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Celecoxib transiently inhibits cellular protein synthesis

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ABSTRACT

To uncover the full spectrum of its pharmacological activities, the selective COX-2 inhibitor celecoxib is routinely being used at concentrations of up to 100 μM in cell culture. At these elevated concentrations, several COX-2-independent effects were identified, although many details of these events have remained unclear. Here, we report a COX-2-independent effect of celecoxib that might have profound consequences for the interpretation of previous results obtained at elevated concentrations of this drug in vitro. We found that celecoxib rapidly inhibits general protein translation at concentrations as low as 30 μM. This appears to be a consequence of endoplasmic reticulum (ER) stress and entails the phosphorylation and inactivation of eukaryotic translation initiation factor 2 alpha (eIF2α). These effects were not achieved by other coxibs (rofecoxib, valdecoxib) or traditional NSAIDs (indomethacin, flurbiprofen), but were mimicked by the COX-2-inactive celecoxib analog, 2,5-dimethyl-celecoxib (DMC), indicating COX-2 independence. Considering the obvious impact of blocked translation on cellular function, we provide evidence that this severe inhibition of protein synthesis might suffice to explain some of the previously reported COX-2-independent effects of celecoxib, such as the down-regulation of the essential cell cycle regulatory protein cyclin D, which is a short-lived protein that rapidly disappears in response to the inhibition of protein synthesis. Taken together, our findings establish ER stress-induced inhibition of general translation as a critical outcome of celecoxib treatment in vitro, and suggest that this effect needs to be considered when interpreting observations from the use of this drug in cell culture.

1. Introduction

Celecoxib is widely prescribed under the trade name Celebrex® for relief of symptoms of osteoarthritis and rheumatoid arthritis and was also approved as an adjunct to standard care for patients with familial adenomatous polyposis (FAP). It is suspected that this drug might be useful for the prevention and treatment of colorectal and possibly other types of cancer, and several clinical trials are ongoing to confirm this expectation. In addition, celecoxib has demonstrated potent anti-cancer activity in various animal tumor models in the laboratory [1]. Despite these promising results, however, the underlying molecular mechanisms by which celecoxib exerts its anti-tumor potential are not completely understood. Particularly intriguing are a number of reports describing potent anti-proliferative and pro-apoptotic effects of this drug

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Abbreviations: CHOP, CCAAT/enhancer binding protein homologous transcription factor; eIF2α, eukaryotic translation initiation factor 2 alpha; ER, endoplasmic reticulum; ESR, ER stress response; GRP78, glucose-regulated protein 78.

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in the absence of any apparent involvement of COX-2 (see Ref. [2], for a review).

When the antitumor effects of celecoxib are studied in cell culture in vitro, concentrations in the range of 30–100 μM are generally required in order to achieve substantial growth inhibition or induction of apoptosis in tumor cells. On occasion, lower concentrations of the drug might be effective as well, although in those cases reduced serum concentrations in the cell culture growth medium or longer incubation times seem to be required [3–6]. It has been well established that celecoxib and other coxibs are able to inhibit their main target, COX-2, at sub-micromolar concentrations in cell culture [7–9]. Remarkably, however, despite efficient inhibition of COX-2, cell proliferation and survival is not affected at these low concentrations. Quite in contrast, much higher concentrations are required to achieve antitumor effects in vitro. This discrepancy suggests that it is very unlikely that those effects of celecoxib that are observed only in the 30–100 μM range are related to the inhibition of COX-2. And indeed, when proper controls were being included in high-dosage in vitro experiments with celecoxib, it generally turned out that the respective effects did not involve the inhibition of COX-2 [2,10,11].

Those in vitro effects of celecoxib that are only observed in the 30–100 μM range are generally discarded as artifacts of the cell culture setting and not considered relevant for the in vivo setting, because such elevated drug concentrations cannot be achieved in the serum of patients or animals [12]. However, there are several recently published examples that clearly demonstrate that specific drug-induced processes, which only take place at 30–100 μM in vitro, can also be detected in tumor tissues from celecoxib-treated experimental animals [13–15]. For example, a minimum of 40–60 μM of celecoxib is required to inhibit the expression of the anti-apoptotic protein survivin and noticeably stimulate apoptosis in glioblastoma cell lines in culture. Nonetheless, and even though drug concentrations below 40 μM are ineffective in vitro, down-regulation of survivin expression and increased apoptosis can also be detected in xenograft glioblastoma tissue from celecoxib-treated animals, clearly indicating that the in vitro and in vivo processes are congruent in this case [15]. Although there is as yet no explanation as to this conundrum between effective in vitro and in vivo concentrations, such results caution against the prevalent tendency of minimizing those drug outcomes that were obtained at elevated celecoxib concentrations in vitro.

