

Research Article

GSH Synthetic Analogue O-Methyl-L-Tyrosinylglutathione Regulates Nrf2-Mediated Expression of GCLc and GCLm

Mats Hansen , Rando Porosk , Riina Mahlapuu , Ceslava Kairane , Kalle Kilk, and Ursel Soomets 

*Institute of Biomedicine and Translational Medicine, Department of Biochemistry,
Centre of Excellence for Genomics and Translational Medicine, University of Tartu, Tartu, Estonia*

Correspondence should be addressed to Mats Hansen; mats.hansen@ut.ee

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A glutathione (L- γ -glutamyl-L-cysteinylglycine, GSH) analogue, UPF1 (O-methyl-L-tyrosinylglutathione), has been shown to increase intracellular concentration of total glutathione (tGSH) in K562 cells. The synthesis of GSH is a two-step process that requires the actions of two distinct enzymes: γ -glutamyl-cysteine ligase (GCL) and glutathione synthetase (GS). Transcription of the GCL is controlled by multiple different factors, among others the nuclear factor (erythroid-derived 2)-like 2 transcription factor (Nrf2), which under the oxidative stress translocates into nucleus, where it binds to the dedicated binding site—antioxidant response element (ARE). In the present study, we investigated if the observed increased concentration of intracellular tGSH is a result of activation of Nrf2 protein—a key transcription factor in the cellular antioxidant response. Two distinct cell lines, adherent human hepatocarcinoma cell line HepG2 and nonadherent human myelogenous cell line K562, were chosen to establish if the increased intracellular tGSH is a universal response to the UPF1 treatment. Western blot analysis demonstrated that, after 3 h, the catalytic subunit of GCL (GCLc) level in HepG2 cells was higher than the modifying subunit of GCL (GCLm), while in K562 cells no change was observed. After 24 h, the GCLc level was higher than GCLm in K562 cells but not in the HepG2 cell line. Reverse-transcriptase PCR experiment demonstrated that no statistically significant difference was found in GCLm or GCLc mRNA levels, while the expression of the mRNA of Nrf2 and GS was elevated in the K562 cell line. Our findings suggest that UPF1 displays unique properties of mobilizing cellular defence mechanisms against reactive oxygen species while it is previously been shown to act as potent antioxidant per se.

1. Introduction

Xenobiotics are removed from the organism in 3 distinct phases—functionalization, conjugation, and excretion [1]. The first two are more conventionally called phase I and phase II of detoxification. In phase I, the xenobiotics are commonly functionalized by the cytochromes P450-mediated redox reactions [2] although there are other enzymes with secondary role, such as alcohol dehydrogenases, aldehyde dehydrogenases, aldo-keto reductases, and flavin-containing monooxygenases participating in phase I as well [3].

Phase II in xenobiotics detoxification may occur in parallel with phase I or take place without phase I ever taking place [1]. In this phase, an endogenous molecule is coupled

to a xenobiotic substrate in order to prepare the latter to move down the metabolic pathway [4]. Although the majority of conjugation reactions in phase II are mediated via different enzymes, such as the methylations, glucuronidations, and acetylations, the nonenzymatic conjugations of xenobiotics with glutathione (GSH) are amongst the very central events in the detoxification processes [5].

In phase III, the GSH-conjugated xenobiotics are eliminated by the transporters [6] like multidrug resistance-associated protein 2, organic anion transporter proteins 1 or 3, organic anion transporting protein [7], or ATP-dependent glutathione S-conjugate export pump [8].

GSH is the most abundant nonprotein thiol in the mammalian cells. It is considered the central antioxidant

molecule, which is involved not only in the metabolism of xenobiotics and eicosanoids, but also in the cellular signalling and thiol exchange reactions [9]. Increased oxidative stress and reduced levels of GSH have been connected to the development of various pathological conditions, including cardiovascular, neurodegenerative, and pulmonary disorders, inflammation, etc. [10].

