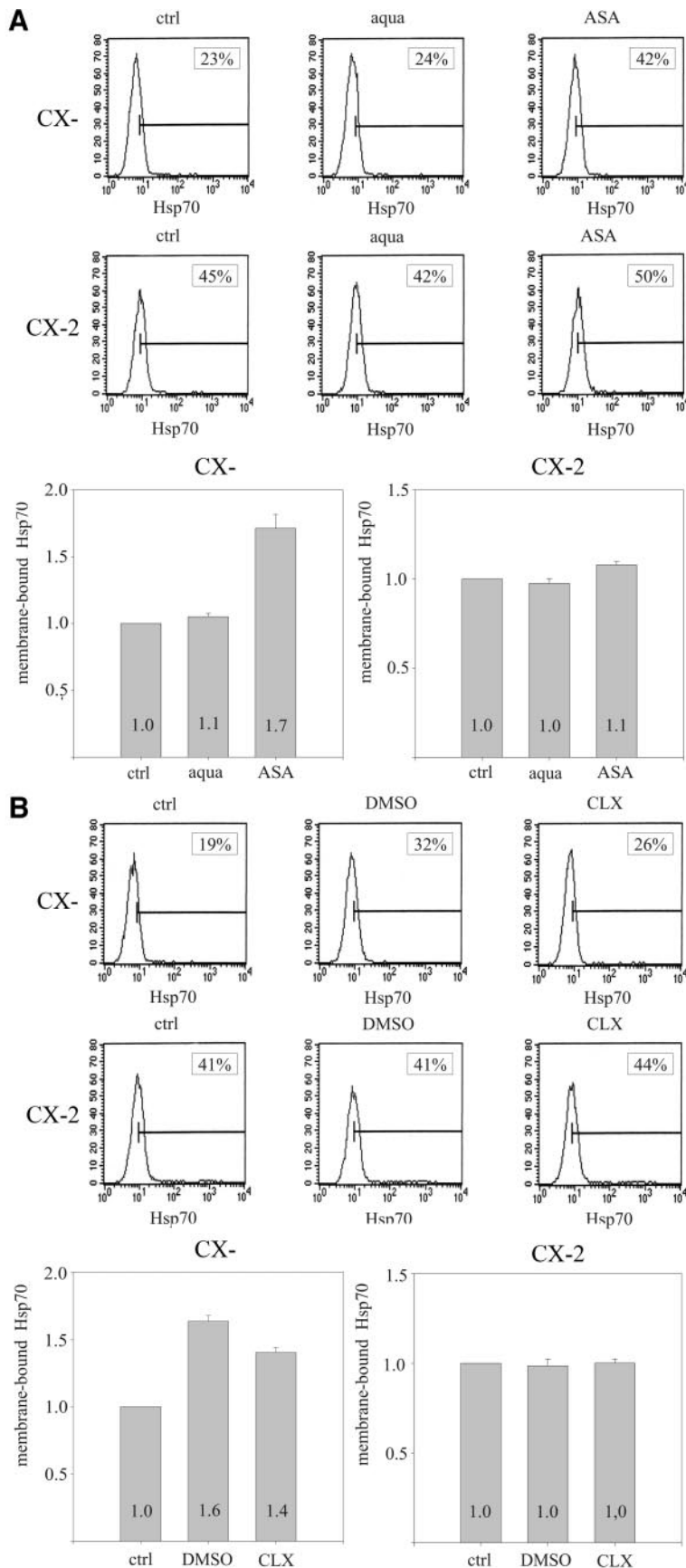


Fig. 5 Flow cytometric analysis of membrane-bound heat shock protein (HSP 70) on CX- and CX-2 tumor cells after treatment with nonlethal concentrations of cyclooxygenase inhibitors. CX- and CX-2 tumor cells were kept untreated (*ctrl*) or pretreated with diluent (*aqua* dest or DMSO) or a nonlethal dose of COX inhibitor. Cells were stained with a FITC-conjugated Hsp70-specific antibody (cmHsp70.1) or an isotype-matched control antibody (*vertical marker*). The percentage of Hsp70-positive cells is shown at the *top right corner*. *A*, representative flow cytometric analysis of CX- and CX-2 cells that were kept untreated or incubated with *aqua* dest (45.5 μ l) or 5 μ M acetylsalicylic acid (ASA; 45.5 μ l). The relative increase in the percentage of Hsp70-positive cells (mean of four to six independent experiments) is shown in the graphs at the *bottom*. *B*, representative flow cytometric analysis of CX- and CX-2 cells that were kept untreated or incubated with DMSO (47.5 μ l) or 500 μ M celecoxib (CLX; 47.5 μ l). The relative increase in the percentage of Hsp70-positive cells (mean of four independent experiments) is shown in the *graphs* at the *bottom*.

