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Regular paper

UV radiation in HCT 116 cells influences intracellular H_2O_2 and glutathione levels, antioxidant expression, and protein glutathionylation

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UV radiation influences cellular levels of hydrogen peroxide (H_2O_2) and glutathione (GSH) and alters the expression of antioxidant genes in the human colorectal cancer cell line HCT116. In this study, cells were irradiated with UV light of different wavelength (A, B, or C). A surge in H_2O_2 concentration and total glutathione (level occurred 6 hours later. Consequently, protein glutathionylation increased above control levels. Expression of the antioxidant enzymes: glutathione peroxidase (GPX) and glutathione reductase (GSR), assessed by real-time quantitative PCR, increased by 1.5–2 times after 24 hours post-irradiation, in comparison to the untreated controls. Glutathionylation of proteins was enhanced after UV radiation and the set of biotinylated glutathione ethyl ester (BioGEE) tagged proteins was detected by Western Blot procedure. This specific glutathione analogue is conjugated with antioxidant proteins during glutathionylation especially under oxidized conditions in cells. A pool of glutathionylated proteins in the treated cells showed peculiar characteristics. These proteins exhibited varying molecular weights. For UVA-irradiated cells, 24 hours after the treatment we observed two additional ~60 and ~72 kDa bands of glutathionylated proteins from NADPH oxidases (NOX family). Total glutathione level in the UV-irradiated HCT116 cells was higher than in the control. This correlates with the detection of glutathionylation in UV-irradiated cells in the first and twelfth hour of post-irradiation, and can be defined as a specific antioxidant element activation for cellular protection.

Key words: ROS, glutathione, HCT116, GSR, BioGEE

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Abbreviations: BioGEE, biotinylated glutathione ethyl ester; GPX, glutathione peroxidase; GSH, glutathion; GSR, glutathione reductase; GSSG, disulfid glutationu; HCT116, clorectal cancer; HRP, horseradish peroxidase; NADPH, reduced form nicotinamide adenine dinucleotide; NO, nitric oxide; NOX, oxidase NADPH; $O_2^{\bullet-}$, superoxide anion; PCR, polymerase chain reaction; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species

INTRODUCTION

Oxidative stress is one of the external factors that influence human health (Cieślar-Pobuda *et al.*, 2017). Maintaining a similar level between the oxidation and reduction processes in tissues and cells is called a redox equilibrium (Buldak *et al.*, 2015). Intracellular redox potential affects cellular functions and its dysregulation is associated with disease (Finkel, 2011; Skonieczna *et al.*, 2017).

Reactive oxygen species (ROS) are radical or non-radical forms of oxygen, which are characterized by high reactivity. In healthy cells, ROS participate in many cellular processes like hormone secretion, drug removal, detoxification, and stimulation of the immune system, and their concentration is physiological. ROS are mainly produced by mitochondrial complexes. The superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are the most common ROS (Puzanowska-Tarasiewicz *et al.*, 2010; Finkel, 2011; Buldak *et al.*, 2013). Overproduction of ROS may lead to oxidative stress, which may result in permanent changes in cells, leading to loss of function of proteins, which in turn can cause disease (Kowalska, 2008; Buldak *et al.*, 2013).

Oxidative stress is the result of an imbalance between ROS and antioxidant activity (Rzeszowska-Wolny *et al.*, 2009). UV radiation is classified as one of the factors causing oxidative stress (Krzywon *et al.*, 2018). It disrupts the redox balance, resulting in irreversible changes in the cell, and causing severe disturbances within the entire metabolic pathways (Widel *et al.*, 2014). Such UV-induced oxidative stress and imbalance may lead to diseases such as diabetes, atherosclerosis, or cancer (Valko *et al.*, 2005; Karpińska & Gromadzka, 2013; Kasperczyk *et al.*, 2013; Buttke & Sandstrom, 2016; Widel, 2016).

