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INVOLVEMENT OF PDIA3 IN OXIDATIVE STRESS RESPONSE

Diploma thesis

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Abstrakt

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Název diplomové práce: Involvement of PDIA3 in oxidative stress response

PDIA3 je členem rodiny protein disulfid isomeráz (PDI) a jedná se o protein, uplatňující se při odpovědi organismu na stres. Dále je zapojen do různých buněčných signálních drah a má v buňce nejrůznější funkce. Jeho nejznámější role je v endoplazmatickém retikulu, kde plní funkci hlavně při skládání a kontrole kvality glykoproteinů. Nicméně jeho existence byla popsána také v mnoha jiných buněčných kompartmentech, například v jádře, mitochondriích, na buněčném povrchu nebo v cytosolu, kde zasahuje do nejrůznějších procesů (fúze spermie s vajíčkem, negenomická odpověď na vitamín D3, příjem vápníku buňkou,transkripční faktor v jádře atd.). Zatímco v některých případech musí být mechanismus jeho působení potvrzen dalšími studiemi, četná pozorování potvrzují jeho účast v přenosu různých signálů z buněčného povrchu (např. kooperce se STAT proteiny) a v regulačních procesech v jádře. V nedávných studiích byla také potvrzena jeho zvýšená exprese při různých patologických procesech.

Cílem naší práce bylo zjistit, jaká je úloha PDIA3 proteinu při vystavení buňky stresu, a to v buněčných liniích MDA-MB 468 a MCF-7. Obě linie jsme po vypočítání optimální koncentrace vzhledem k mortalitě, vystavily oxidačnímu stresu v podobě tert-butyl hydroperoxidu (tBOOH) a poté sledovali pomoci metody Western blot expresi PDIA3 proteinu v časových intervalech 3, 6 a 24 hodin spolu s kontrolním vzorkem. V dalším experimentu jsme buňky před vystavením stresu ošetřili 17ß-estradiolem, protože se předpokládá, že rozdílné hladiny exprese proteinu v obou buněčných liniích po vystavení stresu souvisí právě s tím, zda jsou buňky 17β-estradiol receptor (ERec) pozitivní nebo negativní (MDA-MB 468 jsou ERec negativní, MCF-7 jsou ERec pozitivní). Naše studie tedy rozšiřuje poznatky o proteinu PDIA3 a osvětluje procesy při stresové odpovědi v buněčných liniích MDA-MB 468 a MCF-7. Zatímco exprese proteinu u buněčné linie MCF-7 se po vystavení tBOOH se téměř neliší, u MDA-MB

468 dochází k významným změnám. Rozdíl lze pozorovat také po předchozím ošetření buněk 17 β-estradiolem.

Abstract

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Title of diploma thesis: Involvement of PDIA3 in oxidative stress response

PDIA3 is a member of the protein disulfide isomerase family (PDI) and it is a stress-responsive protein. It is also involved in various cellular signalling pathways and has various functions in the cell. The best-known location is in the endoplasmic reticulum where it plays a major role mainly in the proper folding and quality control of glycoproteins, and participation in the assembly of the major histocompatibility complex class I. However, its existence has also been described in many other cell compartments, such as nucleus, mitochondria, cell surface or cytosol, where it interferes in various processes. While in some instances these roles need to be confirmed by further studies, a lot of observations confirmed its involvement in the signal transduction (for example releated with STAT protein) from the cell surface and the regulatory processes in the nucleus. Recent studies have also confirmed its increased expression in various pathological states.

The aim of our work was to find out what is its role in the exposure of the MDA-MB 468 and MCF-7 cell lines to stress. After calculating the optimal concentration, these cells were exposed to stress in the form of tert-butyl hydroperoxide and we observed the expression of PDIA3 protein after 3, 6 and 24 hour intervals along with the control sample. In the next experiment cells were pre-treated with 17β-estradiol before stress exposure as it is assumed that different levels of protein expression in both cell lines after exposure to stress depend on whether the cells are 17β-estradiol receptor positive or negative (MDA-MB 468 are ERec negative and MCF-7 are ERec positive). Our study therefore extends the knowledge of PDIA3, illuminating the stress response processes in the MDA-MB 468 and MCF-7 cell lines. While the expression of the protein in the MCF-7 cell line is almost unchanged after treatment with tBOOH, the MDA-MB 468 changes significantly. The difference can also be observed after the pre-treatment of cells by 17β-estradiol.

Content

1 List of Abbreviations

ATRA – all-trans retinoic acid

ADP – adenosine diphosphate

ALS – amyotrophic lateral sclerosis

BCIP – 5-bromo-4-chloro-3-indolyl-phosphate

BSA – bovine serum albumin

CLNX – calnexin

CRT – calreticulin

DTT – dithiothreitol

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptorER – endoplasmic reticulum

ERec – estrogen receptor

Gpx – glutathione peroxidase

GRP58 – glucose-regulated protein 58

GSH – glutathione

HSP – heat shock proteins

LDL – low density lipoprotein

MCU – mitochondrial calcium uniporter

MHC I – major histocompatibility complex I

MOMP – mitochondrial outer membrane permeabilization

(mTOR) – mammalian target of rapamycin

NADH – nicotinamide adenindinucleotide

NF-κB – nuclear kappa beta factor

PERK – protein kinase R (PKR)-like endoplasmic reticulum kinase

PDI – protein disulphide isomerase

PKC – protein kinase C

PLC – phospholipase C

PRP – prion protein

RARα – retinoic acid receptor

ROS – reactive oxygen species

URP – unfolded protein response

SDS – sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SOD – superoxide dismutase

TGF-B – transforming growth factor beta

TAP – transporter associated with antigen processing

t-BOOH – tert-butyl hydroperoxide

2 Introduction

Each living system exists under precisely defined physiological conditions. Negative disturbance of these conditions affects an organism that subsequently enters into a state called cellular stress response. The cells use among other things in this process stress proteins which protect cells from damage. The cells vary in the content of these proteins, collectively called the cellular proteome. One of these proteins, protein disulfide-isomerase A3 (PDIA3), is the subject of this study. PDIA3 protein plays a role in many processes, both physiological and pathological. The fact that PDIA3 plays a role in cellular stress has been confirmed by several studies, however, the information is incomplete and further research is needed. The aim is therefore to raise awareness of this protein and its role in cellular stress as this information could potentially be used in the treatment of certain illnesses, for example cancer.

3 Theoretical part

3.1 Cell stress

The existence of every living system is possible only in precisely defined environmental conditions, both physical and chemical conditions. If those factors are suitable for living systems, we call them physiological conditions. As soon as the value of any factor is out of the physiological range, the system will be adversely affected or eventually destroyed.

