*Free Radical Research*, June 2009; 43(6): 572–580

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# Characterization of UPF peptides, members of the glutathione analogues library, on the basis of their effects on oxidative stress-related enzymes

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*(Received 12 January 2009; revised 11 March 2009)*



**Abstract**
Previously the authors have designed and synthesized a library of antioxidative glutathione analogues called UPF peptides which are superior to glutathione in hydroxyl radical elimination. This paper is a follow-up study which investigated the effects of the most promising members of the library (UPF1 and UPF17) on oxidative stress-related enzymes. At concentrations used *in vivo* experiments neither UPF peptide influenced the activity of glutathione peroxidase (GPx) when purified enzyme or erythrocyte lysate was used. At higher concentrations they inhibited GPx activity. UPF peptides had no effect on glutathione reductase (GR) activity. Also they, as well as glutathione itself, slightly increased MnSOD activity in human brain mitochondria and inhibited oxidative burst caused by neutrophil NAD(P)H oxidase. RT-PCR measurements showed that UPF1 and UPF17 have no effect on GPx and MnSOD expression level in human blood mononuclear cells. The results of this study confirm that investigated UPF peptides do not interfere with the enzymatic mechanisms of antioxidative defence and can be used as themselves or as a lead for the protector molecule design against excessive oxidative stress.

**Keywords:** *Glutathione, glutathione analogues, glutathione peroxidase, glutathione reductase, MnSOD, NAD(P)H oxidase, oxidative stress*

**Abbreviations:** *GSH, glutathione; GSSG, glutathione disulphide; GPx, glutathione peroxidase; GR, glutathione reductase; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MnSOD, manganese-containing superoxide dismutase; PDBu, phorbol 12, 13-dibutyrate; PMNs, polymorphonuclear neutrophils*

## Introduction

Glutathione (GSH) is a tripeptide (L-$\gamma$-glutamyl-L-cysteinyl-glycine) that is the most abundant non-protein thiol in the mammalian cells. Its intracellular concentration is 1–8 mM, whereas extracellular concentrations are relatively low (5–50 μmol/l) [1]. Most of the cellular GSH (85–90%) is located in cytosol [2]. Reduced glutathione (GSH) is the central intracellular non-enzymatic water-soluble antioxidant, but additionally it has several other functions in the human body concerning the metabolism of xenobiotics and eicosanoids, cellular signalling and thiol disulphide exchange reactions [3,4]. High grade oxidative stress and decrease of GSH level is related to development of several pathological states including cardiovascular diseases, neurodegenerative diseases, cancer, HIV, inflammation and ischemia/reperfusion injury [5–9]. Compensatory

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ISSN 1071-5762 print/ISSN 1029-2470 online 
DOI: 10.1080/10715760902918691

administration of GSH is not effective because of its rapid degradation, while more stable and non-toxic glutathione analogues could be a more effective alternative [4].

GSH is converted to glutathione disulphide (GSSG) by actions of glutathione peroxidase (GPx), which is the predominate mechanism for eliminating excessive $H_2O_2$ and lipid hydroperoxides [3]. In the literature, there are references to glutathione analogues and antioxidative substances that do not interfere with the GPx-GR system [10,11]. In this work, changes in GPx activity were measured by using both purified enzyme and erythrocyte lysate. The latter was chosen due to the importance of GPx in the protection of haemoglobin from oxidative damage. In addition, GSH can directly inactivate some of the reactive species as hydroxyl radical, hypochlorous acid or peroxynitrite and be converted to GSSG [1]. The ratio of GSH/GSSG in normal cells is high as glutathione reductase (GR) reduces GSSG rapidly back to GSH, whereas NADPH is used as the reducing agent. GSH/GSSG redox couple is playing a principal role in the general redox status of the cell [12]. Decreased GSH/GSSG redox ratio affects several processes in the cells as signalling, growth, differentiation and apoptosis [13]. Consequently, the activities of GPx and GR are therefore partially responsible for the redox state of different tissues [14].

Mitochondrial manganese-containing superoxide dismutase (MnSOD) is the principal antioxidative enzyme converting superoxide radical to hydrogen peroxide and oxygen. Many links between depletion of GSH pool and neurodegenerative diseases have been found, but information about glutathione level as one of the regulators of MnSOD activity in brain mitochondria is quite limited [15,16]. Synthesis of glutathione predominately takes place in cytoplasm. Approximately 15% of total cellular glutathione is localized in mitochondria. Mitochondrial inner membrane is the main barrier for glutathione [17].