Antioxidants are substances that inhibit the oxidation of molecules and cause the conversion of radicals into inactive derivatives (Schmatz *et al.*, 2012; Azqueta *et al.*, 2013; Modrzejewska *et al.*, 2016). Their main task is to protect the cell against oxidative stress and maintain the optimal redox state (Schafer & Buettner, 2001; Czajka, 2006; Kowalska, 2008). Among the main antioxidants that form the antioxidant barrier are glutathione and glutathione peroxidase (Czajka, 2006). Glutathione is a substrate for the S-glutathionylation reaction, which protects proteins from the irreversible effects of oxidation (Peskin *et al.*, 2016). There are two forms of glutathione in the cell: reduced (GSH, about 90%) and oxidized (GSSG, about 10%) (Gómez-Cambronero, 2000). Glutathionylation is a reversible reaction in which glutathione attach-

es to cysteine residues of proteins *via* a disulfide bond (Gómez-Combronero, 2000; Żmijewski *et al*., 2009). Glutathione reductase (GSR) is involved in maintaining the balance between the oxidized and reduced glutathione. The expression of GSR increases in cells with an excess of GSSG (Bilska *et al*., 2007). GSR is an NADPH-dependent enzyme of cytosolic origin that catalyzes the reduction reaction of GSSG to two reduced GSH molecules (Formula 1) (Bilska *et al*., 2007; Zhu *et al*., 2018).

$$NADPH + H^+ + GSSG \xrightarrow{GSR} NADP^+ + 2GSH$$

Formula 1. Reaction of reduction of oxidized form of glutathione GSSG to GSH with the participation of GSR.

Another important antioxidant is glutathione peroxidase (GPX), an enzyme that catalyzes the reduction of hydrogen peroxide to water using reduced glutathione as a substrate (Formula 2). GPX plays a protective role against oxidative stress in the cells (Łukaszewicz-Hussain, 2003).

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

Formula 2. Hydrogen peroxide reduction reaction involving glutathione.

UV radiation, which causes oxidative stress, has a wavelength range of approx. 100 to 400 nm and is not visible to humans. UV radiation is classified as UVA (400–329nm), UVB (320–290nm) or UVC (<290nm) (Diffey, 2002; Grimes, 2015). 90% of the solar UVA radiation reaches the Earth surface. It is the mildest form of UV radiation, yet, it can still penetrate deep into the skin. UVB can penetrate through the outer layer of the epidermis, however, most of the solar UVB is blocked in the atmosphere and only 10% of the radiation reaches the Earth surface. Out of the three types of UV radiation, UVC is the most harmful because at the wavelength range (<290 nm) it can be absorbed by RNA and DNA (245–290 nm) (Soehnge *et al*., 1997; Skórska, 2016), yet it is also mostly blocked in the Earth atmosphere. In the presented study, we investigated the impact of UV radiation on human HCT116 cancer cells, *via* the ROS-induced glutathione target and measurement of its recovery potential.

MATERIALS AND METHODS

Cell cultures. The colorectal cancer cell line, HCT116 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM-F12 medium (PAA, Poland) supplemented with 10% FBS (EURx, Poland) and Antibiotic-Antimycotic Solution (100×; Sigma-Aldrich, Germany). All cultures were carried out in 75 ml flasks (Sarstedt, Germany) under standard conditions (37°C and 5% CO_2, at 80% humidity). Twenty-four hours before irradiation, the cells were seeded in 6-well plates (Sarstedt, Germany) at 1×10^5 cells/well in 2 ml of fresh medium. UV irradiation was carried out using UV crosslinkers (model CL-1000, UVP, Upland, CA, USA) with the following settings: UVA (365 nm), UVB (302 nm), and UVC (254 nm) at doses of 10 kJ/m^2, 5 kJ/m^2 and 100 J/m^2, respectively. After irradiation, cells were collected at five time points (1, 3, 6, 12, and 24 h) and either frozen at –20ºC for further tests (PCR, Western Blot), or stained directly with specific dyes for fluorimetric or cytometric assays.