De novo synthesis of GSH is a two-step ATP-dependent process. In the first step, the γ -carboxyl group of the L-glutamic acid reacts with the amino group of L-cysteine, forming the L- γ -glutamyl-L-cysteine [9]. This step is catalysed by the γ -glutamyl-cysteine ligase (GCL), it is ATP-dependent and rate-limiting [9, 11]. GCL is a heterodimer, with a 72-kDa catalytic subunit (GCLc) and a 30-kDa modifying subunit (GCLm) [12]. The second step in the process, the conversion of L- γ -Glu-L-Cys into GSH by adding L-glycine, is carried out by the homodimeric glutathione synthetase (GS) [9].

The expression of GCL is tightly regulated by the nuclear factor (erythroid-derived 2)-like 2 transcription factor (NFE2L2) or simply Nrf2 [13], which is released from its anchoring protein Keap1 and binds thereafter to the antioxidant response elements (ARE) during the oxidative stress [14]. Nrf2 is a member of Cap'n'Collar proteins from the family of basic leucine zipper transcription factors [13, 14, 15]. It is expressed in different tissues, with the highest levels in muscle, kidney, liver, and lung [15].

Under nonstress conditions, the Nrf2 is attached to its inhibitor protein Keap1, which leads to the ubiquitinylation and degradation of Nrf2 [13, 16] which under normal physiological conditions has half-life less than 15 min and is rapidly degraded by proteasomes [17].

It is generally believed that the cysteine-rich Keap1 acts as a sensor during the oxidative stress and the disruption of intramolecular disulphide bridges causes the conformational change in the Keap1 protein, that leads to its dissociation from Nrf2 [13, 16]. Subsequently, the Nrf2 is free to translocate into the nucleus, where it can bind to ARE and initiate the transcription of GCL.

Nrf2 also regulates the expression of other phase II antioxidant molecules such as glutathione peroxidase, N-acetyltransferase, and glutathione S-transferase [18] and other enzymes related to the drug metabolism and disposition like aldehyde dehydrogenase, alcohol dehydrogenase, or multidrug resistance-associated protein [19].

ARE is a *cis*-regulatory element, found in the promotor region of several genes involved in oxidant defence and redox signalling [6, 19]. Although regulated by the Nrf2, other transcription factors such as small musculoaponeurotic fibrosarcoma proteins (sMAFs), activating transcription factor 4 (ATF4), c-Jun, Jun-B, or Jun-D [6, 20] are necessary to form a heterodimer with Nrf2 in order to activate ARE.

Previously, we have shown that tetrapeptidic GSH analogue O-methyl-L-tyrosinylglutathione (UPF1) increases intracellular GSH levels in the K562 cell line [21]. UPF1 has also been shown to be about 60-fold better hydroxyl radical scavenger than its parent compound GSH [22]. It has also been previously demonstrated that UPF1 is nontoxic to the K562 cells up to concentration of 200 μ M [22].

In the current study, we examined whether UPF1 could be responsible for the activation and nuclear localization of Nrf2 in the K562 and HepG2 cell lines. Since we have previously demonstrated the upregulation of the intracellular levels of GSH in response to UPF1 treatment in K562 cells, the same cell line seemed an obvious candidate for the study. In attempt to establish whether UPF1 could alter the intracellular GSH levels in other cell lines, the HepG2 was chosen, since the Nrf2-mediated increase in GSH levels in the same cell line was recently reported in response to the homocysteine treatment [23].

2. Materials and Methods

2.1. 9-Fluorenylmethoxycarbonyl (Fmoc) Peptide Synthesis.

All Fmoc-L-amino acids were purchased from Novabiochem (Merck-Millipore, Hohenbrunn, Germany) except for Fmoc-L-Tyr(Me)-OH, which was from CBL Patras (Patras, Greece). All the other reagents for peptide synthesis were purchased from Merck Chemicals (Merck-Millipore, Hohenbrunn, Germany).

UPF1 was synthesized manually by solid phase peptide synthesis on a Fmoc-Gly-Wang resin from Novabiochem (Merck-Millipore, Hohenbrunn, Germany) utilizing standard Fmoc solid phase peptide synthesis [24]. Couplings of Fmoc protected amino acids were carried out in a stepwise manner using the standard 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU) and hydroxybenzotriazole (HOBt) activation in dimethylformamide (DMF). The peptide was removed from the resin and simultaneously deprotected with trifluoroacetic acid (TFA) in the presence of scavengers: water 2% (v/v), ethylenediaminetetraacetic acid (EDT) 2% (v/v), and triisopropylsilane (TIS) 2.5% (v/v) for 90 min at room temperature.