Primary reactive species generated in cells are superoxide radicals. Besides being tissue damaging itself, superoxide serves as the precursor for other reactive species such as hydrogen peroxide, hydroxyl radical, hypochlorous acid and peroxynitrite. NAD(P)H oxidase is a major source of superoxide production in phagocytic cells. Thus, our study also focused on the possibility to control superoxide overproduction using UPF peptides as NAD(P)H oxidase inhibitors. This membrane associated multisubunit enzyme catalyses the reduction of molecular oxygen using NADPH as the electron donor. However, phagocytic NAD(P)H oxidase is a critical component of a host defence system, its over-reaction can cause excessive production of free radicals. Emerging evidence suggests that patients with sepsis suffer from severe oxidative stress. According to this, antioxidants have been considered to be a supportive part of sepsis treatment [18,19].

Vascular NAD(P)H oxidase, which releases continually low levels of superoxide radicals and has a regulatory role, is suggested to be the main source of superoxide in vascular cells and its increased activity is related to several cardiovascular diseases such as atherosclerosis, hypertension and heart failure [20–22]. NAD(P)H oxidase inhibitors are potential leads for design of novel anti-inflammatory substances [23]. Molecules such as peptide PR-39, apocynin and its analogues have been tested to decrease NAD(P)H oxidase over-reactivity in pathological conditions [23,24].

Previously we have designed and synthesized a library of antioxidative glutathione analogues called UPF peptides. We demonstrated that all members of the UPF library showed remarkably better hydroxyl radical scavenging properties than glutathione itself [25]. For the current study, we chose a pair of UPF peptides, structural analogues: UPF1 (4-methoxy-L-tyrosinyl-$\gamma$-L-glutamyl-L-cysteinyl-glycine) and UPF17 (4-methoxy-L-tyrosinyl-$\alpha$-L-glutamyl-L-cysteinyl-glycine). These peptides possess identical amino acid sequence but in UPF17, $\gamma$-glutamate residue present in UPF1, is replaced with $\alpha$-glutamate residue, which causes a different spatial arrangement of peptides and different distances between functional groups in the peptides structures. This modification in UPF17 sequence drastically decreased $EC_{50}$ values of hydroxyl radical elimination compared to UPF1 by $\sim$500-times. Despite this, neither UPF1 nor UPF17 showed any toxic effects on the viability of K562 human erythroleucemia cells at concentrations of 200 $\mu$M after 24 h incubation [25]. UPF1 has already been investigated in different *in vivo* experimental models in rats: global brain ischemia, Langendorff model with isolated heart and was shown to possess protective effects [26,27].

Considering the aforementioned results we were interested in whether such a change at glutamate residue could cause different effects on antioxidative enzymes activities. Presuming that UPF peptides could be used as protective molecules, they should not inhibit enzymatic antioxidative defence mechanisms. On the other hand, upregulation of the antioxidative defence could explain their salutary effect in animal models named above. In this paper we have focused on the investigation of whether and how UPF peptides alter the activity of principal antioxidant enzymes GPx, GR and MnSOD. Additionally, influence to the potentially pro-oxidative neutrophil NAD(P)H oxidase activity was studied. The aim of our study was to characterize interactions between the UPF peptides and cellular antioxidative system, to understand mechanisms behind the protective effects of the UPF peptides and to clarify the structure–functional relationships of small molecular weight thiols.

## Materials and methods

### *Peptide synthesis*

UPF peptides were synthesized manually by solid phase peptide synthesis using Fmoc-chemistry and by machine using *tert*-Boc-chemistry. A more detailed synthesis protocol of UPF peptides has been published before [25,28]. The purity of the peptides was $> 99\%$, as demonstrated by HPLC on an analytical Nucleosil 120-3 C18 reversed-phase column (0.4 cm $\times$ 10 cm). The molecular masses of the peptides were determined by a MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass-spectrometry (Voyager DE Pro, Applied Biosystems) and the calculated values were obtained in each case.

### *Assays of GPx and GR activities*

The sources of GPx were: first, commercial purified enzyme from bovine erythrocytes (obtained from Sigma, Sigma-Aldrich, Germany) and second, erythrocyte lysate. Erythrocyte separation and measurement of GPx activity were performed according to the manual of Bioxytech GPx-340 Assay kit (Bioxytech®; OXIS International, Inc., Portland, USA). The reaction mixture of the assay kit contained glutathione, glutathione reductase and NADPH. GPx converts GSH to GSSG (glutathione disulphide), which is immediately converted back to the reduced form by the GR with a concomitant oxidation of NADPH to $NADP^+$. Accompanied decrease in absorbance at 340 nm was measured spectrophotometrically on a Microtiter Plate ELISA Reader. To measure the influence of UPF1 and UPF17 on GPx activity, the solutions of those peptides were added to the reaction mixture with final concentrations of 25–1500 μM. After 5 min preincubation time for peptides, the enzyme reaction was initiated by adding the substrate, tert-butyl hydroperoxide. The change of NADPH concentration was recorded for 3 min with taking a reading at every 30 s. GPx activity is expressed as relative to basal activity (100%) of GPx.