Intracellular ROS levels. ROS levels were measured by flow cytometry using the specific H_2O_2-detecting dye 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Thermo Scientific), a cell-permeable non-fluorescent probe, which, when oxidized by ROS, converts into a highly green-fluorescent form thanks to removal of its acetate groups by intracellular esterases. Three hundred µl of cell suspension in 1×PBS (prepared in triplicates) were stained with carboxy-H2DCF-DA (30 µM). After incubation in darkness (30 min at 37°C), the cells were washed with PBS and kept on ice for 15 minutes. Then, the fluorescence was measured for at least 10000 cells using a flow cytometer Aria III Beckton Dickinson with the FITC configuration (488nm excitation; emission: LP mirror 503, BP filter 530/30). The results from three experiments with three technical repeats each were analyzed using the FlowingSoftware 2.5.0 (Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland) and presented as mean fluorescence.

RNA Isolation. Cells were collected from plates and suspended in Fenozol (203–100, A&A Biotechnology, Gdynia, Poland). RNA was isolated using a total RNA isolation kit (A&A Biotechnology, Gdynia, Poland) following the producer's protocol. RNA purity and concentration were measured using NanoDrop 2000 (Thermo Fisher Scientific). Next, reverse transcription was performed using a dART RT kit (EURx, Gdansk, Poland) to produce cDNA.

Quantitative RT-PCR. The obtained cDNA was used for RT-qPCR with Real-Time 2xPCR Master Mix SYBR A (A&A Biotechnology). The reaction profile was set as follows: after 2 min of cDNA synthesis at 50°C and 4 min of denaturation at 94°C, samples were subjected to 54 cycles of amplification, consisting of 45s at 94°C, 30s at 52,3°C, and 5 min at 72°C, with a final additional extension step with growth temperature from 53°C to 72°C. the readout of results was performed using CFX Manager 3.1 program (Bio-Rad). The RPL41 gene was used as a reference. The transcripts assayed and the sequences of the primers were: GSR gene: 5'AC-CCCGATGTATCACGCAG3' (forward); 5'TTCAT-CACACCCAA-GTCCCTG3' (reverse); GPX4 gene: 5'GCCTTCCCGTGTAACCAGT3' (forward); 5'GC-GAACTCTTTGA-TCTCTTCGT3' (reserve); RPL41 gene: 5'TCCTGCGTTGGGAT-TCCGTG3' (forward); 5'ACGGTG-CAACAAGCTAGCGG3' (reverse).

Results from qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001, Tyburski *et al*., 2008).

Assay of intracellular GSH. Cells were seeded in a 96-well plate (Sarstedt, Germany). Monochlorobimane (MCB; Sigma, Germany), which penetrates cells and reacts with GSH to form the fluorescent compound GSH-monochlorobimane (Webb *et al*., 2006), was added to the culture medium. The samples were incubated with the compound for 30 min, after which the fluorescence at 490 nm (ex 394 nm/em490 nm) was measured using a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

Quantitation of glutathionylated proteins. Cells were seeded in a 96-well plate. BioGEE (178 mM) was added to the culture medium, followed by streptavidin-FITC (Sigma S3762, 1:200 dilution) after 30 minutes of incubation. After 10 min the medium was aspirated, 50 µl of PBS was added to the cells, and fluorescence was measured at 535 nm (ex 485nm/em 535nm) on a plate reader (Infinite 200 PRO, Tecan).

Separation of glutathionylated proteins by SDS-PAGE and Western Blot One µl of BioGEE (Life Technologies), a biotinylated glutathione analogue (178 mM solution) was added to cells 30 minutes before harvesting, to saturate the cells with reagent. The cells were collected and resuspended in 1% SDS, and protein concentration was measured using Bradford reaction (Bio-Rad). Samples containing the same amount of protein (10 µg) were separated on polyacrylamide gradient gels (10%) together with molecular mass markers (Precision Plus Protein™ Dual Xtra Standards, Bio-Rad). Semi-dry transfer to a nitrocellulose membrane was carried out using the Trans-Blot® TurboTM system (Bio-Rad). The membrane was blocked with 5% milk solution (milk dissolved in distilled water) overnight, washed with PBS-Tween, incubated with Streptavidin-HRP conjugate (GE HealthCare) for 1, washed with PBS-Tween 3 times, 10 minutes each, at room temperature. Immunodetection was performed using ECL reagents from the Western Bright Quantum kit (Advansta, San Jose, CA, USA). After air-drying on the membrane, pictures were taken using a G: BOX chemiXX6 (Syngene, Frederick, MD, USA). GAPDH was detected with primary rabbit anti-human antibody (1:5000) followed by HRP-conjugated secondary mouse anti-rabbit antibody (1:10 000) (both from Santa Cruz).