Several recent reports have indicated that treatment of cells with celecoxib leads to the activation of the endoplasmic reticulum (ER) stress response (ESR) [16–23]. One of the features of ESR is a transient inhibition of overall cellular protein synthesis, which is achieved through the inactivation of eukaryotic translation initiation factor 2 alpha (eIF2α) [24,25]. The intensity of ESR-induced translational attenuation can be relatively weak or very strong, depending on the ESR-inducing insult. Naturally, any pharmacologic intervention that interferes substantially with overall protein synthesis may have profound consequences for other processes that are affected by fluctuations in this basic cellular function. Therefore, we have investigated general protein translation in response to treatment of cultured cells with celecoxib. We found that commonly used concentrations of this drug severely (>90%) impaired cellular translation, and this took place similarly in cells expressing or not expressing COX-2 protein. Inhibition of translation involved phosphorylation (i.e., inactivation) of eIF2α, revealing a prominent role of ESR in generating this outcome. We investigated cyclin D protein as an example of a previously reported down-regulated target of celecoxib and found that mere inhibition of protein translation could account for the rapid down-regulation of this crucial cell cycle-regulator. Thus, our results reveal the inactivation of eIF2α as a critical mechanism mediating celecoxib's inhibitory effect on the cellular protein synthesis machinery, and indicate that inhibition of translation should be considered as a potentially significant factor during the interpretation of results obtained from the use of this drug in cell culture.

2. Materials and methods

2.1. Materials

All coxibs and NSAIDs were obtained and used as described previously [15]. The synthesis of 2,5-dimethyl-celecoxib (DMC) was described in Ref. [26]. ^{35}S-methionine (10 μCi/μl; 540 Ci/mmol) was purchased from MP Biomedicals, LLC (Solon, OH).

2.2. Cell lines and culture conditions

HeLa cervix carcinoma and PC-3 pancreatic carcinoma cell lines were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). The glioblastoma cell line U251 was provided by Frank B. Furnari (Ludwig Institute of Cancer Research, La Jolla, CA). MIA-PaCa-2 pancreatic carcinoma cells were obtained from Guido Eibl (University of California, Los Angeles, CA). Mouse embryo fibroblasts (MEFs) harboring a homozygous mutation in the eIF2α gene (resulting in an exchange of serine for alanine at position 51 of the protein [27]) were established in the laboratory of Randal J. Kaufman (University of Michigan, Ann Arbor, MI) and were immortalized with SV40 large T antigen in the laboratory of David Ron (New York University, New York, NY). Wild type MEFs immortalized in the same manner were used as controls. All culture conditions were as described previously [15], except that MEFs received medium supplemented with non-essential amino acids. Unless otherwise stated, the cell culture medium was supplemented with 10% fetal bovine serum (FBS).

2.3. Western blot analysis

Western blot analysis was performed as described [15]. Antibodies against eIF2α and phosphorylated eIF2α (phospho-serine 51) were purchased from Cell Signaling Technologies (Beverly, MA); GRP78, CHOP, and actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); cyclin D antibodies were a kind gift from Bangyan Stiles (University of Southern California, Los Angeles, CA). All immunoblots were repeated at least once to confirm the results.

2.4. Radiolabeling of proteins in cell culture

Cells were grown in 6-cm dishes in the presence or absence of the respective drug. Thirty minutes before cell harvest, the

monolayer was rinsed twice with phosphate buffered saline (PBS), and the growth medium was replaced with methionine-free medium supplemented with ^{35}S-labeled L-methionine to a final activity of 10 μCi/mL of culture medium. The concentration of dialyzed fetal bovine serum and the respective drug treatment was maintained during this labeling period. The cells were cultured in the presence of ^{35}S-methionine for 30 min. Thereafter, the radioactive growth medium was