All the external influences capable of damaging life processes in multicellular organisms influence almost always primary cells or cellular interactions. The knowledge of the mechanism of action is the basis for the cell pathology and pathology in general as it allows us to understand the origin of a disease, its course, treatment and it is a base of any causal therapy. Understanding the mechanisms by which the factors affect the cell also helps us to grasp the current processes in the cell.

3.1.1 Cellular response to the stress

The stress factors (stressors) can be physical (temperature, pH, radiation etc.), chemical (chemical substances) or biological (viruses, parasites etc.). Their mechanism of action can be both specific or non-specific. An example of non-specific mechanism can be high temperature causing denaturation of the proteins. Heavy metals, acids, aldehydes and many other chemical substances act in the same way. They damage macromolecules (proteins, DNA) with no regard for the type of stress. Specific mechanism of action is defined by its ability to influence only some particular structure or function of the cell. It can be provided by enzymatic poisons, which can block the activity of a specific enzyme or microtubular toxins which bind to tubulin and block the polymerisation of microtubules. (*Nečas et al*., 2000). In this study, we focus mainly on stress caused by non-specific mechanism.

There are basically two ways in which the cells react to the stress. Strong stress leads to cellular death (necrosis or apoptosis). On the other hand, weaker stress can cause adaptation of the cell to it. It is a universal mechanism that cells respond to non-physiological conditions. The capacity of the cellular stress response is based on its proteome (set of proteins in particular cells) and it depends on the type of cell and species. (Dietmar Kültz, *et al.,* 2005)

3.1.2 Stress proteins

The key proteins involved in the cellular stress response are conserved in all kinds of organisms, including prokaryotes. This fact shows us that this mechanism is evolutionary thus very old. First, they have been discovered as proteins which are synthesized when a cell is exposed to high temperature, which explains why they are called heat shock proteins (Hsp). Later, it was found that similar proteins occur in response to any cellular stress. They play an important role in protein–protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. (Necas *et al.*, 2000). They are up-regulated not only at the mRNA level but also for example with posttranslational modifications. (Dietmar Kültz, *et al.,* 2005)

Protein disulphide-isomerase (PDI) superfamily can be also classified as a stress-responsive proteins. If stress disturbs the structure of proteins, PDIs can help correct protein conformation. This is achieved by their catalyzation of the formation and breakage of disulfide bonds between cysteine residues. In this study, we are focusing on one in particular, and this is the PDIA3.

3.1.3 Oxidative stress and ROS

Free radicals, also called reactive oxygen species (ROS), are defined as any molecular species which contain an unpaired electron in an atomic orbital which gives them extreme instability and high reactivity. They behave as oxidants and reductants because they can donate but also accept an electron from other molecules such as lipids, nucleic acids or proteins. On the other hand, the molecules that react with free radicals become unstable and search for an electron, triggering a "chain" mechanism (the initial reaction generates a second radical, which in turn can react with a second macromolecule to continue the chain reaction) (Lobo *et al*., 2010). This cascade of reactions can last from seconds to hours and can be stopped only by the presence of antioxidant molecules. The formation of free radicals is either a result of a physiological process in the human body or is caused by external conditions such as exposure to ionizing radiation, X-ray, ozone, cigarette smoking, industrial chemicals and air pollutants (Bagchi, *et al.,* 1998). Alteration of the redox potential of the cell is a major trigger of the cellular stress response. It leads to the activation of many cellular antioxidant systems in which it plays a role in compounds such as ascorbate, glutathione, thioredoxin, and various antioxidant enzymes that protect the DNA, proteins and lipids from damage.

The main source of ROS in cells, where they arise in a process called oxidative phosphorylation, are mitochondria. In this process, highly reactive superoxide anion is also created and then is detoxified to the H_2O_2 by manganese superoxide dismutase and then H_2O_2 is converted to water by catalase. H_2O_2 can also be converted to hydroxyl radical $OH^{\bullet}$. There are also other sources of ROS such as peroxisomes which generate H_2O_2 as a by-product of β-oxidation of fatty acids. Another important source of oxidants are phagocytes. They use the oxidants to protect the central nervous system against infestation by microorganisms (Carmelina Gemma *et al.,* 2007).

These changes can affect the activity of many protein structures and functional units such as enzymes, receptors and membrane transport. The outcome leads to an alteration of signal transduction mechanism, enzyme activity, heat stability, and proteolysis susceptibility (Lobo *et al.,* 2010).

Oxidative stress plays a role in many conditions and can cause many human diseases such as heart diseases, inflammatory conditions, certain cancers and the process of ageing. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases, and many others (Stefanis *et al.,* 1997).

3.1.4 Role of antioxidants

The organisms have developed an antioxidant defense system consisting of both enzymatic and non-enzymatic components. Both enzymatic and non-enzymatic antioxidants occur in the intracellular but also in extracellular environment (Frie *et al.,* 1988). Antioxidants are essential elements for the protection of molecules as they are able to inhibit or delay the oxidation of a substrate.

An antioxidant is a molecule, which is able to donate an electron to a free radical and therefore neutralizes it and reduces its capacity to damage. These antioxidants, through their scavenging property, can delay or inhibit cellular damage and are capable to stop the chain reaction induced by free radicals to prevent damage to macromolecules. Some of the

antioxidants are produced during usual metabolic processes in the body, such as glutathione and uric acid, and the others are provided by the diet (Shi *et al.,* 1999).

Even if there are numbers of diverse enzymes in the body that neutralize the free radicals, the major antioxidants are micronutrients such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene (Levine *et al.,* 1999). The human body is not able to produce those micronutrients and we must get them from our diet.

As mentioned above, the antioxidant defense system include antioxidant enzymes such as superoxide dismutase (SOD), superoxide reductase, catalase, glutathione peroxidase (Gpx), and many heat-shock proteins and non-enzymatic antioxidants including vitamins, glutathione (GSH), ubiquinone, polyphenols and fatty acids.

GSH is the most important intracellular compound that provides the structural integrity of the cell subjected to harmful agents of a different nature. It performs cytoprotective function through two fundamental processes: the maintenance of the reduced status of the sulfhydryl groups of proteins and the inhibition of peroxidation of membrane lipids (Hayer *et al.,* 2005).

SOD, that is present in almost all aerobic cells and in extracellular fluid, catalyzes the conversion of superoxide radical into H_2O_2 (Johnson *et al.,* 2005):

$$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

The H_2O_2 produced in the reaction, which could potentially react and produce other reactive oxygen species, is degraded to water and oxygen by enzymes such as catalase, a common enzyme found in nearly all living organisms (Chelikani *et al.,* 2004):

$$2\ H_2O_2 \rightarrow 2H_2O + O_2$$

and glutathione peroxidase, which uses GSH as a reducing agent:

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

Ascorbic acid, also known as a vitamin C, plays a role in both animal and plant cells. It is a reducing promoter and can reduce, and in this way neutralize, ROS such as hydrogen peroxide. Apart from its antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase (Padayatty *et al.,* 2003).