Statistical analysis. Experimental data are presented as mean from at least three independent experiments, with three technical repeats each, ± standard deviation (± S.D.). Outliers were discarded using Dixon's Q test. Statistical significance was calculated using a *T*-test. The asterisk * in the figures denotes a *p*-value of 0.05 in a comparison of a sample to untreated control from first hour time point.

RESULTS

Intracellular level of reactive oxygen species

Hydrogen peroxide is one of the major reactive oxygen species in cells (Lennicke *et al.*, 2015; Sies, 2017; Jones & Sies, 2015). 1 hour after irradiation with UVA, the level of H_2O_2 was below the control level. Six hours after irradiation, a surge in H_2O_2 concentrations appeared, and this effect was seen in all the UV wavelength treatments. After 24 hours, H_2O_2 in the irradiated cells returned to the control levels (Fig. 1).

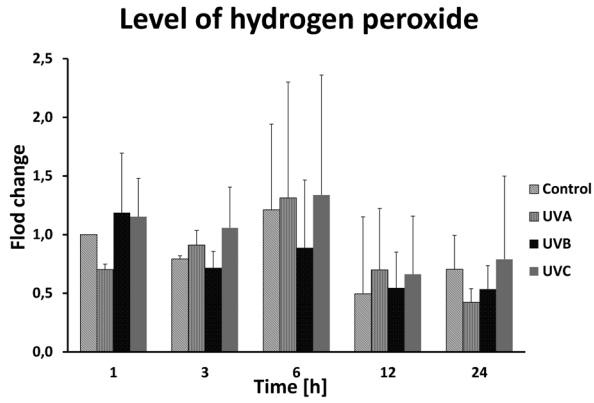

Figure 1. Intracellular H_2O_2 levels after UV irradiation, shown as fold-change relative to the untreated control from the first hour. Data shows mean ± S.D.

Expression levels of antioxidant genes

UVB irradiation caused a significant increase (p=0.05) in expression of GPX4 gene in HCT116 cells 24 h after the treatment. However, in UVC irradiated cells, the expression was down-regulated over the 24-hours of observations (beyond 6 h, Fig. 2A).

The level of *GSR* mRNA was down-regulated directly after UVB and UVC exposure until 12 h post-irradiation. UVA stimulated *GSR* expression but only at 6 and 24 hour post-irradiation time points (Fig. 2.B).

Level of intracellular glutathione

Intracellular glutathione levels were assayed using monochlorobimane, which is essentially nonfluorescent until conjugated with glutathione, or another low molecular weight thiols (Kamencic *et al.*, 2000; Webb *et al.*, 2006). The levels of reduced glutathione increased in irradiated cells at all measuring points up to 24 h after treatment (Fig. 3).

Protein glutathionylation process

Plate-reader fluorometric measurements

Incorporation of glutathione into proteins can be detected using biotinylated glutathione ethyl ester (BioGEE), a cell-permeant biotinylated glutathione ana-