The crude peptides were purified by the reversed-phase HPLC on a Jupiter 5 μ C18 250 \times 21.2 mm column (Phenomenex, Torrance, CA, USA) employing an acetonitrile-water mixture (containing 0.1% TFA) as an eluent at a flow rate of 4 ml/min and absorbance of 218 nm. The fractions were pooled together and lyophilized.

The purity of the peptide was >99% as demonstrated by HPLC on an analytical Nucleosil 120-3 C18 reversed-phase column (0.4 cm \times 10 cm), and the peptide was identified by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass-spectrometry (Voyager DE Pro, Applied Biosystems, USA).

2.2. Cell Lines. The human cell lines K562 (DSMZ no. ACC10) and HepG2 (DSMZ no. ACC180) were grown in T75 cell culture flasks in RPMI 1640 supplemented with 2 mM glutamine (PAA, Austria), 7.5% foetal calf serum, streptomycin (100 μ g/ml), and penicillin (100 U/ml) (all from Invitrogen, USA) at 37°C in a humidified 5% carbon dioxide atmosphere. Cells were seeded at concentration of 1.0×10^6 per ml either into 6-well plates or 100 mm Petri dishes for western blot analysis. Experiments were conducted 24 h after passage.

The cellular lines of K562 and HepG2 were incubated with DPBS (PAA, Austria) as control or with UPF1 solution of 0.1 mM final concentration for 3 or 24 h at 37°C. After treatment, the cells were washed twice with DPBS and were lysed in water by keeping at -20°C overnight. Samples were sonicated for 30 sec and centrifuged ($12000 \times g$) for 10 min, and supernatants were transferred for experiments. The protein concentration in the supernatants was quantified by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

2.3. Measurement of Intracellular GSH Content. Concentration of glutathione (GSH) was assessed in cellular lysates by an enzymatic method using Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Briefly, the cellular lysates were deproteinated by 10% solution of metaphosphoric acid (Sigma-Aldrich, Germany) in water and centrifuged at $10000 \times g$ for 5 min. The enzymatic reaction was initiated by the addition of NADPH, glutathione reductase, and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in buffer containing EDTA. The change in optical density was measured after 25 min at 412 nm spectrophotometrically (Sunrise, Tecan). The amounts of GSH were calculated based on lysate protein concentration and expressed as nM per mg protein.

2.4. Western Blot. On a 100 mm Petri dish, 1.5×10^6 HepG2 cells were incubated with UPF1. Thereafter, cells were washed with 2×5 ml DPBS and harvested by scraping with soft blade cell scraper. Cells were then centrifuged at $300 \times g$ for 10 min. Supernatant was discarded and cell pellet was suspended in 200 μ l of hypotonic buffer (10 mM HEPES; 10 mM KCl; 1 mM EDTA; 1 mM $MgCl_2$; 0.5 mM DTT; 0.5% NP-40; 4 mg/l leupeptin; 20 mg/l aprotinin; 0.2 mM PMSF) [25]. Cells were incubated on ice for 15 min and centrifuged at $6000 \times g$ for 15 min at 4°C. Supernatant, containing cytoplasmic proteins, was removed, and its protein concentration was measured with Bio-Rad Protein Assay kit (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. Remaining pellet was further suspended in 50 μ l of hypertonic buffer (10 mM HEPES; 400 mM KCl; 1 mM EDTA; 1 mM $MgCl_2$; 0.5 mM DTT; 10% glycerol; 4 mg/l leupeptin; 20 mg/l aprotinin; 0.2 mM PMSF) and incubated on ice for 30 minutes [25]. Suspension was then centrifuged at $10000 \times g$ for 15 min at 4°C. Supernatant, containing nuclear proteins, was removed, and its protein concentration was measured with Bio-Rad Protein Assay kit (Bio-Rad, Hercules, USA).

Equal amounts (20 μ g) of cytoplasmic and nuclear proteins were separated in 10% SDS-polyacrylamide gels and electrotransferred to Amersham Hybond (TM)-P PVDF membrane (Amersham, Buckinghamshire, UK).