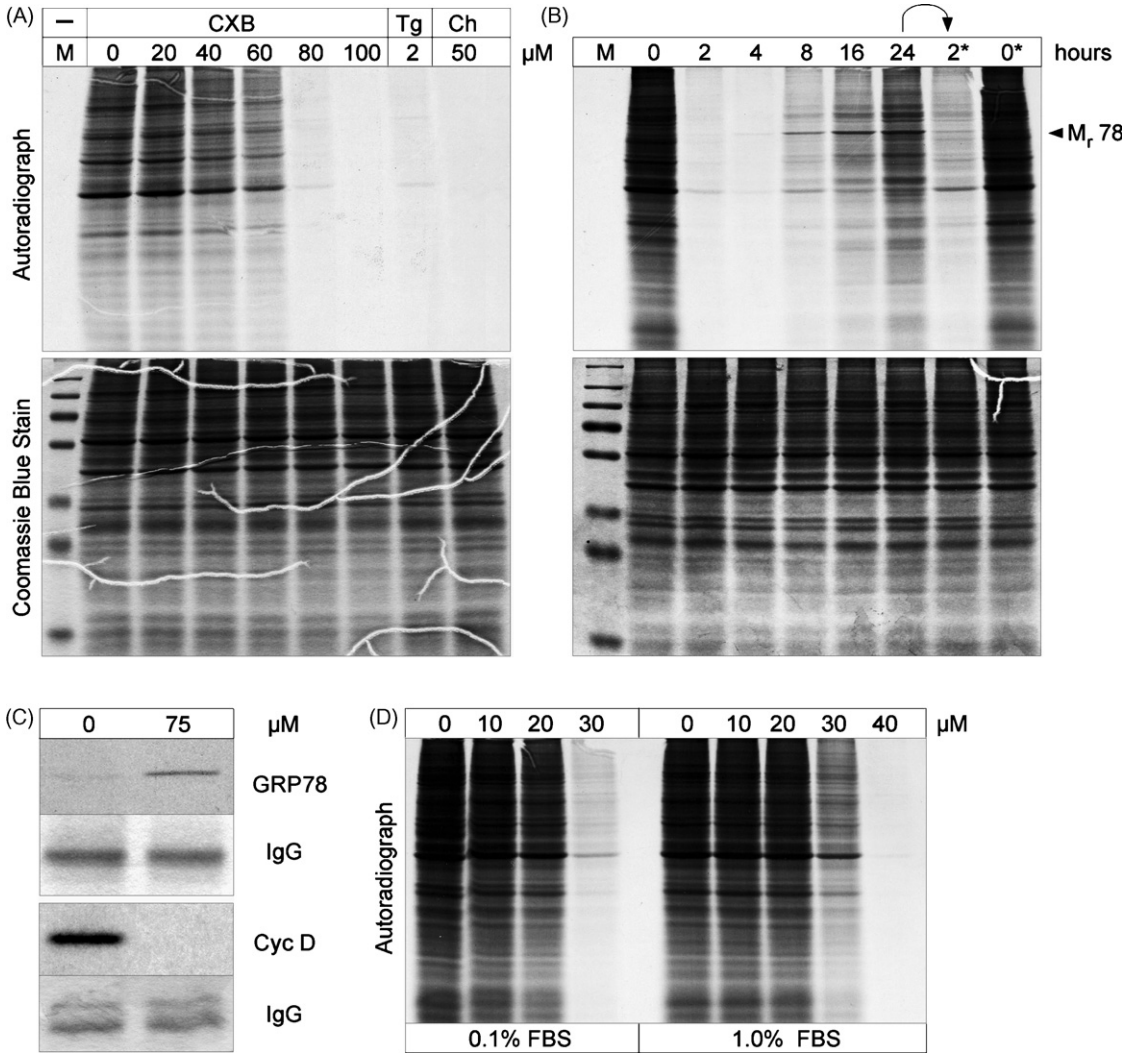

Fig. 1 – Inhibition of general protein synthesis by celecoxib. U251 glioblastoma cells were cultured in the presence of celecoxib (CXB), and overall cellular protein synthesis was determined via the incorporation of ^{35}S-methionine into newly synthesized proteins. (A) Cells were treated with increasing concentrations of celecoxib for 1.5 h. Paralllel cultures received thapsigargin (Tg) and cycloheximide (Ch). Top: autoradiograph of gel with lysates from the different treatment conditions, which is indicative of newly synthesized proteins. Bottom: Coomassie blue staining of the same gel, confirming that the same amounts of total cell lysates were loaded in each lane. (B) Cells were treated with 80 μM celecoxib for the time points indicated. To determine whether the drug maintained its potency during the 24-h incubation period, the cell culture medium (including celecoxib) from treated cells was recovered after 24 h and transferred to fresh, not previously drug treated cells for 2 h (2*; curved arrow); note that celecoxib was still able to potently suppress protein synthesis under these conditions, as compared to a non-drug-treated culture (0*). The arrowhead to the right points to a prominent band of 78 kDa that appears at 8, 16, and 24 h of drug treatment. It should be noted that during the recovery of protein translation, there was no substantial cell death (yet), i.e., increasing rates of translation between 8 and 24 h were not due to the survival and selective accumulation of drug-resistant cells. (C) GRP78 and cyclin D proteins were immunoprecipitated (IP) from cells treated with 0 or 75 μM celecoxib for 12 h and labeled with ^{35}S-methionine during the final 30 min. The top panels are autoradiographs showing the amount of radioactive (i.e., newly synthesized) GRP78 and cyclin D (cyc D), respectively, wherease the bottom panels show Coomassie blue staining of the antibodies (IgG) in the same gel, verifying equal amounts of antibodies used in each IP. Note that GRP78 synthesis is stimulated by drug treatment, whereas cyclin D synthesis is completely blocked. (D) Cells cultured in the presence of 0.1 or 1.0% fetal bovine serum (FBS) were incubated with increasing concentrations of celecoxib, labeled with ^{35}S-methionine, and analyzed by PAGE. Shown is the autoradiograph only; the gel was also stained with Coomassie blue to confirm equal loading in each lane (not shown). M: molecular weight marker.

removed and discarded, the monolayer rinsed twice with ice-cold PBS, and the cells scraped in 1 mL of PBS and transferred to a microcentrifuge tube. After a 30-s spin at 10,000 rpm, the buffer was aspirated and discarded, and the cell pellet frozen at −80 °C until further use.

2.5. PAGE of radiolabeled proteins

Freshly harvested or frozen cell pellets were lysed in 70–100 μL RIPA buffer and analyzed by polyacrylamide gel electrophoresis (PAGE). In experiments where all points had been harvested at the same time, 30% of total cell lysate was used for PAGE. When cells had been harvested at different time points, the protein concentration of each sample was determined first, and 40 μg of total cell lysate was used for PAGE. All samples were separated on a 10% acrylamide gel. After the run, the gel was stained with Coomassie blue, dried, and exposed to CL-XPosure X-ray film (Pierce) for 3–10 days. The amount of ^{35}S-methionine incorporation in the various lysates was quantitatively determined via trichloroacetic acid (TCA) precipitation and Cherenkov counting of aliquots from total cell lysates. All ^{35}S-methionine incorporation experiments were repeated at least once.