3.2 Introduction of protein PDIA3

The protein PDIA3 (another name is ERp57) is a member of PDI family. It is 58-kDa stress-responsive protein localized maily in endoplasmic reticulum (ER). It plays an important role during post-translation folding as a quality control of newly synthetized proteins, participates in major histocompatibility complex I (MHC I) assembly and is important in regulation of gene expression in some particular cases.

Further studies showed that this protein occurs not only in ER but also in other compartments of the cell, such as the cell surface, cytosol, mitochondria, but also nucleus and is involved in many cellular processes. Recent studies show that PDIA3 is also associated with many pathological states including cancer and Alzheimer's disease. This is subject of present observations (Turano *et al.,* 2002, Montibeller L, de Belleroche J. 2018).

PDIA3 was first discovered as a stress-responsive protein, after glucose depletion its level in the cells increased and that explains why this protein is also called GRP58 (glucose-regulated protein 58) (Lee, *et al.,* 1981).

In recent studies it was also proposed that PDIA3 is involved, with its chaperonic and redox function, in repairing misfolded proteins after cell stress (Turano *et al.,* 2002).

3.2.1 PDIA3 as a member of PDI family

The first mammalian protein disulfide isomerase, best-known member and family namesake, is PDI. It is one of the total twenty members of the PDI family. It is present in most of the mammalian tissues.

This protein catalyzes the formation and reduction of disulfide bonds between two cysteines, but also provides isomerization of the disulfide bonds, and therefore, this enzyme activity can catalyze the formation of the protein. It was observed that PDI has also chaperonic activity independent of the redox status (Turano *et al.,* 2002). As was mentioned before, this function is mainly located in ER.

PDI activation is one of the mechanisms of unfolded protein response (UPR). This system is activated by cellular stress and also leads to transitional suppression of protein translation. The initial process which leads to UPR activation is the activation of the ER transmembrane kinase protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK).

Despite the fact that PDIA3 and PDI belong to the same family of luminal ER oxidoreductases, they have exactly the opposite role in the regulation of PERK function. Meanwhile, depletion of PDIA3 causes oxidation of PDI and it acts as a PERK activator. The depletion of PDI means inhibition of PERK signalling (Chelikani *et al.,* 2004).

However, the PDIA3, unlike most PDI family members, does not contain C-terminal especially responsible for effectively limiting the localization to retain protein in the lumen of ER, and therefore it can also occur in other cell compartments. PDI is a part of the thioredoxin (TRX) superfamily. Thioredoxin is a small ubiquitous protein with disulfide oxidoreductive function, it interacts with many variable proteins and it is involved in many cellular processes by its reversible oxidation of the two thiol groups of cysteine. Unlike PDI family members, which have two or three similar active sites formed by two cysteines, thioredoxin has only one active site (Hatahet, F., Ruddock, L. W, 2009).

3.2.2 Structure of PDIA3

PDIA3 is a human protein, which is coded by the gene *PDIA3* on chromosome 15 and contains 505 amino acids. The first 24 amino acids represent signal sequence. The structure is created by four main domains called a, b, b', a', together with C-terminal (ER retention motif composed) and N-terminal sequences. Each domain consists of thioredoxin-like fold, having both alpha-helices and beta-strands structures (Figure 1) (Ferrari & Soling, 1999; Silvennoinem *et al.,* 2004).

The a and a' domains are catalytically active, they carry a thioredoxin-like active site consisting of amino acid sequence Cys-Gly-His-Cys and this gives PDIA3 its redox properties. The b and b' domains are catalytically inactive, but they have a special function in binding and folding of the proteins. The shape of a protein is best described as a U-shape structure which allows the a and a' domain to get closer and this way they can interact with proteins. B and b' sites also contain binding sites for calreticulin a calnexin (CRT and CLNX), which are proteins occurring in ER. It was observed that these two proteins bind much more to the PDIA3 than other proteins of the PDI family. The b' domain has also the lowest similarity between PDIA3 and PDI. That explains the fact that PDIA3 and PDI have a different specificity to bind proteins. The similarity between PDI and PDIA3 is higher for catalytic a and a' (around 50%) and lower for b and b' domains (around 20%) (Kozlov *et al.,* 2004). However, PDIA3 also binds a variety

of small ligands (Gaucci *et al*., 2008) but also macromolecules (Dick *et al.,* 2002; Grillo *et al.,* 2006).

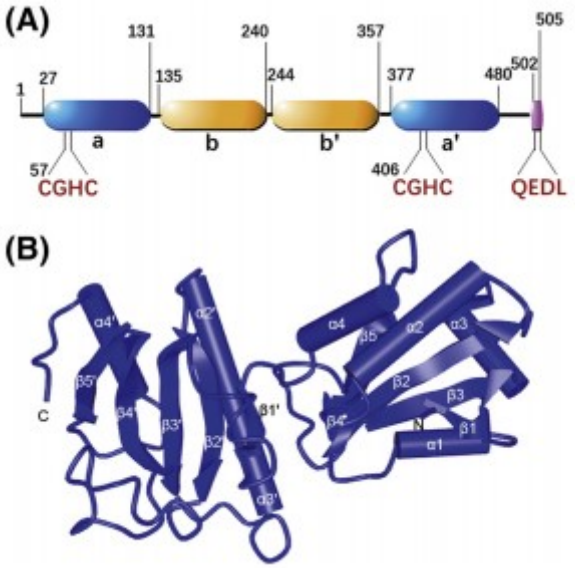

Figure 1: Structure of PDIA3 (Hettinghouse et al., 2018)

3.2.3 PDIA3 location and function

Although abundant in ER, PDIA3 was also found in a smaller amount in other cell compartments.

3.2.3.1 PDIA3 in ER

The main function of ER is to control the quality and posttranslational modification of newly synthetized proteins during maturation and it is critical for cell survival. ER presents huge site of processes and modifications of newly synthetized proteins or of misfolded proteins (caused for example by stress). The first identified specific function of PDIA3 in ER was its participation in correct folding of newly synthetized proteins. (High *et al.,* 2000). For the proper function of this process, participation of chaperone molecules CRT and CLNX that bind through their P-domains to the PDIA3 is necessary. CRT/CLNX selectively recognize and bind misfolded proteins and stop their secretion from ER. This binding is situated on the b and b' domain of PDIA3 and therefore the a and a' domain is free to interact with protein by disulfide isomerase activity. (Turano *et al*., 2002; Wearsch & Cresswell, 2008). PDIA3's b' domain which binds CRT and CLNX contains lysine and arginine residues that provides positive surface charge essential for binding negatively charged lectin P-domain of CRT/CLNX. The B

domain, on the other hand, provides additional lysin residues conducive to the stability of interaction (Kozlov *et al*., 2006; High *et al.,* 2000).