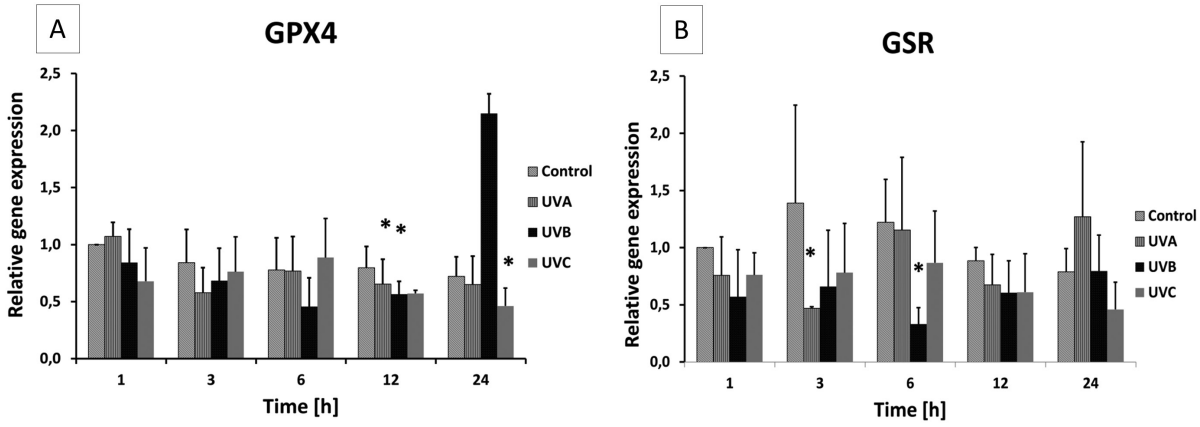

Figure 2. Relative levels of GPX4 (A) and GSR (B) mRNA in HCT116 cells after UV irradiation, fold-change relative to untreated control at 1h timepoint.
*Indicates statistical significance calculated with *T*-test in comparison to untreated control at 1 h timepoint, *p*-value <0.05.

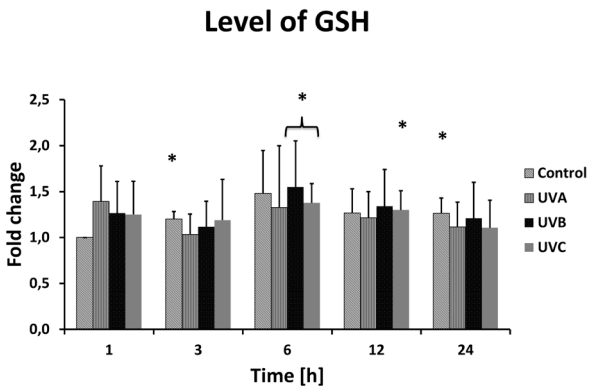

Figure 3. Levels of GSH after UV irradiation, fold-change relative to untreated control at 1h timepoint.
*Indicates statistical significance calculated with *T*-test in comparison to the untreated control at 1 h timepoint, *p*-value <0.05.

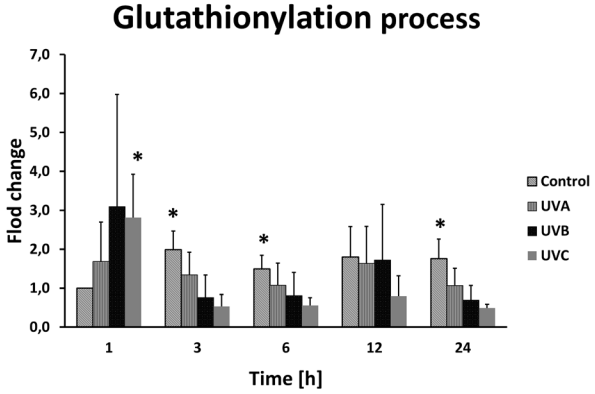

Figure 4. Level of glutathionylated (BioGEE tagged) proteins after UV irradiation, fold-change relative to untreated control at 1 h timepoint.
Measurements from plate reader (ex 485 nm/em 535 nm). *Indicated a statistical significance calculated with *T*-test in comparison to untreated control at 1 h timepoint, *p*-value <0.05.

log which is incorporated into proteins (García-Giménez *et al.*, 2013). Cells exposed to UVA, UVB and UVC responded with over-production of glutathionylated antioxidant proteins, which could be the first protective reaction against protein damage and oxidation (Fig. 4) (Yang *et al.*, 2010). The highest level of glutathionylation occurred in the first hour after irradiation. After that time, the level of glutathionylated proteins oscillated around the control level (Fig. 4). The activation of GSH at 1 hour (Fig. 3), together with a glutathionylation process (Fig. 4), protected the proteins from the irreversible effects of oxidation. The glutathionylation increased for a second time at 12 h, in correlation with an increase in GSH.