The membranes were blocked in the blocking buffer (5% nonfat dry milk in TBS, containing 0.1% Tween 20) for 1 h at room temperature. Thereafter, the membranes were incubated with rabbit monoclonal anti-Nrf2 primary antibody

(1:1000, ab62352, AbCam, Cambridge, UK), γ -GCLm rabbit polyclonal antibody (1:1000, sc-22754, Santa Cruz biotechnology, USA), or γ -GCLc rabbit polyclonal antibody (1:1000, sc-22755, Santa Cruz biotechnology, USA) overnight at 4°C on a shaking platform. Next day, the membranes were washed three times with TBS, containing 0.1% Tween 20 (TBST); the membranes were blotted with horseradish peroxidase-conjugated secondary antibody (1:5000, Jackson ImmunoResearch Europe Ltd, Suffolk, UK) for 1 h; and immunoreactive bands were detected by chemiluminescence detection (SuperSignal West Pico, Thermo Scientific, Rockford, USA) with ImageQuant RT ECL imager (GE Healthcare, Buckinghamshire, UK). Quantification of band intensity was performed with ImageJ software (<http://imagej.nih.gov/ij>).

For Western blot with nonadherent K562 cells, the method was slightly modified. 3×10^6 K562 cells were seeded to the 6-well cell culture plate and after incubation with UPF1 cells were harvested by centrifugation at $300 \times g$ for 10 min and washed twice with DPBS. Then, the cells were lysed as described previously.

2.5. Reverse-Transcriptase PCR. RNA from the K562 cells, incubated with 0.1 mM UPF1 for 30 min, was extracted with Trizol reagent according to the manufacturer's instructions. cDNA was synthesized from 250 ng of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instructions and was kept at -80°C until analysed with PCR.

PCR analysis of the gene expression was performed using 5 μ g of total RNA, on an Eppendorf MasterCycler with Solis BioDyne PCR reagents. Specific primers for the target genes were used (Table 1).

PCR products were separated with 1% agarose gel electrophoresis, and the band intensities were analysed using ImageJ software.

2.6. Statistical Analysis. All the data were analysed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Results are presented as mean \pm standard error of the mean (SEM) and compared with the *t*-test.

3. Results

3.1. UPF1 Alters GSH Levels in K562 and HepG2 Cells. K562 and HepG2 cells were incubated with 0.1 mM UPF1 for 3 and 24 h. After the incubation, the total GSH level was measured in the cells.

In K562 cells, statistically significant ($p < 0.05$) increase in the GSH concentration was detected after 3 h incubation with the peptide (Figure 1). When incubation time was increased to 24 h, the intracellular GSH level in K562 cells remained unaltered. On the other hand, when K562 cells were incubated with 0.1 mM hydrogen peroxide, the concentration of tGSH was not changed during the first three hours after incubation and decreased slightly over 24 h period, although not reaching statistical significance.

TABLE 1: Primers used for the RT-PCR analysis.

| Gene | Forward primer | Reverse primer |
|-------|------------------------------|-------------------------------|
| Nrf2 | 5'-TTTCCTCTGGGGCCGTCA-3' | 5'-TCCACCCGTACCCCAATCAA-3' |
| GS | 5'-AGCAAACCTTTTCCAGTGCTC-3' | 5'-GACGAGGCATGTAGCCATCC-3' |
| GCLC | 5'-TGTCGCTGGGGAGTGATTTC-3' | 5'-CAATGCCTTCCTGCAACAGC-3' |
| GCLM | 5'-TGGCCTAGGTATCAGGGTAATG-3' | 5'-AGTAAATCCCAGCTACTCCAGTT-3' |
| GAPDH | 5'-GAGAAGGCTGGGGCTCATTT-3' | 5'-AGTGATGGCATGGACTGTGG-3' |
| POLG | 5'-ATCTCATCCTGGGACTGGCT-3' | 5'-TCGCATTGGGGAAAAGGTGT-3' |
| LAMP1 | 5'-CCAAGAGTGGCCCTAAGAACA-3' | 5'-TCGCATTGGGGAAAAGGTGT-3' |