The second function of PDIA3 in ER is its participation in the immune system. It plays an important role in the folding of major histocompatibility complex class I (MHC I) (Lindquist *et al.,* 1998). Glycosylated heavy chains in the early stage bind to both calnexin and PDIA3 and this complex catalyzes the creation of disulfide bonds between heavy chains and later subsequently contributes to the formation of the MHC I protein kinase C (PKC) (Wearsch & Cresswell, 2008). This complex consists of heavy chain class I protein, beta2-microglobulin, calreticulin, PDIA3, tapasin and transporter associated with antigen processing (TAP). TAP transports the peptides which are generated by proteasome in cytosol to the ER where they are recognized by MHC I. The role of PDIA3 in this process is to create and stabilize PKC, so PDIA3 in that case has a structural role more than catalytic one (Turano *et al*., 2002). The redox activity is not required for the assembly of the peptide loading complex (Lindquist *et al*., 1998).

Together with calreticulin, PDIA3 was also found to modulate activity of calcium intake by sarcoendoplasmic reticulum calcium ATPase (SERCA) placed in membrane of ER. PDIA3 regulates the redox state of sulfhydryl groups of SERCA. PDIA3 overexpression reduces the frequency of Ca^{2+} oscillations enhanced by SERCA (Li & Camacho, 2004).

Another ability of PDIA3 was discovered in the participation in viral infection. In fact, PDIA3 can uncoat the virus SV40. This virus enters the cell by endocytosis and then penetrates the ER where PDIA3 dissociates the protein capsid. Uncoated virus can than enter the cytosol and after that to the nucleus. This process is independent of CRT/CLNX binding. The mechanism of action is based on catalyzation the isomerisation of the disulfide bonds of virus homopentamers. (Schelhaas *et al.*, 2007).

3.2.3.2 PDIA3 at the cell membrane

Finding protein in different cell compartments than in ER was unexpected because PDIA3 holds the Gln-Glu-Asp-Leu sequence in the C-terminal which is similar to the true ER retention signal Lys-Asp-Glu-Leu. One of the possible explanations of PDIA3 occurrence in variable cell compartments can be saturation of the protein holding mechanism, removal of the C-terminal or by the interaction with other macromolecules and forming complexes with them (Johnson *et al.,* 2005).

It turned out that PDIA3 is fundamental for fusion of the sperm and the egg. Using specific inhibitors, it was demonstrated that there is a need for the presence of the protein on the surface of the sperm head (Ellerman *et al.,* 2006).

Another function of the protein PDIA3 on the cell surface is in the binding of the hydroxylated vitamin D3 i.e. 1α,25-dihydroxycholekalciferol (calcitriol) and it is followed by fast activation which leads to a rapid non-genomic response (Nemere *et al.,* 2004). After stimulation with calcitriol numerous cascades of signal transduction are activated, involving many signaling proteins such as phospholipase C (PLC) and A2 (PLA2), PKC and extracellular response activated kinase (ERK). All of them are shown to respond to the formation of the PDIA3-calcitriol complex (Boyan *et al*., 2007). Through this process, PDIA3 and calcitriol are responsible for many processes. Together they represent an important initiator of membrane signaling pathways. One of them is the role of this complex in steroid hormone-stimulated uptake of calcium in mammalian intestinal cells (Nemere *et al.,* 2010). In the breast cancer cells PDIA3 modulate anti-cancer activity mediated by vitamin D. PDIA3 can increase the sensitivity of MCF-7 (breast cancer cell line) to agents related to vitamin D (Cynthia *et al.*, 2010).

According to other studies, PDIA3 plays a key role in platelet aggregation, intervening in the fibrin production process. In fact, the protein is secreted during a vascular lesion accumulating in the thrombus, where it induces the activation and the recall of the platelets (Holbrook *et al*., 2012).

3.2.3.3 PDIA3 in the cytosol

PDIA3 was also found in the cytosol mainly related to the STAT3 protein. This protein is a member of the family of STAT signaling and transcription factors. Those factors are activated by kinase associated with receptors for cytokines, growth factors and other hormones. These are involved in the processes of cell differentiation, proliferation and survival. Following binding to extracellular activation signals, the members of the STAT family are phosphorylated and enter the nucleus where they recognize specific gene sequences which activate their transcription. PDIA3 acts here as an accessory scaffolding protein (Ndubuisi et al., 1999).

Similarly, PDIA3 has been shown to interact with another factors during translocation from the cytosol to the nucleus, including nuclear kappa beta factor (NF-κB). PDIA3 together

with NF-κB and a transcription factor are involved in monocyte/macrophage differentiation (Wu *et al.,* 2010).

The equivalent function of the PDIA3 protein appeared in interaction with the retinoic acid receptor (RARα) and all-trans retinoic acid (ATRA). In this case, it is necessary for nuclear appearance of RARα, a transcriptional regulator implicated in multiple developmental processes in the nucleus. It is believed that in this case the role of the oxidoreductase activity of PDIA3 on the cysteine residues of RAR-α plays a role which is necessary for the binding of the ATRA-RAR-α complex and subsequent import of the complex into the nucleus (Zhu *et al*., 2010).

PDIA3 further mediates the arrangement and modulates the activity of the mammalian target of rapamycin (mTOR) complex that regulates cell growth and survival.There are two mTOR complexes and PDIA3 preferentially interacts with mTOR complex 1. PDIA3 promotes kinase activity and also participates in the mechanism by which mTORC1 detects its stimulatory signals, such as insulin or nutrients (Ramirez-Rangel *et al*., 2010).

PDIA3 also interacts with p53 through an indefinite mechanism to inhibit p53-mediated stimulation of mitochondrial outer membrane permeabilization (MOMP) and consequent apoptosis. These findings assure us that PDIA3 plays a role in p53 and mTOR mediated cellular proliferation, redox sensing, and apoptosis (Fingar& Blenis, 2004).

3.2.3.4 PDIA3 in mitochondria

A critical function reported by some laboratories is the role of PDIA3 in influencing calcium intake and apoptotic signaling pathways in mitochondria. Calcium intake is partially stimulated by PDIA3, which has a stimulatory effect on the mitochondrial calcium uniporter (MCU) transcription. However, transcription of regulatory components of the MCU complex are not responsive to alteration of PDIA3. The exact mechanism of the PDIA3 and MCU relationship remains to be clarified (He *et al*., 2014). Additionally, PDIA3 binds to mitochondrial m-calpain, and the calcium dependent cysteine protease to break them apart and activate apoptosis inducing factor (Ozaki *et al.,* 2008).