In erythrocyte lysate, kinetics of inhibition was also followed. We studied the effect of UPF17 as it showed more expressed inhibition of GPx activity compared to UPF1. Peptides were used in the following concentrations: GSH as substrate with concentrations of 0.5, 1.0 and 1.5 mM and UPF17 as inhibitor with concentrations of 0.1 and 0.15 mM. In order to confirm the type of inhibition of GPx by UPF17, the Lineweaver–Burk plot was constructed.

Also the activity of GR (from baker's yeast; obtained from Sigma, Sigma-Aldrich, Germany) was measured spectrophotometrically according to the Bioxytech GR-340 Assay (Bioxytech®; OXIS International, Inc., Portland, USA). Its activity was determined by the time-dependent change in the added NADPH concentration. Final enzyme concentration in the assay was 8.4 mU/mL. The final concentrations of GSH, UPF1 and UPF17 were from 0.2 to 1.0 mM. Preincubation time was 5 min and 24 h for peptides. GR activity is expressed as relative to the basal activity (100%) of GR.

### *MnSOD activity in brain cortex mitochondria*

Human brain tissue was obtained from the Huddinge Brain Bank, Huddinge University hospital, Sweden. The mitochondria were isolated from post-mortem human temporal cortex (aged 86–92 years) according to Rajapakse et al. [29]. The protein content in the enzyme preparations was determined by the method of Lowry et al. [30]. MnSOD activity was measured with the commercially available Ransod kit (Randox Laboratories Ltd. Ardmore, UK). To inhibit possible CuZnSOD activity, 5 mM KCN was added to the reaction mixture. GSH was used at concentration 10 nmol/mg protein with incubation time 5 and 40 min. UPF1 and UPF17 were used at concentrations 0.5, 1.0 and 5.0 nmol/mg protein with the incubation times 5 and 40 min.

### *PCR analysis of GPx and MnSOD*

Human blood mononuclear cells were separated using BD Vacutainer® CPT tubes (Becton Dickinson, Franklin Lakes, NJ) in which the whole blood was centrifuged at 1500*g* for 30 min at 22°C. Isolated mononuclear cells were washed twice with phosphate-buffered saline and centrifuged at 200*g* for 10 min at 22°C. Mononuclear cells were divided into three even amounts. One of them was cultivated in the presence of 0.5 μM UPF1, the second was cultivated in the presence of 0.5 μM UPF17 and the third portion was cultivated without UPF peptides, using RPMI-1640 medium (includes 10% foetal calf serum and 1% penicillin/streptomycin). Incubation time was 12 h.

RNA was extracted from the mononuclear cells with the Trizol method according to the manufacturer's protocol (Invitrogen, San Diego, CA) and stored at $-80$°C until cDNA synthesis. cDNA was synthesized using 250 ng (RNA from mononuclear cells) of total RNA, oligodT18 primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

Gene expression levels were detected using the TaqMan-QRT-PCR method (ABI Prism 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA) using TaqMan Assay-On-Demand (FAM-labelled MGB-probe) FAM-MGB labelled gene expression primer mix (20× gene expression

assay mix) and TaqMan® universal Master Mix. HPRT-1 (Hypoxanthine Phosphoribosyl-Transferase-1) expression level was detected by using specific primers (HPRT-1 exon 6, 5′-GACTTTGCTTTC CTTGGTCAGG-3′; HPRT-1 exon 7, 5′-AGTCTG GCTTATATCCAACACTTCG-3′; with final concentration 300 nM) and VIC-Tamra labelled probe (VIC-5′-TTTCACCAGCAAGCTTGCGACCTTG A-3′-TAMRA; with final concentration 200 nM). Reactions were carried out in 10 μl reaction volumes in four replicates.

For quantification of mRNA we used a comparative Ct method ($\triangle$Ct value), where the amount of target transcript was normalized according to the level of endogenous reference HPRT-1 (hypoxanthine phosphoribosyl-transferase-1). The assay mixes used were Hs00167309_m1 (MnSOD) and Hs00829989_gH (GPx).