3. Results

3.1. Celecoxib transiently inhibits general translation

When studied in cell culture, celecoxib is most commonly used at concentrations ranging from 10 to 100 μM. In the vast majority of reported cases, the observed drug effects become most apparent towards the upper limit of this concentration range. To determine whether such concentrations might impinge on general protein synthesis, we treated the human glioblastoma cell line U251 with increasing concentrations of celecoxib and determined the ongoing rate of translation via the incorporation of ^{35}S-methionine. As shown in Fig. 1A, 80 μM celecoxib reduced overall translation by >90%, and 100 μM of the drug blocked translation as effectively as the potent protein synthesis inhibitor cycloheximide. For comparison purposes, cells were also treated with thapsigargin (Tg), the model inducer of calcium-triggered ER stress response, and this drug as well caused rapid and potent inhibition of translation in these cells. Moreover, the translation-inhibitory effects of celecoxib were transient. As shown in Fig. 1B, maximum inhibition became apparent at 4 h of drug treatment, but thereafter protein translation slowly recovered and normalized after 24 h. This recovery was not due to loss of drug activity, as drug-supplemented medium incubated with cells for 24 h was still able to strongly reduce translation when transferred to fresh cells (Fig. 1B).

3.2. Celecoxib induces characteristics of ER stress

Interestingly, a prominent band of apparent 78 kDa molecular weight appeared under conditions of greatly reduced protein translation (Fig. 1B), and this band was identified as glucose regulated protein 78 (GRP78; Fig. 1C), a well-established indicator of the ER stress response (ESR). The transient nature of protein synthesis inhibition, combined with elevated levels of GRP78, are typical features of the ER stress response (ESR) [24,25,28], and are consistent with other previously reported effects of celecoxib on this cellular response system [16–23]. Parallel to the increased translation of GRP78, synthesis of cyclin D protein, a cell cycle regulator and known target of celecoxib, was blocked by celecoxib (Fig. 1C), in keeping with the drug's general attenuation of translation.

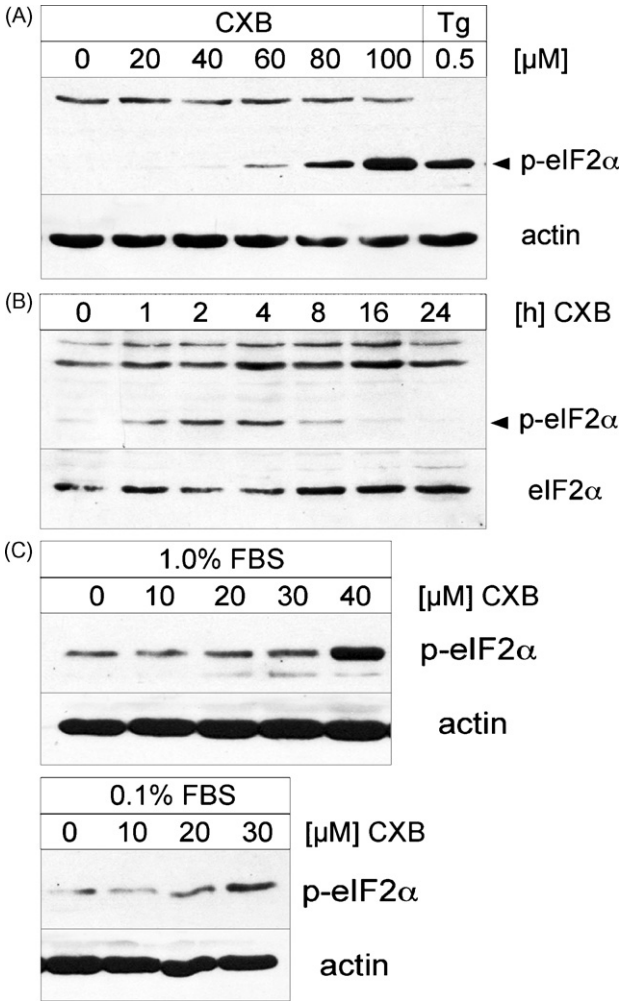

Fig. 2 – Celecoxib treatment causes phosphorylation of eIF2α. U251 cells were cultured in the presence of celecoxib (CXB), and the phosphorylation status of eIF2α protein was determined by Western blot analysis of total cell lysates. (A) Cells were treated with increasing concentrations of celecoxib for 1.5 h. As a positive control, cells were also treated with thapsigargin (Tg), a model inducer of the ER stress response that is known to potently induce the phosphorylation of eIF2α. (B) Cells were treated with 75 μM celecoxib for the time points indicated. (C) Cells were treated with increasing concentrations of celecoxib in medium containing 1.0 or 0.1% fetal bovine serum (FBS). In all blots, phosphorylated eIF2α protein is indicated by p-eIF2α. As a loading control, actin or the total amount of eIF2α protein (eIF2α) was determined either in parallel blots or on stripped blots.

3.3. *Inhibition of translation is exacerbated at low serum concentrations*

Because of earlier findings that reduced serum concentrations in the cell growth medium substantially enhance the cytotoxic effects of celecoxib [29], we investigated protein synthesis under lower serum concentrations. We found that celecoxib as low as 30–40 μM potently inhibited cellular translation in medium with 0.1 or 1.0% FBS (Fig. 1D). Different batches of serum resulted in slightly varied drug potencies (not shown), but overall the IC50 for inhibition of general translation by celecoxib was in the range of 50–65 μM at 10% FBS, approximately 30 μM at 1.0% FBS, and <30 μM at 0.1% FBS.

3.4. *Inhibition of translation involves phosphorylation of eIF2α*

It is known that severe ER stress transiently inhibits translation via the phosphorylation (and resulting inactivation) of translation factor eIF2α at position serine-51 [27]. We therefore investigated this component of the ESR via Western blot analysis with phospho-serine-51-specific antibodies. As shown in Fig. 2, treatment of cells with celecoxib caused prominent phosphorylation of this translation factor. In all instances, the kinetics of eIF2α phosphorylation mirrored the inhibition of protein synthesis under the different conditions shown in Fig. 1.