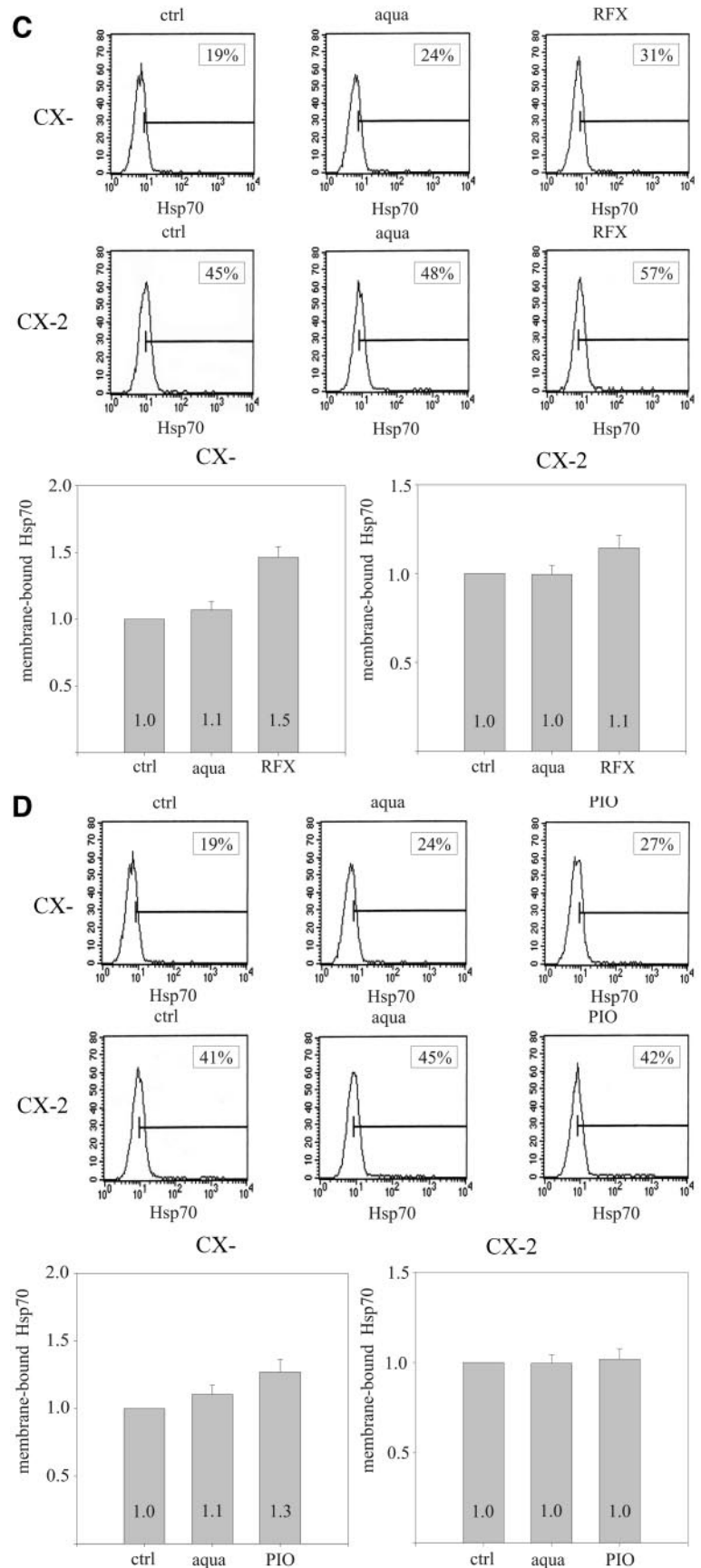


Fig. 5 (Continued) C, representative flow cytometric analysis of CX- and CX-2 cells that were kept untreated or incubated with aqua dest (192.6 μ l) or 100 μ M rofecoxib (RFX; 192.6 μ l). The relative increase in the percentage of Hsp70-positive cells (mean of six independent experiments) is shown in the graphs at the bottom. D, representative flow cytometric analysis of CX- and CX-2 cells that were kept untreated or incubated with aqua dest (192.6 μ l) or 150 μ M pioglitazone (PIO; 192.6 μ l). The relative increase in the percentage of Hsp70-positive cells (mean \pm SE of four to six independent experiments) is shown in the graphs at the bottom.