### *Measurement of superoxide radical generation in isolated human PMNs*

Venous blood with EDTA supplement was obtained freshly from volunteered blood donors of local donor centre. Erythrocytes were separated by sedimentation with methylcellulose-25. White blood cells containing supernatant were diluted with $Ca^{2+}$ and $Mg^{2+}$ free Hank's Balanced Salt Solution (HBSS) and centrifuged on Ficoll-Paque Plus density gradient (Amersam Biosciences AB, Uppsala, Sweden) for 30 min at 400*g* and 20°C. The remaining erythrocytes were removed by hypotonic lysis. PMNs (polymorphonuclear neutrophils) pellet was resuspended in HBSS and counted automatically with Sysmex-XE2100. Neutrophil count in reaction mixture was kept between 60 000–160 000 cells. UPF peptides were used at concentrations of 10 and 100 μM. Superoxide production was quantified using 200 μM L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; Wako Chemicals, Japan) as a chemiluminescence dye. After 3 min of incubation at 37°C in the presence of UPF peptides followed by 10 min of dark adaption, the superoxide burst was initiated using 1 μM phorbol 12,13-dibutyrate (PDBu; obtained from Sigma, Sigma-Aldrich, Germany) as a NAD(P)H oxidase activator. Validation of this assay has been reported previously [31–33].

### *Data analysis*

Data were analysed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). For statistic analysis of PCR results, Mann-Whitney U-test was used. Other results are presented as the mean ± standard error of the mean (SEM) and compared with the *t*-test.

## Results

### *Results of GPx and GR activity assays*

Figure 1 represents the effects of UPF peptides on the activity of GPx measured by using purified enzyme or erythrocyte lysate. Results are expressed at each concentration as the percentage of basal GPx activity. In the range of concentrations used *in vivo* experiments [26,27] there is no significant inhibition of GPx by UPF peptides. For example, the 50 μM UPF1 inhibited GPx activity 1% and UPF17 3% in the erythrocyte lysate. At higher concentrations, UPF1 and UPF17 decreased the activity of GPx in a concentration-dependent manner. Experiments with purified enzyme and erythrocyte lysate showed similar inhibition dynamics. GPx activity after incubation with 500 μM UPF1 or UPF17 was $71 \pm 5\%$ and $51 \pm 3\%$ of the basal activity of the purified enzyme. Respective effects on GPx activity by UPF1 and UPF17 in erythrocyte lysates under the same conditions were $76 \pm 2\%$ and $61 \pm 1\%$.

According to these results, at the same concentration UPF17 inhibits GPx more than UPF1 and this difference is statistically significant and is expressed in the case of both purified enzyme and erythrocyte lysates. In addition, the curves of the same peptide are statistically different, comparing the figures of purified enzyme or erythrocyte lysate.

The Lineweaver–Burk plot analysis revealed that inhibition of GPx by UPF17 at the used concentrations is competitive (Figure 2). Preliminary experiments with UPF1 showed the same mechanism of inhibition (data not shown). The concentration of GSH as the substrate was varied from 0.5–1.5 mM and the concentrations of UPF17 were 0.1 and 0.15 mM.

Neither UPF1 nor UPF17 at concentrations of 0.2–1.0 mM caused any noticeable change in purified GR enzyme activity (Figure 3).

### *MnSOD activity in mitochondria of human brain cortex*

To investigate the influence of GSH and UPF peptides on MnSOD activity, mitochondria isolated from human temporal cortex were used. The concentration of added GSH solution was 10 nmol/mg of protein. Five and 40 min were used as incubation times. Five minutes of incubation enhanced remarkably MnSOD activity: $21.9 \pm 5.8\%$ (Figure 4). After further incubation (40 min), MnSOD activity was decreased back to the same level with control.

Influence of glutathione analogues UPF1 and UPF17 on MnSOD activity was investigated at three different concentrations—0.5, 1.0 and 5.0 nmol/mg of protein. Incubation times were 5 and 40 min; 0.5 nmol/mg concentration of UPF peptides was the most effective for MnSOD activation. Changes in MnSOD activity from basal value for UPF1 after 5

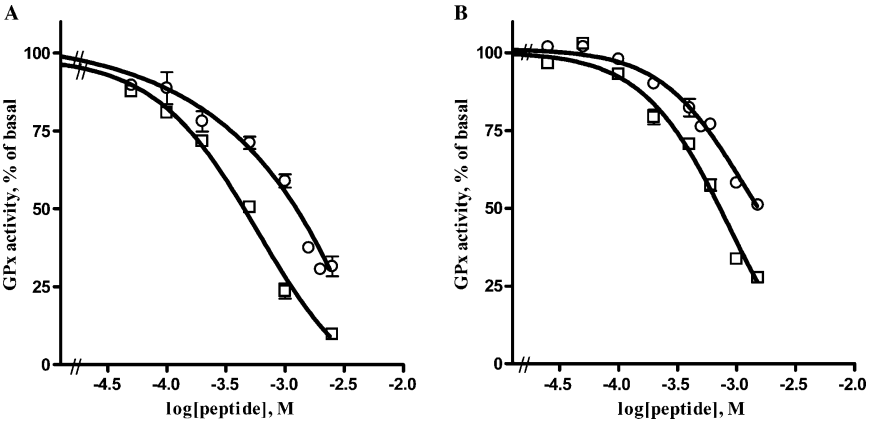


Figure 1. Activity of GPx measured with (A) purified enzyme and (B) enzyme from erythrocytes. Influence of (○) UPF1 and (□) UPF17 on GPx activity. 100% corresponds to the basal GPx activity. For purified enzyme, $n=6$; for enzyme from erythrocytes, $n=4$.