To further evaluate the contribution of eIF2α phosphorylation to translational blockage induced by celecoxib, we used mouse embryonic fibroblast (MEF) cells derived from animals with homozygous mutation at the eIF2α phosphorylation site (S51A: exchange of serine for alanine) [27]. As shown in Fig. 3, celecoxib was unable to stimulate phosphorylation of eIF2α in these cells. In parallel, drug-induced attenuation of protein synthesis was less severe, although still noticeable. In wild type cells, 80 and 100 μM celecoxib reduced protein synthesis by 76 and 89%, respectively, whereas in eIF2α-mutant cells this reduction was only 38 and 52%. Thus, these results point to a major role of eIF2α in translational inhibition by celecoxib, but also suggest that other factors might contribute as well.

3.5. *Inhibition of translation by celecoxib is COX-2 independent*

We next investigated whether the above described effects of celecoxib required inhibition of its best-known target, the COX-2 enzyme. First, we determined protein synthesis in three widely used tumor cell lines, namely HeLa cervix carcinoma, PC-3 prostate carcinoma, and MIA-PaCa-2 pancreatic carcinoma cells; the latter are known to be COX-2 negative [30]. In all three cell lines, celecoxib potently blocked cellular translation (Fig. 4A) and caused increased phosphorylation of serine-51 of eIF2α (Fig. 4B). Evidently, the absence of COX-2 expression in MIA-PaCA-2 cells had no influence on this outcome, i.e., phosphorylation of eIF2α and inhibition of translation by celecoxib took place in these cells in the same way as compared to the other cells that were COX-2 positive. Thus, these results indicate that these drug effects do not require the presence of this enzyme.

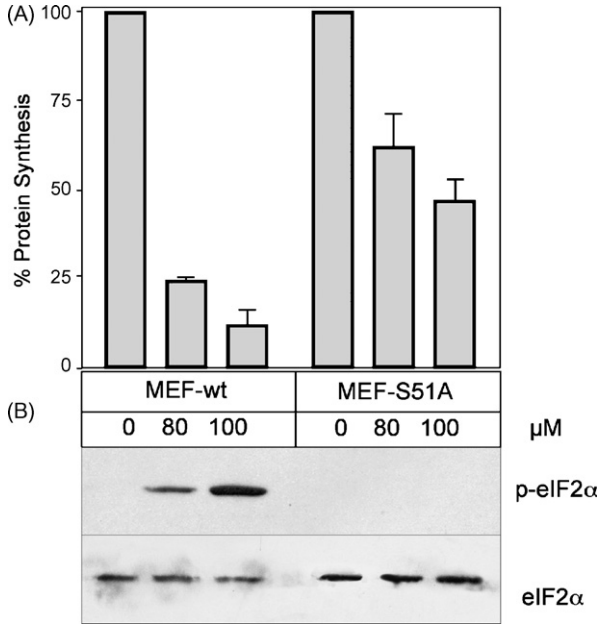

Fig. 3 – Protein synthesis and phosphorylation of eIF2α in eIF2αmutant cells. Mouse embryo fibroblasts (MEFs) with mutated eIF2α (S51A), or the respective wild type MEFs in comparison, were treated with 80 and 100 μM of celecoxib for 2 h. (A) The rate of general translation was analyzed by ^{35}S-methionine labeling. Shown is the amount of incorporation of radioactivity during drug treatment (average of three measurements, ±S.D.). (B) The level of eIF2α phosphorylation (p-eIF2α) was determined by Western blot.

To further exclude a role for COX-2, we included additional drugs in our analysis. We used rofecoxib and valdecoxib (selective inhibitors of COX-2), indomethacin and flurbiprofen (non-selective dual COX-1/COX-2 inhibitors), and 2,5-dimethyl-celecoxib (DMC; a COX-2-inactive analog of celecoxib [31]). As shown in Fig. 5, only celecoxib and DMC, but none of the other drugs, caused phosphorylation of eIF2α and potent inhibition of translation at 100 μM or less. Furthermore, only celecoxib and DMC caused substantial inhibition of cell growth and survival at these concentrations (Fig. 5B). Thus, the drugs' effect on protein translation correlated closely with their impact on overall cell growth and survival, but did not at all correlate with their COX-2-inhibitory potential.

3.6. *Celecoxib down-regulates cyclin D synthesis, but increases expression of ER stress proteins*

Can diminished translation in itself serve as an explanation for some of the other previously reported effects of celecoxib? As an example, we selected cyclin D, a critical cell cycle-regulatory protein that is known to be down-regulated by celecoxib. Cells were treated with celecoxib or with the protein synthesis inhibitor cycloheximide for up to 24 h, and cyclin D protein levels were analyzed. As shown in Fig. 6, both drugs caused rapid loss of cyclin D protein. In the case of celecoxib, this effect was transient, and cyclin D protein reappeared after