Table 1 Summary of the Hsp70^a-inducing effects of anti-inflammatory drugs

Cytoplasmic and membrane-bound Hsp70 were measured in CX- and CX-2 cells after treatment with diluents (aqua dest and DMSO), COX inhibitors (ASA, CLX, and RFX), and insulin sensitizer (PIO).

Treatment	CX-		CX-2	
	Cytoplasm	Membrane	Cytoplasm	Membrane
Aqua dest	+ ^b	+	+	+
ASA	++	+++	+++	+
DMSO	++	+++	++	+
CLX	++	++	++	+
Aqua dest	+	+	+	+
RFX	++	+++	+++	+
Aqua dest	+	+	+	+
PIO	++	++	++	+

^a HSP, heat shock protein; COX, cyclooxygenase; ASA, acetyl-salicylic acid; CLX, celecoxib; RFX, rofecoxib; PIO, pioglitazone.

^b +, 1.0–1.1-fold increase; ++, 1.2–1.4-fold increase; +++, 1.5–1.7-fold increase.

Table 2 Measurement of Hsp70^a membrane expression concomitant with apoptosis in CX- tumor cells

CX- cells were untreated (control) or pretreated with granzyme B alone (10 ng/ml, 12 h), a nontoxic dose of ASA (5 μM, 6 h), ASA + granzyme B (10 ng/ml, 12 h), a nontoxic dose of RFX (100 μM, 6 h), RFX + granzyme B, a nontoxic dose of PIO (150 μM, 6 h), PIO + granzyme B, or camptothecin (4 μg/ml, 12 h). After treatment, Hsp70 membrane expression was determined by flow cytometry using the Hsp70-FITC-specific monoclonal antibody cmHsp70.1. Apoptosis was determined by measuring the percentage of annexin V-FITC-positive cells on a FACSCalibur instrument.

Treatment	CX-	
	Hsp70+ (%)	Apoptotic cells (%)
Control	19	17
Granzyme B	18	16
ASA	47	20
ASA + granzyme B	46	32
RFX	nt	14
RFX + granzyme B	31	34
PIO	nt	15
PIO + granzyme B	27	28
Camptothecin	21	33

^a HSP, heat shock protein; ASA, acetyl-salicylic acid; RFX, rofecoxib; PIO, pioglitazone.

^b nt, not tested.

tive target structure for the cytolytic attack mediated by NK cells (4). Interaction of NK cells with Hsp70 presented on the cell surface of tumor cells causes production and secretion of high amounts of the proapoptotic enzyme granzyme B (19). Furthermore, the amount of membrane-bound Hsp70 correlated with sensitivity to lysis mediated by NK cells (4, 5).

In the present study, we analyzed the effects of anti-inflammatory drugs on their capacity to modulate Hsp70 synthesis in the cytosol and on the plasma membrane. There is evidence that nonsteroidal anti-inflammatory drugs exert antineoplastic effects through induction of apoptosis and inhibition of proliferation induced by up-regulated PPAR-γ expression (33). In addition to classical COX-1/COX-2 inhibitors (ASA, CLX, and RFX), the insulin sensitizer PIO was also tested (34–38).

Recently, it has been shown that PIO, a natural ligand for PPAR-γ, exhibits its anti-inflammatory effects by suppression of interleukin-1β, tumor necrosis factor α, COX-2, and inducible nitric-oxide synthase and by up-regulation of Hsp70 in a gastric ulcer rat model (39). In line with these data, Ethridge *et al.* demonstrated an inverse correlation of COX-2 overexpression and Hsp70 induction (40).

Our data clearly show a significant up-regulation of cytoplasmic Hsp70 in tumor sublines CX-2 and CX- by any of the tested reagents at nonlethal concentrations. However, in contrast to the compounds ASA, RFX and PIO, CLX was dissolved in DMSO. Because this membrane-interactive compound stimulated Hsp70 synthesis by itself, the effects induced by CLX and DMSO could not be separated. In another set of experiments, we addressed the question of whether enhanced cytoplasmic Hsp70 levels correlated with an increased amount of Hsp70 on the