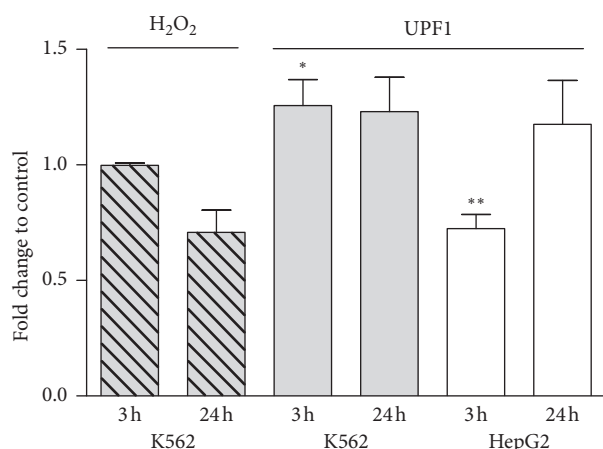


FIGURE 1: Total GSH levels of K562 (gray bars) and HepG2 (white bars) cells after incubation with 0.1 mM H_2O_2 or UPF1. Both graphs are normalised to the cells treated with Dulbecco's PBS (control). * $p < 0.05$; ** $p < 0.01$; $n = 6$.

When HepG2 cells were incubated with 0.1 mM UPF1, a statistically significant ($p < 0.01$) 25% decrease of the intracellular GSH level was observed after 3 h. After 24 h incubation with UPF1, the GSH level in HepG2 cells normalised to the level of the untreated control.

3.2. UPF1 Increases the Protein Expression Levels of γ -Glutamyl-Cysteine Ligase Subunits. K562 and HepG2 cells were incubated with 0.1 mM UPF1 for 3 and 24 h. After the incubation, GCLC and GCLM levels were quantified using Western blot analysis. Statistically significant increase was observed in the protein concentration of GCLC in K562 ($p < 0.01$) as well as HepG2 ($p < 0.001$) cell lines after 3 hour incubation (Figure 2). After 24 h incubation, the levels of the catalytic subunit remained increased from the in both K562 ($p < 0.001$) and HepG2 ($p < 0.01$) cells, when compared to the untreated control. The concentrations of GCLM after UPF1 treatment were also significantly higher in both K562 ($p < 0.01$) and HepG2 ($p < 0.001$) cell lines after 3 h incubation, but no increase in GCLM levels was observed after 24 h incubation.

The GCLC levels were also significantly higher than the levels of GCLM after 3 h incubation with UPF1 in HepG2 cells ($p < 0.01$). In K562 cells, there was no apparent difference in the catalytic and modifying subunit after 3 h incubation. However, a statistically significant increase in

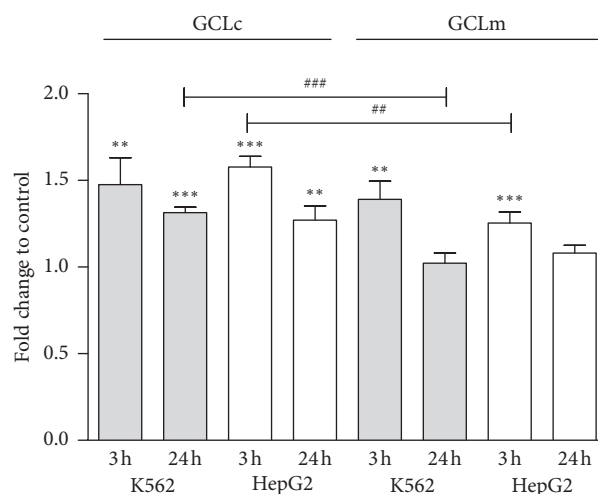


FIGURE 2: Western blot analysis of the concentration of GCLC and GCLM in the K562 (gray bars) and HepG2 (white bars) cells. Normalised to the cells treated with Dulbecco's PBS (control). ** $p < 0.01$; *** $p < 0.001$; $n = 6$. ## $p < 0.01$; ### $p < 0.001$.

the GCLC levels was found after 24 h incubation with UPF1 ($p < 0.001$).