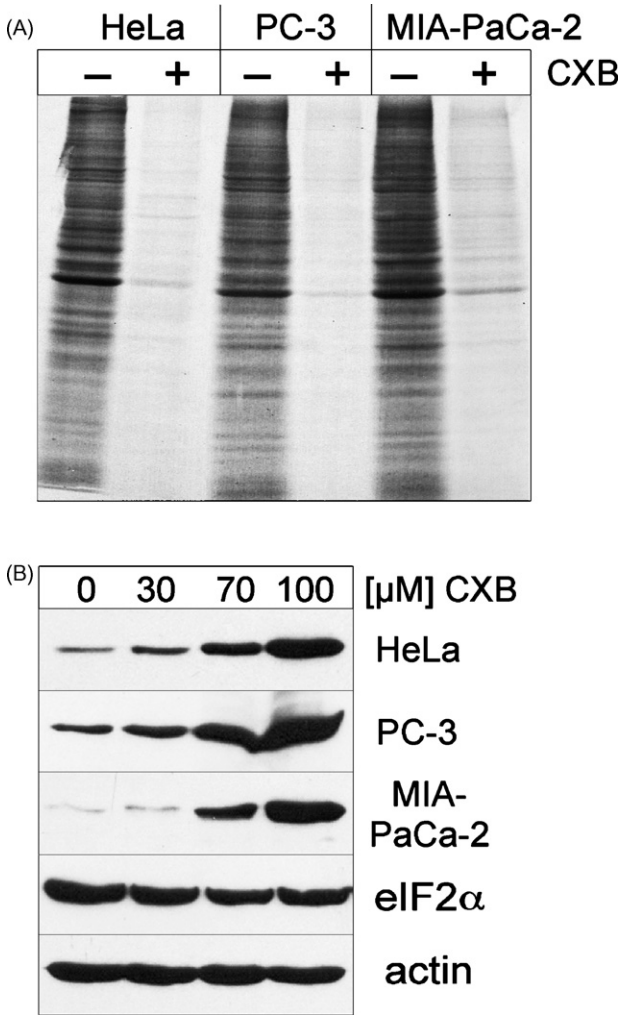

Fig. 4 – Protein synthesis and phosphorylation of eIF2α in various tumor cell lines. Three different types of established human tumor cell lines were treated with celecoxib for 1 h. (A) Cells were treated with 80 μM of drug, and the rate of ongoing protein synthesis was determined by ^{35}S-methionine labeling as outlined in the legend to Fig. 1. Shown is an autoradiograph of the gel. To ensure equal loading, the gel was stained with Coomassie blue (not shown). (B) Cells were treated with increasing concentrations of drug, and the phosphorylation status of eIF2α was determined by Western blot analysis of total cell lysates. The top three panels show the amount of phosphorylated eIF2α protein in the various tumor cells as indicated. Each blot was also subjected to hybridization with antibodies recognizing actin or the total amount of eIF2α as a loading control (only one example each is shown in the bottom two panels).

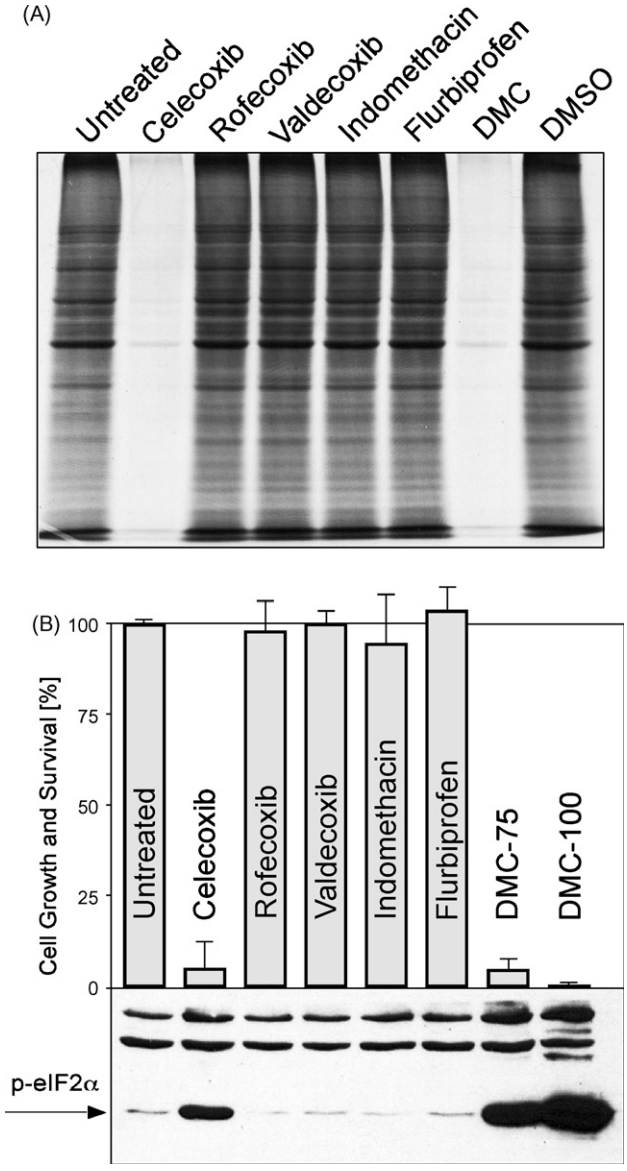

Fig. 5 – Protein synthesis and phosphorylation of eIF2α in the presence of NSAIDs. U251 cells were treated with 100 μM of various coxibs and traditional NSAIDs as indicated for 2 h. In parallel, cells were also treated with vehicle alone (DMSO) or with 75 μM DMC (a non-coxib analog of celecoxib). (A) Autoradiograph of drug-treated cells, indicating the rate of ongoing protein synthesis via the incorporation of ^{35}S-methionine. The gel was stained with Coomassie blue to confirm equal loading (not shown). (B) Western blot analysis of phosphorylated eIF2α (p-eIF2α) (2 h treatment) in combination with MTT cell growth and survival assay (48 h treatment). DMC was used at two different concentrations, 75 and 100 μM, which are indicated as DMC-75 and DMC-100.