and 40 min were $94.1 \pm 0.7\%$ and $116.2 \pm 14.7\%$. Respective values for UPF17 were $111.5 \pm 14.7\%$ and $110.4 \pm 10.4\%$. Both UPF peptides induced statistically non-significant elevation of MnSOD activity (Figure 5). MnSOD was less activated when higher concentrations were used, for example UPF17 at 5 nmol/mg of protein had no activating effect at all (data for 1 and 5 nmol/mg of protein UPF peptides not shown).

### *GPx and MnSOD expressions*

Examining the effect of UPF1 and UPF17 on the expression of antioxidant enzymes, we found that these glutathione analogues have no statistically significant effect on GPx and MnSOD expression in human mononuclear blood cells (Figures 6 and 7).

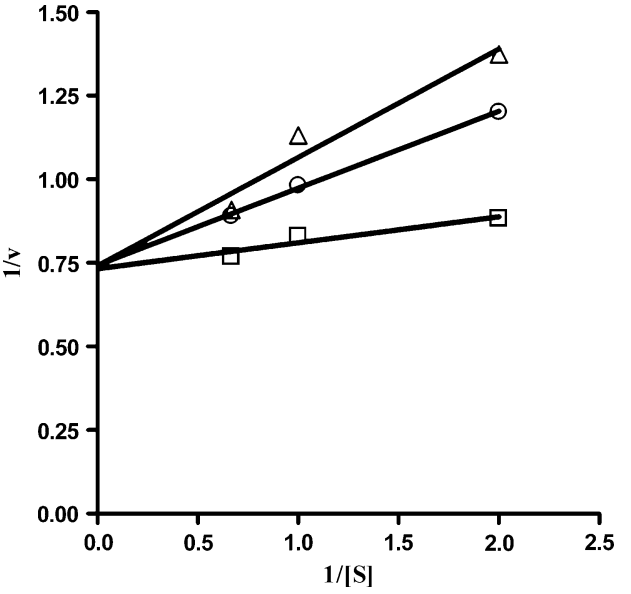


Figure 2. Inhibition of GPx activity by UPF17 using GSH as substrate. The concentration of GSH was varied from 0.5–1.5 mM. (□) Enzyme alone; (○) 0.1 mM UPF17; (△) 0.15 mM UPF17. The graph is a example of typical experiment of GPx inhibition.

Concentration of UPF peptides was 0.5 μM and incubation time was 12 h.

### *Effects of UPF peptides on superoxide radical production by NAD(P)H oxidase in isolated human PMNs*

UPF peptides were added to the separated PMNs at concentrations of 10 and 100 μM. All tested peptides (GSH, UPF1, UPF17) showed a statistically significant inhibitory effect against PDBu stimulated oxidative burst by neutrophil NAD(P)H oxidase. PDBu stimulated signal in absence of peptides was considered to be 100%. Incubation with 10 μM GSH, UPF1 and UPF17 decreased the signal to $52.9 \pm 4.7\%$, $40.8 \pm 4.5\%$ and $50.2 \pm 3.8\%$, respectively; 100 μM peptide concentrations followed analogical differences in remaining activity ($19.3 \pm 5.1\%$, $13.5 \pm 2.5\%$, $18.1 \pm 2.9\%$), indicating that UPF1 is a slightly more potent NAD(P)H oxidase inhibitor than GSH or UPF17 (Figure 8). Change of $\gamma$-glutamyl moiety to $\alpha$-glutamyl moiety rather attenuated the inhibitory effect of UPF peptides (UPF1 compared to UPF17), but it was not statistically significant.

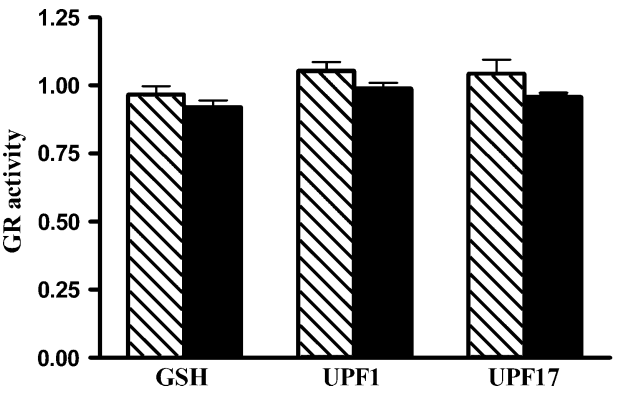


Figure 3. Activity of GR, measured with purified enzyme. Effects of 1 mM GSH, UPF1 and UPF17 on GR activity. Pre-incubation time is 5 min (shaded bars) and 24 h (black bars). 1.0 corresponds to the basal GR activity. Values are expressed as mean ± SEM, $n = 6$, $p > 0.05$.