3.2.3.5 PDIA3 in the nucleus

PDIA3 can act in the nucleus in a dual pathway, either by direct interaction with DNA or by affecting the activation and/or binding of transcription factors to DNA. In DNA binding, PDIA3 interacts with regulatory parts involved in cellular adhesion, intracellular traffic, and cellular stress response. Although PDIA3 affects transcription in target regions, it does not bind with such affinity as transcription factors. Importantly, PDIA3 has been detached as a part of certain multiprotein nuclear complexes engaged in DNA binding which may influence nuclear transcription (Aureli *et al.,* 2013).

PDIA3 interacts with a nuclear protein complex that targets the correct DNA in cells with defective DNA mismatch repair. This is a system for recognizing and repairing erroneous insertion, deletion, and misincorporation of bases that can arise during DNA replication and recombination, as well as repair some forms of DNA damage (Krynetski *et al*., 2003).

As mentioned above, PDIA3 interacts with STAT3 and in this case reacts in the nucleus with the STAT3-DNA complex. Inhibition of PDIA3 can reduce binding of STAT3 to DNA (Eufemi *et al*., 2004).

PDIA3 can indirectly affect gene expression due to its redox potential, which activates transcription factors. On the other hand, PDIA3 has negative regulatory effects on transcriptional activators through its reduction of endophilin A2 proteins, promoting heterodimerization of the transcription factor and by that preventing DNA binding and transcription of B cells differentiation genes (Markus & Benezra, 1999).

These studies assume that stress-responsive PDAI3 may operate to affect transcription of genes relevant to cell survival and differentiation either through direct or indirect interaction with DNA targets and/or transcriptional factors (Grillo *et al.,* 2006).

3.2.3.6 PDIA3 as a stress-responsive protein

Although PDIA3 is generally accepted to be involved in stress response, no specific reviews about this topic exist. Up today, only several recent articles on the involvement of PDIA3 in several stress type and cellular pathologies are available.

As a stress-responsive protein, the first studies identified PDIA3 as over-expressed protein in the K12 cell line under stress due to glucose depletion. This same overexpression was afterwards confirmed in various cell types and various stress-inducing agents (Murray *et al.,* 2004). The mechanism by which this response to stress occurs at the PDIA3 gene promoter

has not yet been defined. However, one study described the presence of sequences for transcription factors of the Smad family (structurally similar proteins that are the main signal transducers for receptors of the transforming growth factor beta ⟨TGF-B⟩ superfamily, which are critically important for regulating cell development and growth) involved in stress, in the rat gene promoter, also present in the human gene (Rohe *et al*., 2007).

The redox and chaperone activities of overexpressed PDIA3 prevent some harmful effects of cellular stress, such as protein misfolding or damage caused by ROS products. A series of studies confirms this behaviour, although they reveal large differences in the response and protection given, depending on the type of stress and the type of cell examined (Turano *et al.,* 2002, Montibeller L, de Belleroche J. 2018., Lee, *et al.,* 1981).

In any case, the knowledge of these effects could be used for a therapeutic intervention, as proposed by a study in which the mechanism of action of anticancer agents was examined. Indeed, it has been shown that the two proapoptotic drugs, bortezomib and fenretinide, act through a stress mechanism. While fenretinide induces stress through the production of reactive oxygen species, bortezomib does so by inhibiting the proteasome. In both cases, the homeostatic mechanism, a mechanism which leads to the overexpression of stress-responsive proteins, is only partially able to counteract the apoptotic target induced by drugs, a desirable outcome for their therapeutic action. Apoptosis is also significantly increased by knockout of the gene encoding PDIA3 or genes related to other PDIs (Corazzari *et al*., 2007).

One study examined the expression of proteins in Alzheimer's disease and found that expression of PDIA3 protein increased in this stress state. This study assumes that the condition of Alzheimer's disease activates transcription factors that induce gene expression inter alia for the PDIA3 protein (Montibeller L, de Belleroche J. 2018).

Expression of the PDIA3 was also investigated in connection with Amyotrophic lateral sclerosis (ALS). Although the aetiology of this fatal neurodegenerative disorder remains unclear, protein misfolding, ER stress, protein and neuronal apoptosis are implicated. It was previously established that PDIA1 is protective against mutation of ER stress and apoptosis in neuronal cells. Then it was claimed that mutations in PDIA1 and PDIA3 are connected with ALS state. This study also examined that over-expression inhibited inclusion formation, ER stress, UPS dysfunction and apoptosis, whereas silencing of PDIA3 expression enhanced mutant SOD1 inclusion formation, ER stress and toxicity, indicating a protective role for PDIA3 against SOD1 misfolding. PDIA3 also

inhibited the formation of mutant SOD1 inclusions and apoptosis in primary cortical neurons, thus confirming results obtained from cell lines. Thus, PDIA3 is linked to correction of protein misfolding and therefore has a protective role against pathological events induced by mutant SOD1. (Parakh *et al*., 2018)

Another study showed that the PDIA3 is up-regulated in the brain of Creutzfeldt-Jakob disease patients. However, the actual role of PDIA3 in prion protein (PrP) biogenesis and the ER stress response remained poorly defined. The results revealed that PDIA3 modulates the biosynthesis and maturation of PrP but, surprisingly, does not contribute to the global cellular reaction against ER stress in neurons. They claimed that PDIA3 could be potential therapeutic target in PrDs and other protein misfolding disorders (Supulveda *et al.,* 2016).

3.2.3.7 PDIA3 in a process of cancer

PDIA3 protein levels vary in different types of cancer and expression of this protein has been clinically evaluated as a prognostic marker, either upregulation or downregulation, it can correlate with poor prognosis depending on tissue types. The relationship between the two-way variation of PDIA3 expression and prognosis in many different types of cancer suggests potential for involvement in complex cellular and molecular processes underlying the etiological heterogeneity of cancer. As mentioned above, PDIA3 could potentially be used in the cancer treatment for knockdown of the PDIA3 gene, resulting in various changes in the pathways involved in the physiological functions of PDIA3. PDIA3 has been implicated as a potential target for the therapeutic modulation of apoptotic signalling and metastasis through its indirect regulation of EGFR and through many other ways (Gaucci *et al.,* 2013).

4 Aim of work

The main aim of our work was to find out whether the expression of PDIA3 protein in the cells is influenced by chemically induced stress, and whether PDIA3 protein levels are different in two cell lines (MCF-7 and MDA-MB 468). Another objective was to investigate the effect of 17β-estradiol pre-treatment before exposure to the chemically induced stress and describe a potential mechanism of these processes.

In details:

- To evaluate the effect of chemical stress (t-BOOH) on PDIA3 protein expression in MCF-7 and MDA-MB 468 cell lines.
- To evaluate the effect of 17β-estradiol pre-treatment before chemical stress, to describe its effect on PDIA3 protein expression.