16–24 h, coinciding with the recovery of cells from translational inhibition (compare to Fig. 1C). Thus, the kinetics of cyclin D inhibition by celecoxib closely correlated with overall translational activity, and general inhibition of protein synthesis with cycloheximide mimicked the loss of cyclin D.

The difference in the underlying mechanisms that generate the translational block in the case of celecoxib versus cycloheximide is indicated in Fig. 6 as well. In the case of cycloheximide, phosphorylation (i.e., inhibition) of eIF2α did not take place during the first 4 h of drug treatment, and therefore is unlikely to be involved in the early down-regulation of cyclin D by this particular drug. On the other

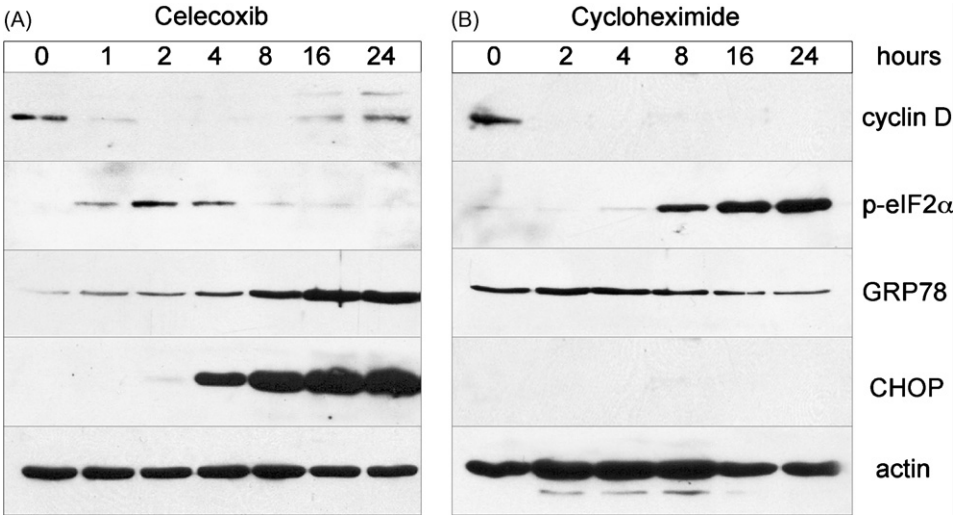

Fig. 6 – Expression levels of cyclin D and ER stress proteins during treatment with celecoxib or cycloheximide. U251 cells were treated with 75 μM celecoxib or with 50 μM cycloheximide for the indicated times. Total cellular lysates were prepared and analyzed by Western blot with antibodies as indicated. It should be noted that during the time course of this experiment, there was no substantial cell death; therefore, the differential drug effects shown in this figure were not due to the survival and selective accumulation of drug-resistant cells over time.

hand, treatment of cells with celecoxib resulted not only in the rapid phosphorylation of eIF2α, but also in the pronounced induction of GRP78 and CHOP (Fig. 6), which are reliable indicators of ER stress. The synthesis of these two proteins is known to be selectively exempt from the general inhibition of translation that takes place during ER stress, and their induction therefore further underscores that the translational block induced by celecoxib is part of the ER stress response mechanism.

4. Discussion

Investigations with the use of elevated concentrations (10–100 μM) of celecoxib in vitro have been met with skepticism, because such high concentrations cannot be achieved in vivo. Nonetheless, it is remarkable that several of celecoxib's in vitro effects, which can only be detected at 30–100 μM, can also be verified in xenograft tumor tissue in vivo, where drug concentrations remain well below 5 μM. For instance, inhibition of 3-phosphoinositide-dependent protein kinase-1 (PDK1), down-regulation of cyclins, or inhibition of survivin by celecoxib requires relatively high concentrations in vitro, but has also been documented in experimental tumor tissue in vivo, where much lower drug concentrations are present [13,15,19,32]. Similarly, induction of tumor cell death/apoptosis by celecoxib in cell culture generally requires drug concentrations well above 30 μM; however, when the same cells are studied as xenografts, cell death/apoptosis can also be detected in vivo subsequent to treatment of animals with celecoxib [15,19]. Taken together, such results caution against the tendency to disregard those effects of celecoxib that are observed at elevated concentrations in vitro.

One of the recently documented effects of celecoxib in vitro is the stimulation of the ER stress response (ESR), which appears to be triggered by the rapid drug-induced inhibition of ER transmembrane calcium pumps (SERCAs) and the resulting leakage of calcium into the cytosol [16–23]. In this regard, celecoxib resembles thapsigargin, a sesquiterpene lactone that is frequently used as a model inducer of calcium-stimulated ESR, due to its potent inhibition of SERCAs [33]. In general, ESR is characterized by mild to severe, but transient, down-regulation of general protein synthesis, which is accomplished by the inactivation of the central translation initiation factor eIF2α (through phosphorylation of serine-51). At the same time, several ER stress proteins, such as GRP78 and CHOP/GADD153, are selectively exempt from translational inhibition [24,25,28]. Because the degree and duration of translational inhibition during ESR can vary widely, depending on the respective trigger, our study addressed two questions: (i) does celecoxib inhibit protein synthesis via ER stress, and, if yes, (ii) could this effect perhaps explain some of the previously reported COX-2-independent effects of this drug? We determined that the answer to both queries is: Yes!