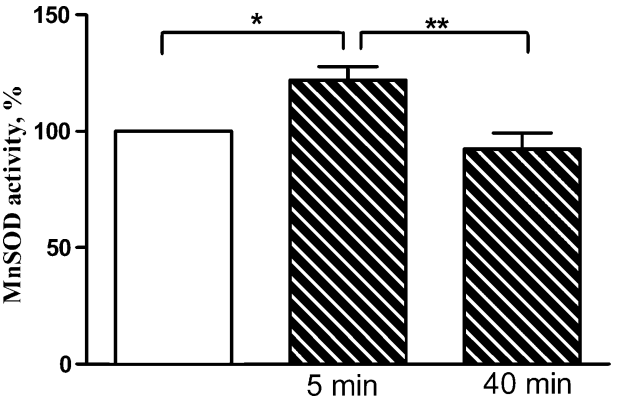


Figure 4. Effect of 10 nmol/mg protein GSH (shaded bars) on MnSOD activity of *post mortem* human brain *in vitro* at different pre-incubation times. 100% corresponds to the basal MnSOD activity (open bar). Values are expressed as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, $n = 6$.

## Discussion

One of the aims of this work was to clarify that antioxidative UPF peptides do not attenuate the natural enzymatic defence against oxidative stress. We also used higher, non-pharmacological concentrations of UPF peptides to better investigate the structure-related effects. The additional purpose was to explain the protective mechanisms of UPF peptides, shown in previous *in vivo* experiments with UPF [26,27].

At low UPF peptides concentrations (up to 50 μM), similar to those used in *in vivo* experiments, UPF peptides did not inhibit GPx activity. At higher concentrations, both UPF peptides inhibited GPx activity concentration-dependently, whereas the α-peptide bond containing UPF17 had the strongest inhibitory effect on GPx activity, which can be caused by a more available carboxylic acid group at glutamate residue. *In silica* modelling is further needed to study spatial rearrangements at the enzyme. In the case of purified enzyme the inhibitory effects of UPF peptides

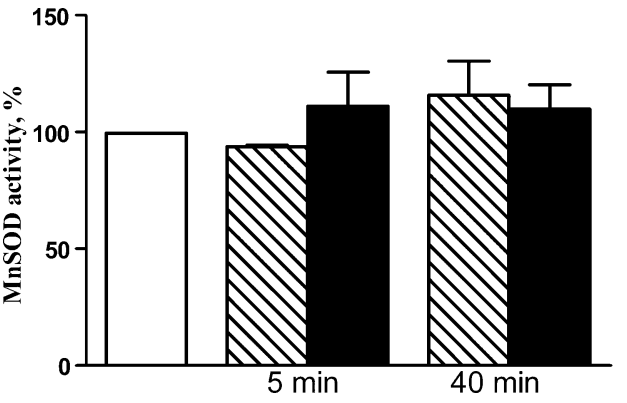


Figure 5. Effects of 0.5 nmol/mg protein UPF1 (shaded bars) and UPF17 (black bars) on MnSOD activity of *post mortem* human brain *in vitro* at different pre-incubation times. 100% corresponds to the basal MnSOD activity (open bar). Values are expressed as mean ± SEM, $n = 6$.

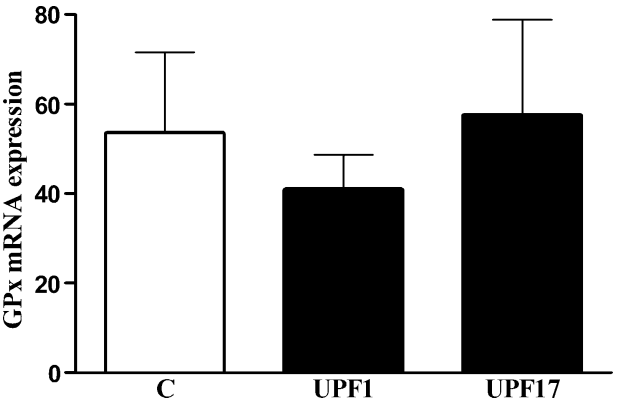


Figure 6. Influence of UPF1 and UPF17 on GPx expression compared to control in human blood monocytes. Values are expressed as mean ± SEM, $n = 4$, $p > 0.05$.

were more expressed than in the erythrocyte lysate and this tendency is more pronounced at lower concentrations. This could be explained with an additional number of interfering components in the erythrocyte lysate reducing the acting concentration of the peptide. As the measurement of GPx activity comprises two reactions—creating GSSG by GPx and further conversion of GSSG by GR, which depletes NADPH, and leads to signal decrease, the exact cause of signal decrease due to UPF peptides must be further investigated. The results shown on the Lineweaver-Burk plot can be caused by the competitive reaction of UPF17 with enzyme.