5 Materials and methods

5.1 Manipulation with cell cultures

Two cell lines, MDA-MB 468 and MCF-7, were used for our research. Both of which were mammalian breast cancer cells. The major difference between the cell lines is that MDA-MB 468 is estrogen receptor (ERec) negative and MCF-7 is ERec positive. Different cell culture media were used for each cell line. DMEM (Dulbecco's Modified Eagle Medium) low glucose, which is a widely used basal medium for supporting the growth of many different mammalian cells, was used for MDA-MB 468 cell line. RPMI (Roswell Park Memorial Institute) 1640 (With L-glutamine and sodium bicarbonate) medium was used for MCF-7 cell line. The following additives were added to both media:

- FBS (bovine fetal serum) 10%
- Antibiotic penicillin 100U/ml
- Antibiotic streptomycin 100g/ml
- Sodium pyruvate 1mM
- Glutamine 2mM

All the chemicals were purchased from the company Sigma-Aldrich, USA.

The cells were grown in flasks and kept in a CO_2 incubator (37 °C, 5 % CO_2). During this incubation, the cells adhered to the flasks up to a concentration of about 60-70 % of the flask capacity. This process is controlled by Thoma cell counting chamber – described below and it takes usually 3-4 days. Subsequently, they were detached by 3 ml of trypsin (0,25%) to peel away from the bottom of the flask (cells are attached to the bottom of the flask by lysine bonds and the role of the trypsin is to break this bonds). The cells are then kept in an incubator for a variable time, depending on the cell line (it is recommended for 5 minutes) to let the trypsin work. After the separation of the cells from the flask bottom, the trypsin was inactivated adding the medium with a volume equal to three times the amount of trypsin used (9 ml). Suspension of the cells in medium was transferred to the test tube and centrifuged at 1100 rpm for 5 minutes. The medium was discarded and the cells stayed at the bottom. The pellet at the bottom was resuspended in a fresh medium, and finally the cells were planted in the new flasks.

5.2 Thoma cell counting chamber

To ensure to have the same number of cells for each experiment amount of cells in one ml of suspension must be calculated.

The cell count is carried out using the Thoma chamber (figure 2), consisting of a thick glass, in which a capillary chamber is created. The frame of the counting chamber contains a large central square divided into 2 main quadrants each with 16 medium squares and each with 25 small squares inside (9 of them are divided in half).

The procedure for counting is as follows:

- the chamber is cleaned with ethanol and then filled up with 100 µl of the cell suspension in the capillary line

- the cells present inside the two quadrants and those on two sides of the choice must be counted under a microscope

- the number of cells counted is divided by 2 and then multiplied by 10^4, thus obtaining the number of cells contained in 1 ml of suspension.

Figure 2: Thoma chamber (author: Lucie Ženklová)

5.3 Cell culture treatment

5.3.1 Treatment with tert-butyl hydroperoxide

Six-well plates were used for treatment by tert-butyl hydroperoxide, two wells for each experiment, one for control cells without treatment. In each well approximately 450 000 cells were added in incubation medium (1,9 ml). Cells were then treated by tert-butyl hydroperoxide (5 µl tHBOOH 7,2M). At interval of 0, 3, 6 and 24 hours spending in incubator the cells (treated and control) were removed and subjected to other approaches.

5.3.2 Treatment with tert-butyl hydroperoxide and 17β-estradiol

The cells were pre-treated with 20nM 17β-estradiol first and then same protocol was followed as in case of tert-butyl hydroperoxide treatment (chapter 5.3.1). Again 450 000 cells for each well and periodical collection of samples after 0,3,6 and 24 hours.

5.4 Preparation and analysis of protein extracts

5.4.1 Collection of total protein extract

First, medium from the wells was removed and then 1100µl of PBS 1X (Phosphate Buffer Saline, concentration of PO_4^{3-} is 10mM) added. The cells were mechanically craped from the wells with a plastic scrape (it is more appropriate than detachment by trypsin, because trypsin causes another stress to cells). Afterwards the samples were centrifuged at 1000 rpm to obtain a pellet. A special buffer named RIPA was used for cell membrane lysis. Composition of the RIPA is as follows (we used the finished purchased buffer):

- tris HCl pH 7.4 50mM
- NaCl 150mM
- Igepal 1%
- Sodium deoxycholate 0.5%
- SDS (sodium dodecyl sulfate) 0.1%
- EDTA (ethylenediaminetetraacetic acid) 1mM
- DTT (dithiothreitol) 1mM

RIPA is completed at the time of need by adding the following ingredients:

- DTT 1mM 5µl
- Na_3VO_4 (sodium orthovanadate) 2mM 3µl
- Protease inhibitor 1:100 ratio 3µl

The cell lysis continued by sonication of each sample for 20 seconds. (Figure 3)

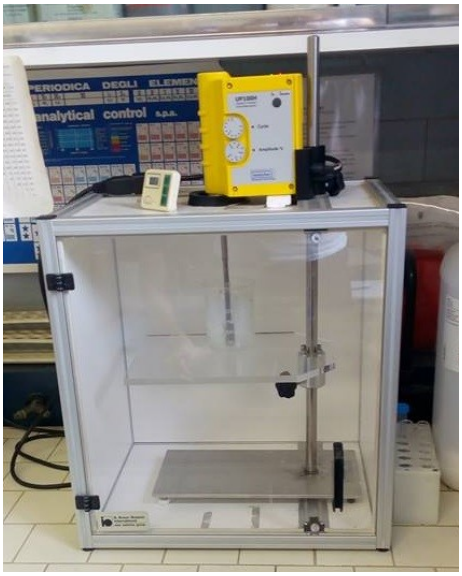

Figure 3: Sonicator (author: Lucie Ženklová)

5.4.2 Bradford assay, the protein quantitation

The protein quantitation is carried out with the Bradford method, which is implemented by the addition of the Bradford Reagent 1X. It is acidic dye added to the solution containing the protein. This dye interacts with the protein and forms a coloured complex, which is spectrophotometrically detected at a wavelength of 595 nm. Measuring the absorbance provides a quantitative amount of protein. Composition of Bradford 1X Reagent:

- Coomassie Brilliant-Blue-G 250
  (BIO-RAD solution 5X) 0.01% (w/v)
- 96% EtOH 4.7% (w/v)

- H_3PO_4 8.5% (w/v)

To obtain the protein amount it is crucial to establish a calibration curve. This is obtained by preparing standard solutions of bovine serum albumin (BSA); 5 concentrations of standard are required (0 µg/µl, 0.025 µg/µl, 0.05 µg/µl, 0.1 µg/µl, 0.2 µg/µl).

The standards and the samples are diluted 1:20. All of them prepared in duplicates.