3.3. Intracellular Relocalization of Transcription Factor Nrf2 is Facilitated by UPF1. K562 and HepG2 cells were incubated with 0.5 mM UPF1 for 15, 30, and 60 minutes at 37°C. Followed the incubation, the nuclear and cytoplasmic fractions of cells were analysed for the concentration of the transcription factor Nrf2. In the cytoplasmic fraction of HepG2 cells, a statistically significant increase in the Nrf2 level ($p < 0.01$) was observed after 30 min incubation with the peptide, while, at the same time point, the nuclear fraction did not show significant changes (Figure 3). In the cytoplasmic fraction of K562 cells, the tendency for maximum levels of Nrf2 was observed after 15 min incubation, while in the nuclear fraction, the trend of minimal expression was also observed after 30 min incubation (Figure 4). Although the differences in Nrf2 levels in K562 cells did not reach the statistical significance in cytoplasmic or nuclear fractions, the tendency for Nrf2 levels changes are quite similar for the two cell lines.

3.4. UPF1 Increases the mRNA Levels of the Nrf2 and GS in K562 Cells. In order to investigate whether the elevated levels of Nrf2 were recorded due to increased translation of

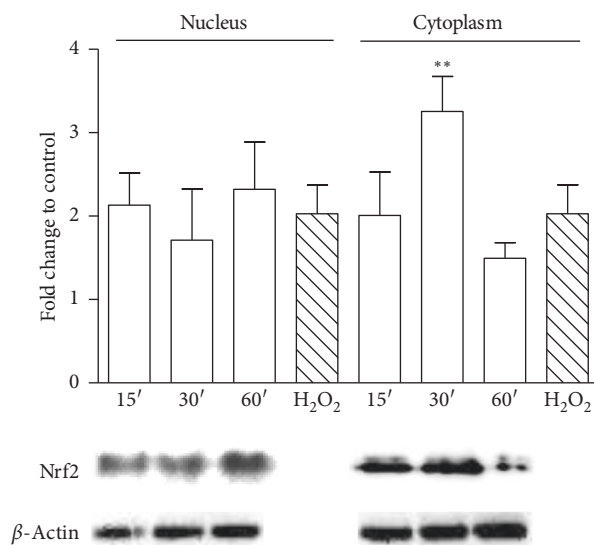


FIGURE 3: Western blot analysis of Nrf2 concentration in the nuclear and cytoplasmic fractions of HepG2 cells. Cells were treated with 0.1 mM UPF1 or DPBS (control) for indicated time periods. As a positive control of Nrf2, activation cells were incubated with 0.1 mM H₂O₂ for 60 min. Then, the nuclear and cytoplasmic fractions were separated and analysed for Nrf2 concentration using 10% SDS-polyacrylamide gel. Representative western blot bands are inserted under the x-axis at their respective time points with β -Actin as a loading control. Images were analysed using ImageJ software. ** $p < 0.01$.

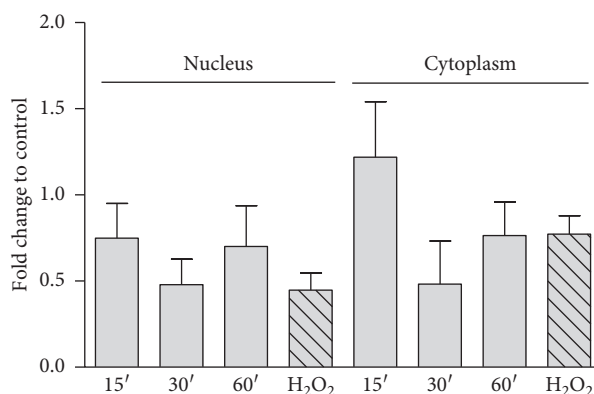


FIGURE 4: Western blot analysis of Nrf2 concentration in the nuclear and cytoplasmic fractions of K562 cells. Cells were treated with 0.1 mM UPF1 or DPBS (control) for indicated time periods. As a positive control of Nrf2, activation cells were incubated with 0.1 mM H₂O₂ for 60 min. Then the nuclear and cytoplasmic fractions were separated and analysed for Nrf2 concentration using 10% SDS-polyacrylamide gel. Images were analysed using ImageJ software.

mRNA, a reverse-transcriptase PCR experiment was conducted. The mRNA expression levels of Nrf2 and GS were significantly increased ($p < 0.01$ and $p < 0.05$ relatively to control, respectively) during the 30 min incubation with 0.1 mM UPF1 (Figure 5). At the same time, no statistically significant changes in mRNA levels of GCLc and GCLm were observed. No changes occurred in mRNA expression