Western Blot analysis

Total cell proteins conjugated with biotinylated-BioGEE were detected with Western Blot following SDS-PAGE (Fig. 5). In control HCT116 cells a set of

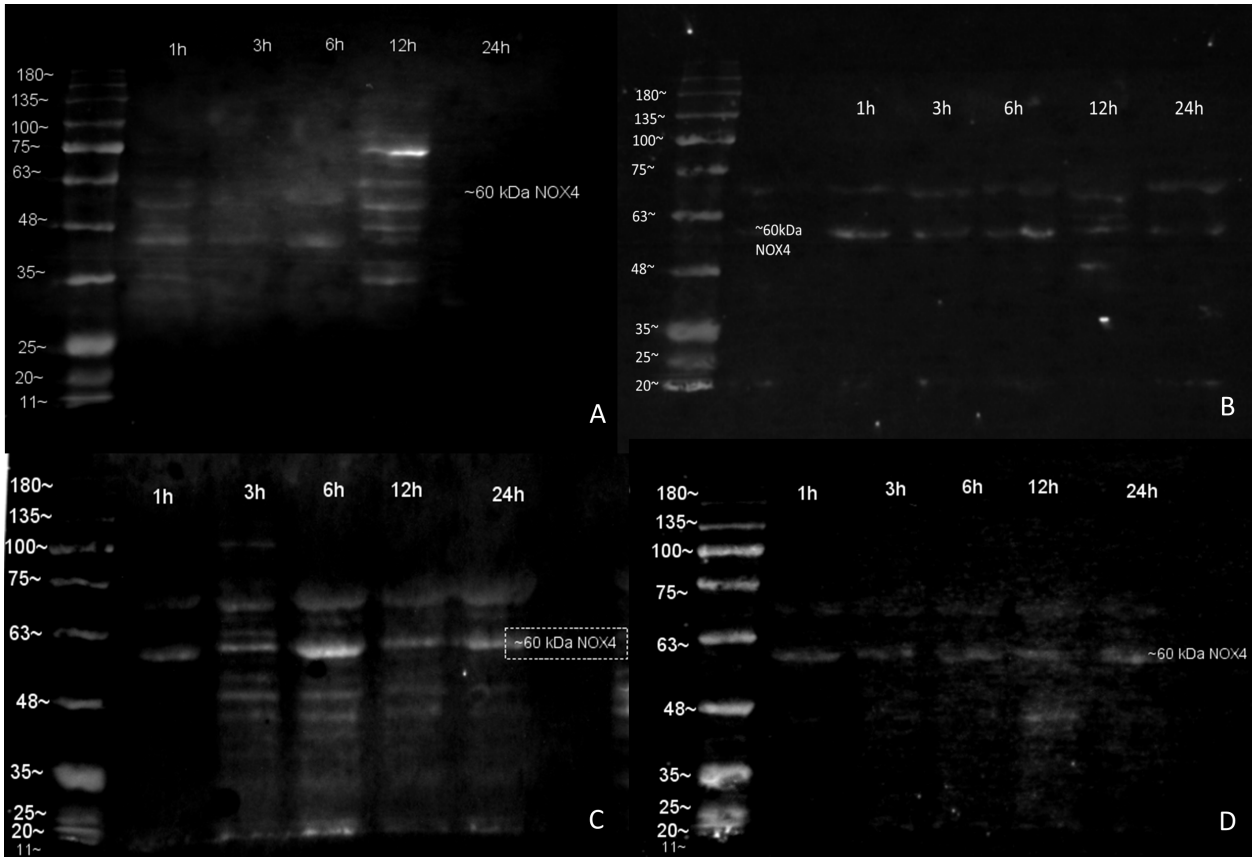

Figure 5. Glutathionylated proteins in control HCT116 cells (A), UVA-irradiated (B), UVB-irradiated (C) and UVC-irradiated (D) at time points (1–24 h).
Ten µg of protein were loaded in each lane. Left lanes show mass marker ladder (Bio-Rad).