96 wells plate with round bottom was used for the protein quantitation. To each well 200 µl of Bradford 1X reagent and 10 µl of each sample or standard were added. Afterwards, the well plate was incubated in the dark on a 3D analog shaker for about 15 minutes (time necessary for the formation of the complex of dye - proteins complex).

A spectrophotometer Appliskan microplate reader, Thermo Scientific, was used for the absorbance measurement at 595nm of wavelength. Values of the absorbance of the calibration curve were used to extrapolate the total protein quantities of each sample.

Detecting the total amount of protein is necessary for normalization of sample amount added on the acrylamide gel.

5.4.3 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis) is polyacrylamide gel electrophoresis in the presence of SDS. This technique allows to separate a mixture of proteins based on their molecular weight. The proteins are exposed to the anionic detergent SDS before and during the electrophoretic run. SDS is applied to protein samples to coat proteins in order to impart two negative charges (from every SDS molecule) to every two amino acids of the denatured protein. Due to the SDS treatment, all the proteins have the same negative charge and therefore the separation is based only on their molecular weight. During the migration across the gel a constant electrical potential is applied that allows all the proteins to migrate in the same direction and to separate based on their molecular weights. The process can be visually detected by simultaneous migration of a marker. This marker contains standard proteins with known molecular weight.

Electrophoretic chamber for the electrophoretic run was used. The chamber must be appropriately assembled before the use: the two slides are cleaned with ethanol, properly mounted on a suitable support and between the two slides, acrylamide gel gel is added.

The gel used in this experiment is a 10% acrylamide gel. It is composed of the following ingredients:

- Running gel: acrylamide 2ml, lower buffer (Tris-HCl 1M pH 8.8) 1.5 ml, H_2O 2.45ml, SDS 10% 60µl, APS 75µl and TEMED 5µl;

- Stacking gel: acrylamide 0,5ml, upper buffer (Tris-HCl 0,5M pH 6.8) 1,25ml, H2O 3,25ml, SDS 10% 50µl, APS 50µl, TEMED 5µl.

First, the running gel is prepared and allowed to stand for about 40 minutes until the polymerization is complete. A portion of the gel remains in the tube where we can easily control its polymerization. After the polymerization of running gel the stacking gel is added, which takes about 30 minutes to be prepared for the run. Before the polymerization, a comb is inserted into the liquid stacking gel. This comb is necessary for the formation of the wells (where the protein samples will be loaded). 10 wells were needed for each of our experiment.

The polymerization takes place thanks to the presence of the two catalysts, added in the following order: TEMED (tetramethylethylenediamine) and APS (ammonium persulfate). The TEMED reacts with the persulfate ion ($S_2O_8^{2-}$), thus determining the formation of the corresponding free radical. This radical reacts by chain radical reaction with the acrylamide

monomers, leading to the formation of long chains held together by cross-links formed by the insertions of the bis-acrylamide.

Once the polymerization has taken place, the samples are loaded. We can check this state with the gel we left in the test tubes.

Then the gel is placed in the appropriate electrophoretic tray containing the run buffer and connected to the electrodes. The electrophoretic run takes place at a constant voltage (200 V) for about 45 minutes. The marker can be followed during the run to see if everything works properly.

5.4.4 Western Blot

Western Blot is an analytical method that allows detection of specific a protein in a protein mixture. After electrophoretic separation, the proteins are subsequently transferred from the gel to the surface of the membrane. Subsequently, they are detected by specific antibodies.

After the electrophoresis, at the time when the color of the control marker is at the end of the gel, the proteins present on the gel are transferred onto a nitrocellulose membrane using the TRANS-BLOT TURBO transfer system (BIORAD) for 30 minutes at 0.8A. The transfer is carried out by positioning the anode of the device from the bottom upwards: three sheets of filter paper soaked in the appropriate solvent, membrane, gel, three sheets of filter paper soaked in the appropriate solvent. In our case, an older method was used and all the layers were cut individually.

The paper and the membrane, before assembling the sandwich, are soaked with the CAPS solvent with the following composition:

- H_2O 400ml
- CH_3OH 100ml
- CAPS (3-cyclohexylamino-1-propansulfonic acid) 1.1g
- 1M NaOH 3.6ml

It is positioned above the cathode and finally a transverse electric field is applied that allows the transfer of proteins from the gel to the membrane. To avoid problems during the transfer it is necessary to check that there are no air bubbles between the gel and the membrane.

It can be done using a special roller. At this point the transfer can be started (30 minutes at 0.8A).

Once the transfer is complete, the membrane is incubated overnight at 4°C with a milk solution (prepared from non-fat dry milk powder) in TBS 1X (prepared from TBS 10X: Tris HCl 0.5M pH 7.5 and NaCl 1,5M) on a 3D analog shaker. This serves to block non-specific sites.

5.4.5 Immunoblotting analysis

With this technique, the membrane is incubated with two different antibodies: the primary, which is specific to the protein of interest, and subsequently the secondary, which recognizes the primary antibody and is bound to the enzyme detector.

The primary antibodies we used are the following:

Anti PDIA3: is a rabbit antiserum the laboratory prepared few years ago and is available from Merck-Millipore (ABE1032 - Antri-PDIA3 antibody)

Anti actin: is from Sigma (A5316 - Monoclonal Anti-beta actin produced in mice)

This identification can be performed by two techniques, the first one is based on chemiluminescence (ECL) and the second one is colorimetric. In our case the ECL technique was used for the identification of PDIA3 and the colorimetric one for the identification of ß-actin, housekeeping protein used for the standardization of the results.

5.4.5.1 Chemiluminescence (ECL: enhanced ChemiLuminescence)

The blocking with 3 % milk is followed by detection:

- The membrane is incubated with the primary antibody prepared in TBS 1X with a 1:2000 dilution, for one hour under stirring
- Then washed with TBS 1X: two one-minute washes, a fifteen-minutes wash and three five-minutes washes
- The membrane is incubated with the secondary antibody, prepared in TBS 1X with a 1:5000 dilution, for one hour under stirring. This antibody is specific for the primary one and conjugated with a peroxidase.

- Washings are carried out as previously.

The secondary antirabbit antibody is conjugated with a peroxidase, that in the presence of the luminol substrate, forms the products and causes the emission of photons. The membrane is treated with a LumiGLO ReverseTM solution, this is prepared at the time of use and is obtained by mixing a part of solution A, containing Luminol, and a part of solution B, containing hydrogen peroxide (H_2O_2). Then the membrane is placed on a glass plate, treated with this mixture and left to incubate in the dark for 3 minutes. After the incubation, a light signal is observed due to the oxidation reaction of the Luminol by the peroxidase: the latter converts the Luminol into an excited dianionic intermediate, which emits light returning to the ground state. The light emission reaches its maximum intensity within five minutes and is detected through the ChemiDocTMMP Imaging System (BIORAD).