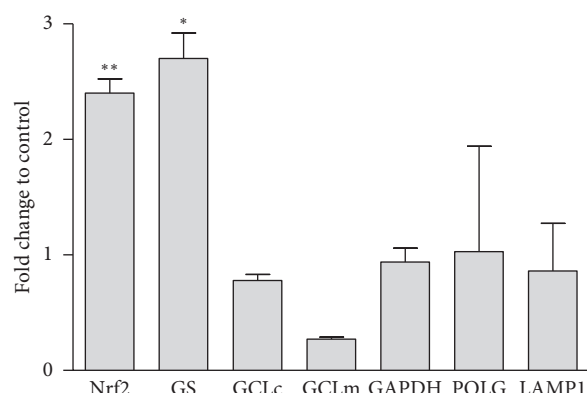


FIGURE 5: Reverse transcriptase PRC analysis of the mRNA expression in the K562 cells. Cells were treated with 0.1 mM UPF1 for 30 min. Then the mRNA expression was analysed and normalised to the cells treated with Dulbecco's PBS (control). * $p < 0.05$; ** $p < 0.01$.

levels of Nrf2 in HepG2 cells after 30 min incubation with UPF1 (data not shown).

4. Discussion

In recent years, several powerful antioxidant molecules have been developed in order to ameliorate the cellular injuries caused by oxidative stress. Oftentimes, these antioxidants have been shown to have great potential to reverse or prevent the cellular damage [26, 27, 28].

GSH is widely distributed in human body, with the highest (mM) concentration in red blood cells, liver, brain, etc., where it is involved in many cellular processes: DNA methylation [29, 30], protein synthesis [31], prostaglandin synthesis [32, 33], immune system enhancement [34, 35], and activation of various enzymes [31].

One of the most important functions of GSH is the protection against oxidative stress that is generated by the emission of reactive oxygen species (ROS) both in normal metabolism and pathological conditions. This is also the main reason why there has been an increasing interest in GSH, the most abundant nonprotein thiol in mammalian cells, as the decreased levels of GSH and increased levels of ROS species are related to a prolonging list of pathologic conditions.

In this study, we first focused on the effect of UPF1-induced increase in the intracellular GSH levels in two commonly used cell lines, K562 and HepG2. In a previous study, we observed an increase in the intracellular GSH concentration when K562 cells were treated with UPF1 [21]. So far, it was unknown whether this behaviour is universal to other cell lines as well.

A statistically significant increase in GSH concentration was detected in K562 cells after 3 h incubation with the UPF1 (Figure 1). When K562 cells were incubated with hydrogen peroxide, a decreasing tendency in the GSH levels was observed suggesting that the mechanisms of action are different for UPF1 and hydrogen peroxide.

A statistically significant decrease in GSH concentration was observed in HepG2 cells after 3h incubation with UPF1

(Figure 1), indicating that the UPF1-mediated effect of increased GSH levels is not universal for different cell types.

Previously, we have established that the UPF1 is not able to cross the plasma membrane and reach the cytoplasm of the BEAS-2B cell line [11]. In the present study, we further investigated if the observed increase in GSH levels [21] was due to activation of the synthesis of phase II detoxifying enzymes, GCLm and GCLc.

Statistically significant increase in the concentration of both catalytic and modifying subunit of GCL was detected by western blot analysis when the cells were treated for 3 h with UPF1. After 24 h incubation with UPF1, the concentrations of catalytic subunit were increased in both cell lines, whereas the concentrations of modifying subunits remained unaltered in both cell lines at that time point.

We also observed the statistically significant difference in the levels of GCLc and GCLm in HepG2 cells after 3 h incubation with UPF1 (Figure 2).

It is previously known that the catalytic subunit is capable to synthesize the γ -glutamate-cysteine even without the modifying subunit, albeit with lower efficiency [36, 37]. This could be also true for the HepG2 cell line, since after 3 h incubation with UPF1, the GSH levels in the cells were significantly lower than the control (Figure 1). At the same time, we did not observe any significant change in mRNA levels of GCLc or GCLm, although there was a notable decreasing tendency (Figure 5).