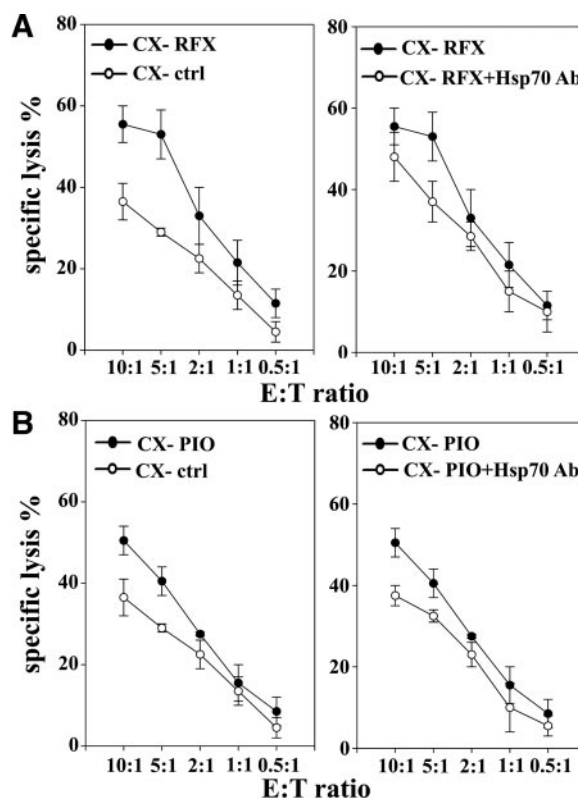


Fig. 6 Cytotoxicity assays (4 h) using heat shock protein (HSP) 70 peptide-activated natural killer (NK) cells as effector cells and untreated, rofecoxib (RFX)-treated, and pioglitazone (PIO)-treated CX- cells as target cells. The percentage of Hsp70-positive cells before and after treatment using RFX and PIO is shown in Table 2. A, CX- cells were kept untreated (CX- ctrl) or pretreated for 6 h with a nonlethal dose of RFX (CX- RFX; 100 μM). NK cells were used at E:T ratios ranging from 10:1 to 0.5:1. Specific lysis was blocked by preincubation of the RFX-treated target cells with Hsp70 antibody (CX- RFX+Hsp70 Ab). B, CX- tumor cells were kept untreated (CX- ctrl) or pretreated for 6 h with a nonlethal dose of PIO (CX- PIO; 150 μM). NK cells were used at E:T ratios ranging from 10:1 to 0.5:1. Specific lysis was blocked by preincubation of the PIO-treated target cells with Hsp70 antibody (CX- PIO+Hsp70 Ab). The experiments illustrate the mean of two independent experiments; the experiments were repeated four times with identical results.

plasma membrane. Previously, we have shown that a nontoxic concentration of the alkyl-lysophospholipid derivative ET-18-OCH₃ resulted in an increase in the amount of plasma membrane-bound Hsp70 in tumor cells, but not in normal cells (5). As mentioned previously, the quantity of membrane-bound Hsp70 was associated with an enhanced NK cell-mediated killing activity (1, 4) and thus might have further immunological relevance. Tumor cells that differed in initial Hsp70 membrane expression were incubated with nonlethal doses of COX-1/COX-2 inhibitors and PIO. An up-regulated Hsp70 membrane expression was detected selectively in CX- tumor cells; membrane expression in CX-2 tumor cells remained unaltered. These data led us to speculate about conversion of a Hsp70-negative phenotype into a Hsp70 membrane-positive phenotype in cancer cells by anti-inflammatory drugs. Transport of Hsp70 to the cell surface appeared to be independent of the cytoplasmic amount of newly synthesized Hsp70. It is worth mentioning that Hsp70 membrane localization in general was restricted to tumor cells; nonmalignant cells did not exhibit any cell surface localization under physiological conditions or after stress (1, 4).

The immunological consequences of an increased Hsp70 membrane expression were determined by using granzyme B as a selective inducer for apoptotic cell death in Hsp70 membrane-positive tumors. In accord with previous results with stressed tumor cells (5), apoptosis was higher in CX- cells that had been pretreated with ASA, RFX, and PIO and thus exhibited elevated Hsp70 membrane expression. In line with these results, we also showed an increased cytotoxic response of Hsp70 peptide-activated NK cells against RFX- and PIO-pretreated CX- cells with an enhanced Hsp70 membrane expression. Interestingly, this increased lysability could be blocked by a Hsp70-specific antibody, indicating that Hsp70 is the relevant target structure for the cytolytic attack mediated by NK cells. Taken together, our findings might have future clinical implications in the case of a combined therapy consisting of anti-inflammatory drugs, *e.g.*, selective COX-2 inhibitors or glitazones, and NK cell-mediated immunotherapy.

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