UPF peptides had no influence on GR activity, which means they do not disturb the conversion of GSSG back to GSH. It also made it possible to measure GPx activity with the indirect method described above. Still, there is the question of whether the dimeric form of UPF peptides or their heterodimer with GSH could be a substrate for GR. The relevance of this information is as follows: can the UPF peptides be recycled back to monomeric form, which supposedly carries the bioactivity. Previously, the dimerization of UPF peptides and formation of heterodimer with GSH has been

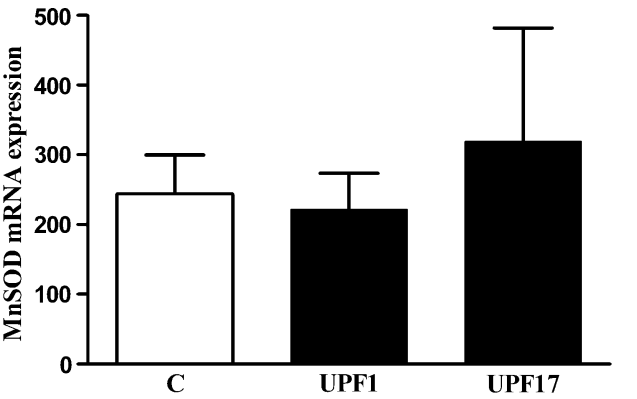


Figure 7. Influence of UPF1 and UPF17 on MnSOD expression compared to control in human blood monocytes. Values are expressed as mean ± SEM, $n = 4$, $p > 0.05$.

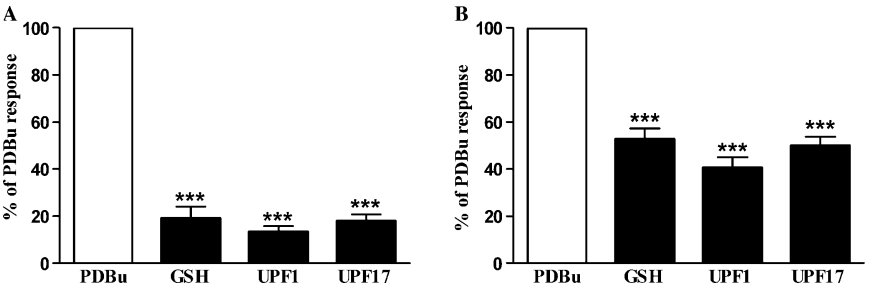


Figure 8. PDBu induced neutrophil NAD(P)H oxidase activity after incubation with GSH, UPF1 and UPF17 on concentrations of 100 μM (A) and 10 μM (B). Incubation with PDBu alone corresponds to the maximal superoxide radical production (100%). Values are expressed as mean ± SEM, $n = 4$. Significance of difference compared to controls, *** $p < 0.001$.

followed by us using capillary electrophoresis and MALDI-TOF mass spectrometry [34,35]. We have shown that the appearance of dimeric form of UPF1 was observed after 2 days in physiological solution and after 4 h in phosphate buffer [35]. These results indicate that spontaneous dimerization of UPF peptides is too slow a process to influence the results of GPx and GR measurements.

MnSOD in brain mitochondria is less investigated than its isoenzyme in the kidneys and liver. A study performed by Matsuda et al. [36] indicated that human liver MnSOD is composed of four identical subunit in which one cysteine (Cys196) is readily reactive towards thiols whereas the other (Cys140) is hidden inside the molecule. In light of these results, some predictions about the UPF peptides and brain MnSOD interaction mechanisms can be given. Incubation of the mitochondrial fraction with 10 nmol/mg GSH gives a statistically significant increase in MnSOD activity after 5 min. After 40 min, the MnSOD activity was equal to basal again. Speculatively, this could be explained by cleavage or by leaving GSH from the activating site after a longer time of incubation. UPF1 and UPF17 also showed a tendency of MnSOD activation, whereas the lowest used concentration (0.5 nmol/mg of protein) gave the best activating effect. This may be a result of different affinities of thiol groups in MnSOD. Higher concentrations may react with additional thiol groups that change the conformation and through it, the activity of the enzyme. An alternative explanation to the loss of activity in 40 min incubation with GSH may also be the circumstance that this time is sufficient for reacting with additional thiol groups, which leads to enzyme inhibition. It seems that γ-glutamyl moiety containing UPF1 needed a longer time for MnSOD activation, whereas α-glutamyl moiety containing UPF17 exerted its influence already on the 5$^{th}$ min, but distinctly from glutathione, MnSOD activation by UPF peptides was not statistically significant.