In the K562 cell line, there was no statistical difference between the GCLc and GCLm concentrations after 3 h incubation with UPF1, but instead a significant change after 24 h incubation (Figure 2). This, however, did not result any change in intracellular GSH levels at the respective time points (Figure 1).

In order to confirm that the observed changes in the intracellular GSH is indeed due to activation of translation by Nrf2, we investigated if UPF1 has an effect on the intracellular accumulation of free Nrf2. Results show that HepG2 cells, when incubated with the UPF1, show significantly increased ($p < 0.01$) concentration of free Nrf2 in the cytoplasmic fraction after 30 min. In K562 cells, the Nrf2 concentration tends to increase in the cytoplasm after 15 min incubation. At the same time, Nrf2 concentrations in the nucleus show the tendency of maximal decrease in both cell lines after 30 min incubation with the peptide. The results, although not conclusive, indicate that the levels of free Nrf2 might indeed be quite rapidly elevated in the cytoplasm in response to the UPF1 treatment in both cell lines.

When UPF1 was added to the K562 cells, a statistically significant increase in the mRNA levels was recorded for Nrf2 and GS (Figure 5). At the same time, the mRNA levels of the both subunits of rate-limiting enzymes GCLc and GCLm remained unaltered, although showing the tendency to decrease. In order to ensure that the differences in mRNA expression are not an experimental artefact, the common housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), DNA polymerase subunit gamma (POLG), and lysosomal-associated membrane protein 1 (LAMP1) were included. Expectedly, the mRNA levels of

housekeeping genes remained unchanged during the experiment. No difference in Nrf2 mRNA levels was observed in the HepG2 cell line after 30 min incubation with UPF1 (not shown).

These results indicate that the activation of Nrf2 by UPF1 peptide is not a universal response in different cell types. HepG2 response to the UPF1 is a rapid increase in the free intracellular Nrf2, but not the mRNA synthesis, which leads to the unequal synthesis of the GCL subunits and subsequent decrease in the cellular GSH content in the first 3 h, which normalizes over 24 h period.

In the K562 cell line, there is instead a rapid response in the Nrf2 translation, which also leads to the increased intracellular Nrf2. In this cell line, however, the initial response in GCL subunit synthesis is more equal, leading to the increase in GSH concentration in the cell.

These findings are in unison with an earlier study by Pöder et al., where best neuroprotective effect of UPF1 was demonstrated on male Wistar rats when administered 20 min before inducing a global brain ischaemia [24]. Also, the significant protective effect of the peptide was observed when administered immediately before the reperfusion [24]. Similar results were observed in a study by Kals et al., where administration of UPF1 10 min prior to ischaemia, but not at reperfusion, significantly improved heart function [38].

Biosynthesis of phase II detoxifying enzymes is regulated by the release of Nrf2 from its anchoring protein Keap1 and subsequent activation of ARE. This leads to the increased concentration of free Nrf2 in the cytoplasm and allows Nrf2 to translocate to the nucleus where it activates the transcription of phase II detoxifying enzymes [39, 40].

Another possible mechanism of Nrf2 regulation is proposed involving the modulation of the Nrf2 transcription by Myc or Jun [13, 41].

It is plausible and also supported by our findings that different cell types deploy different activation mechanisms of Nrf2. One of the mechanisms could rely on the upregulation of the Nrf2 expression in the mRNA level, while the other mechanism increases the cytoplasmic free Nrf2 by detaching it from the anchoring Keap1 protein. It seems however that no matter how the Nrf2 levels in the cytoplasm are elevated, it leads to the upregulation of GCLc in distinct cell lines.

Although our study does not reveal the exact mechanism by which the UPF1 leads to the change in intracellular GSH, it indicates that the Nrf2-mediated change in the expression of GCLc and GCLm is a crucial event in the process.

Abbreviations

| | |
|-------|--|
| ARE: | Antioxidant response element |
| GCL: | Glutamate-cysteine ligase |
| GCLc: | Catalytic subunit of glutamate-cysteine ligase |
| GCLm: | Modifying subunit of glutamate-cysteine ligase |
| GS: | Glutathione synthetase |
| GSH: | Glutathione |
| Nrf2: | Nuclear factor (erythroid-derived 2)-like 2 transcription factor |
| ROS: | Reactive oxygen species |
| UPF1: | O-methyl-L-tyrosinylglutathione. |

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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