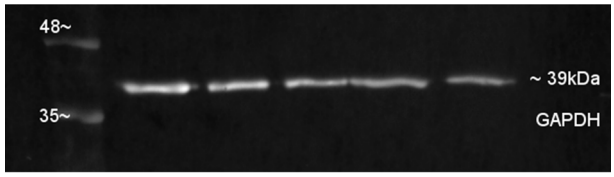

Figure 6. Western blot showing signals detected for reference GAPDH protein (39 kDa) from the membrane for UVA-irradiated HCT116 cells at given time points (1–24 h) after standard stripping procedure.
Membrane signals for reference from control, UVB- and UVC-irradiated HCT116 cells were similar (not shown).

glutathionylated proteins was detected, especially noticeable for the first 12 h, with a small amount of proteins detected at the 24th hour of observation (Fig. 5A).

In the case of UVA-irradiated cells (Fig. 5B), a similar amount of proteins was glutathionylated at each of the time-points of our observation, except for 12h post-irradiation, where more bands could be seen. The glutathionylation process was specific to proteins with characteristic molecular weight: 60 kDa and 72 kDa, relating to NOX oxidases, which was confirmed using antibodies against NOX-4 (not shown).

UVB radiation resulted in an increase in the number of different glutathionylated proteins at 3, 6 and 24 hours after irradiation (Fig. 5C). The glutathionylated proteins had different molecular weights, which became clearly visible at the timepoints of 3+ hours and was still noticeable after 24 hours. In control cells, at the same 24 h timepoint, almost no glutathionylated proteins were detected (Fig. 5A).

UVC exposure results were similar to UVA (Fig. 5B). Signals for glutathionylated proteins in each lane for all time points were similar, and showed specific glutathionylation for ~60 and ~72 kDa sized NOX proteins (Fig. 5D). These results may indicate the same profile of glutathionylation for analogous proteins (the same mass and bands from UVA and UVB membranes). However, at 12 h timepoint, additional proteins of a lower mass were observed. GAPDH (Fig. 6) was made as a reference to check that the same amount of protein was applied.

DISCUSSION

Cells possess natural protective mechanisms against oxidation and rapid damage of proteins, DNA, lipids and other macromolecules (Jones & Sies, 2015). The natural "shield" consists of antioxidants, responsible for protecting the cells from action of free radicals (Modrzejewska *et al*., 2016; Dziaman *et al*., 2018). The most important antioxidant found in the cells is glutathione, which protects proteins against the irreversible oxidation (Peskin *et al*., 2016). The mechanism of action for GSH is to participate in the reversible S-glutathionylation reaction, which creates disulfide bonds between protein's cysteines and forms glutathione and a protected protein (Drozd *et al*., 2016). Measurements of hydrogen peroxide levels in cells after UV exposure (Fig. 1) showed a significant increase of this radical at the sixth hour after irradiation. The response to the increased H_2O_2 was prevalent at twelve hours post-irradiation. We observed higher levels of glutathionylated proteins (Fig. 5) and an increase in total glutathione (GSH and GSSG) in the cells (Fig. 2). After the expected reduction of hydrogen peroxide to water within the next measured timepoint (24h), we noticed an increase in the expression of glutathione reductase (GSR) and glutathione peroxidase (GPX) (Ciesielska *et al*., 2019). These enzymes are responsible for the reduction of glutathionylation products. GSR restores the level of reduced glutathione (GSH), and in the cell more than 90% of the GSH pool comes from the reduction of its oxidized form (GSSG) (Sabens Liedhegner *et al*., 2012; Mieyal & Chock 2012). In contrast, GPX reduces glutathione attached to proteins. Analysis of glutathione-related products after UV exposure showed the presence of NOX-family proteins, which were involved in the glutathione target that provided protection to HCT116 cells against oxidative stress. The reaction of glutathionylation is reversible, which allows the reduced/oxidized pool of glutathione to remain balanced *via* activation/deactivation of antioxidative enzymes – crucial players in protective feedback loops: glutathione reductases and glutathione peroxidases (Sullivan *et al*., 2002; Yang *et al*., 2010; Mailloux *et al*., 2011; Mailloux *et al*., 2013). Expression levels for both enzymes were measured on mRNA. Additionally, a pool of reduced glutathione, together with glutathionylation process were estimated on the post-translational process. Obtained results showed important roles of all the investigated elements in UV-induced oxidative stress prevention in HCT116 cancer cells. These findings can be used further research aimed at weakening the antioxidative barrier in cancer cells in future UV-based therapies.

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