In general, stimulation of antioxidant enzymes should be considered as a positive effect for a potential antioxidative protector molecule. At the same time, it must be considered that antioxidative defence is cooperation of several systems. Superoxide dismutases catalyse the dismutation of superoxide radical into hydrogen peroxide, but if hydrogen peroxide is not further eliminated by GPx or by catalase, it could be a source of oxidative stress. The hypothesis that increasing the concentration of SOD alone might enhance oxidative stress resistance is disconfirmed by several studies [37,38]. In light of these results, it is advantageous that UPF peptides do not alter the expression level of MnSOD in the human blood monocytes and had only a minimal stimulating effect on MnSOD activity in the isolated mitochondria of human brain temporal cortex. However, increased MnSOD gene expression induced by small molecular weigh thiol has been shown before in lung tissue by N-acetylcysteine [39].

S-thiolation is accompanied with stimulation of respiratory burst and is correlated with respiratory burst intensity [40]. Many NAD(P)H oxidase inhibitors mediate their influence through the conjugation with thiol groups essential for NAD(P)H oxidase activation, for review see Cross [41]. This led to the thought that NAD(P)H oxidase inhibition by UPF peptides could also be mediated by bounding the thiol groups vicinal for the enzyme activation. Controversially, research has shown that binding of thiol groups does not always lead to an inhibition of NAD(P)H oxidase. Moriguchi et al. [42] induced S-thiolation by pre-treating the neutrophils with a direct thiol oxidizing compound diamide and showed the enhanced release of superoxide anion by NAD(P)H oxidase after binding the thiol groups. Different effects of thiol binding could be explained by the exact location of the modification and previous redox status of the enzyme. It is generally recognized that thiol oxidants inhibit phagocytic NAD(P)H oxidase, but in the case of vascular enzyme it depends on used thiol and NADPH- or NADH-driven signal. For example, thiol group containing glutathione precursor N-acetylcysteine had been shown to inhibit vascular NAD(P)H oxidase when used alone, but has some recovery effect on enzyme activity when used together with other NAD(P)H oxidase inhibitors [43]. The results obtained with UPF peptides from experiments with neutrophil NAD(P)H oxidase can not be automatically expanded to the vascular

enzyme, although these isoenzymes share several common inhibitors. Widely accepted NAD(P)H oxidase inhibitor apocynin inhibits NAD(P)H oxidase in phagocytic cells, but increases reactive oxygen species generation in vascular tissues because it requires conversion into active form [44]. Comparing structural similarities, UPF1 and apocynin contain both methoxy moiety in aromatic structure. It is not known whether the UPF peptides are able to penetrate through the neutrophil membrane, hence at the moment more exact location of binding can not be predicted. Thiolation of free sulphydryl groups by UPF peptides can still be questioned as a potential inhibition mechanism. Previously we have excluded that UPF peptides could be potential non-specific superoxide radical scavengers (data not published). Generating superoxide via xanthine/xanthine oxidase reaction, UPF peptides failed to significantly decrease the superoxide radical production. It confirms that the signal decreases after the incubation of PMNs with UPF peptides is caused by interaction with NAD(P)H oxidase.

The enzyme free hydroxyl radical scavenging assay showed that substitution of $\gamma$-glutamyl moiety (UPF1) with $\alpha$-glutamyl moiety (UPF17) improved hydroxyl radical scavenging activity $\sim$ 500-fold [25]. The results of the current investigation suggest that this modification gives no considerable difference in influencing GR, MnSOD and NAD(P)H oxidase activities. Statistical difference between effects of UPF1 and UPF17 was found only in the case of GPx inhibition.

The results obtained from current research confirm that investigated UPF peptides can be used as themselves or as a lead for the protector molecule design against excessive oxidative stress. They do not influence principal antioxidative enzyme activities or shift their activity towards elevated protection. Moreover, UPF peptides suppress the activity of potentially pro-oxidative NAD(P)H oxidase.



## Acknowledgements

This work was supported by grants No. 6503, 6574, 6588, 7856 from the Estonian Science Foundation, by targeted financing PARBK 0906 from University of Tartu, by targeted financing from Ministry of Education and Science of Estonia (SF0180105s08) and by European Union through the European Regional Development Fund.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 7 May 2009.