Consistent with earlier reports [18,20–22], we found that celecoxib strongly stimulated the expression of the ER stress markers GRP78 and CHOP (Fig. 6), confirming that this drug triggered severe ER stress in our cell systems. In parallel, we found that overall translational activity was attenuated (Figs. 1 and 4) and this closely correlated with the inactivation (phosphorylation) of eIF2α (Figs. 2, 4 and 5). The critical involvement of this translation factor was established with the use of cells harboring a mutant form of eIF2α that cannot be phosphorylated at serine-51; in these cells, the ability of celecoxib to inhibit translation was greatly diminished (Fig. 3).

Depending somewhat on the particular batch of serum that is used for cell culture, we found that the IC50 for inhibition of protein synthesis by celecoxib is approximately 45–65 μM, and at concentrations of 80 μM and above the translational block is

essentially complete (Fig. 1). Notably, the IC50 can be lowered substantially (to below 30 μM) under conditions of reduced serum supplementation in the growth medium. Considering that in vitro studies of celecoxib frequently employ the concentration range of 10–100 μM, sometimes combined with reduced or no serum supplementation [3,4,6], it is conceivable that some of the previously reported effects of celecoxib might have been a consequence of translational inhibition. To explore this supposition, we investigated the cell cycle-regulatory protein cyclin D, a well-established target for down-regulation by celecoxib (for example, Ref. [5]), as proof-of-principle. Our experiments show that mere inhibition of translation, such as with cycloheximide, mimics the rapid disappearance of this protein (Fig. 6), indicating that blockage of protein synthesis by celecoxib may have profound implications for at least some of its previously identified non-COX-2 targets. These results are also in agreement with earlier findings that ER stress causes translational inhibition of cyclin D via phosphorylation of eIF2α [34]. In view of cyclin D's critical role during the cell cycle, translational blockage of this protein would also suffice to explain other known effects of celecoxib, such as the reduction of cyclin-dependent kinase activity and the resulting loss of retinoblastoma protein phosphorylation (see details in Ref. [2]), all of which depend on the presence of cyclin D.

Whether or not other known targets of celecoxib are affected through the inhibition of general translation remains to be established, although potential candidates are proteins (or their modifiers) with relatively short half-lives, such as the anti-apoptotic proteins survivin and Mcl-1 (myeloid cell leukemia 1), which are known to be down-regulated by this drug [15,35]. Additionally, nuclear factor kappa B (NF-κB) is a target of celecoxib that might be affected via reduced translation rates. For instance, it has been shown that conditions of ER stress activate NF-κB [36], and this appears to be due to the phosphorylation of eIF2α, possibly resulting in translational repression of IkBα, a short-lived repressor of NF-κB [37,38]. Based on this, our finding that celecoxib induces the phosphorylation of eIF2α and results in translational repression might suggest an explanation for earlier studies that reported activation of NF-κB by this drug [7,39–41]. It should be noted, however, that in other investigations celecoxib was found to prevent the activation of NF-κB by tumor necrosis factor (TNF) and cigarette smoke condensate [42,43]. The reason for these discrepant findings is unclear, but emphasizes the need for further study of these drug actions with special consideration of translational activity.

Celecoxib-induced calcium release from the ER can be detected within seconds after drug exposure [16,17,19,20,23], and inhibition of translation constitutes a very early event as well (Fig. 1). Considering the well-established severe impact of ESR on cellular function, one could surmise that some of the other known COX-2-independent effects of celecoxib might be a consequence of ER stress. The loss of cyclin D protein was introduced as one example further above. Induction of apoptosis could be another example. In general, when cellular stress is sustained, as is the case when drug treatment is maintained, the protective component of the ESR is being overwhelmed and its pro-apoptotic module gains dominance. This situation is reminiscent to what has been established for thapsigargin, an inhibitor of SERCA and well-studied model inducer of calcium-stimulated ESR [33]. Like celecoxib, thapsigargin causes severe, yet transient, inhibition of translation [44]. The attenuation of general protein synthesis appears to be part of the protective component of the ESR and provides the cell with an opportunity to cope with the initial stressful insult. Eventually, however, protein synthesis has to recover, whether or not the cellular defense was successful in neutralizing the insult. At this point, the cells' further fate rests with the levels of the pro-apoptotic protein CHOP, which is induced during ER stress and represents a predictor of cell death [45]; if the stressful insult could not be alleviated (i.e., if thapsigargin or celecoxib remain chronically present), the stress-induced increase in CHOP levels will be maintained (Fig. 6) and the ESR will switch from protection to apoptosis.

In the above context, it might not be unreasonable to postulate that ESR might constitute a decisive mechanism by which celecoxib causes cell death in vitro. Whether this scenario takes place under in vivo conditions as well is uncertain, although we and others have shown that the ESR markers GRP78 and CHOP are elevated in xenograft tumor tissues from animals treated with celecoxib [19,22]. In any case, our present study demonstrates severe inhibition of general protein synthesis due to ER stress triggered by celecoxib at concentrations that are quite commonly used by many laboratories; these effects are likely to bear upon some of the other non-COX-2 targets that are known and therefore should be considered when interpreting observations from the use of this drug in cell culture.

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