5.4.5.2 Colorimetric analysis

The cut membrane is incubated with I-block necessary for blocking all remaining binding surfaces of the membrane that is not occupied by the coated protein. The I-block buffer (Thermo Fisher scientific) binds to all potential sites of nonspecific interaction without altering or obscuring the epitope for antibody binding.

The I-block has the following composition:

- H_2O 450ml
- TBS 10X50ml
- I-block powder 1g
- Tween 20 500µl

The preparation starts with the hot solubilization of the powder, then the solution is let to cool down before adding the Tween 20 (Sigma-Aldrich, USA).

After the blocking process, colouring was proceeded:

- The membrane was incubated with the primary antibody, prepared in TBS 1X with a 1:2000 dilution, for one hour under stirring.

- Three washes were performed, each of ten minutes, with I-block.

- The membrane was incubated with the secondary antibody conjugated with the alkaline phosphatase enzyme, prepared in I-block with a 1:5000 dilution, for one hour under stirring.

- Two washes were done with I-block for ten minutes each and a third wash with a solution of 10ml of alkaline phosphatase buffer (Tris HCl 0.1M pH 9.2, MgCl2 0.05M, 0.1M NaCl) and 10µl of levamisole.

- Substrates for alkaline phosphatase were added:

  A solution of 10ml of alkaline phosphatase buffer was prepared containing 10µl of levamisole (inhibitor of any other phosphatases present) and 40µl of 4-nitro-blue-tetrazolium (NBT 3mg/ml in DMF) and 140µl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP 50mg/ml in DMF), which is an artificial chromogenic substrate used for the sensitive colorimetric detection of alcaline activity.

The localization of the protein-antibody complex was evidenced by the colour of the membrane in the position in which the protein's part of interest was located. The alkaline phosphatase catalyzes the hydrolysis of the phosphoric group of the BCIP. The resulting BCI reacts spontaneously with the NBT to give two intensely coloured and insoluble products: the indigo and the formazan. These, precipitating on the membrane, colour it at the point where the reaction occurred.

The membrane was left in the dark for the time long enough for the reaction to take place and subsequently washed with water to stop the reaction. The colour obtained was detected through the ChemiDocTMMP Imaging System (BIORAD).

5.4.5.3 Densitometric analysis

The densitometric analysis was performed using the BIORAD Image Lab program. This program enables to calculate the area and the intensity of each band on the membrane. The data obtained can be exported and analyzed using statistical software and graphs such as GraphPad and MS Excel. Density values related to the housekeeping gene, ß-actin, were used to normalize the intensity values related to the PDIA3 protein. The analyses were conducted using the mean of the values obtained in four trials.

6 Results

This study was a pilot study. Each experiment was performed twice.

6.1 Expression levels of PDIA3 in MDA-MB 468 cells after treatment with tert-butyl hydroperoxide by Western Blot

MDA-MB 468 cells were treated with 75µM tert-butyl hydroperoxide for 3, 6, and 24 hours. The peroxide concentration was experimentally determined so that the cell mortality was 50 %.

From this chart, the results of the Western Blot show that after 3 hours of treatment with tBOOH the protein levels of PDIA3 decrease drastically, reaching a percentage of expression of 15 %. After 6 hours of treatment, the PDIA3 levels instead return to an increase of about 60 %, and then reach a 200 % expression after 24 hours.

What we can observe in the control samples is a slight but progressive increase in PDIA3 expression levels, resulting from the normal cellular replication cycle.

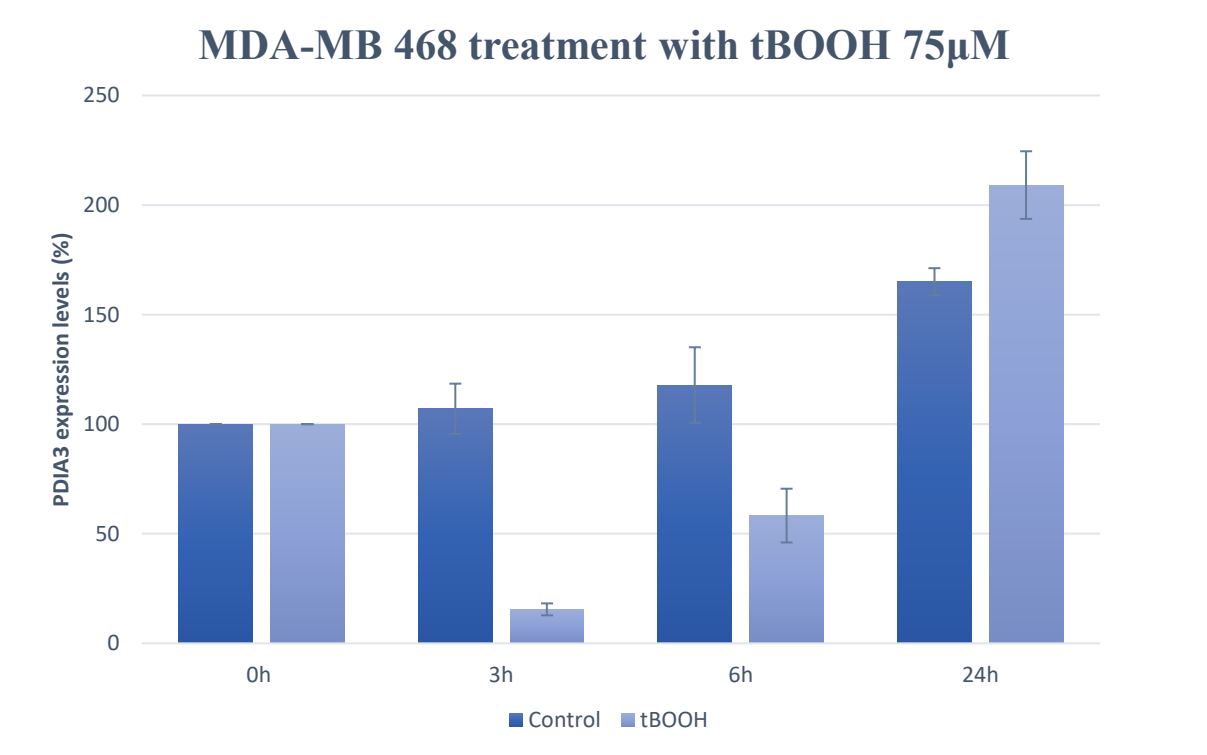

Figure 4: Levels of expression PDIA3 protein in MDA-MB 468 with 75µM tBOOH

The data represent the mean ± S.D. (n=2)

6.2 Expression levels of PDIA3 in MCF-7 cells after treatment with tert-butyl hydroperoxide by Western Blot

The MCF-7 cells were treated with 50µM tert-butyl hydroperoxide for 3, 6 and 24 hours. The concentration of the tert-butyl hydroperoxide was experimentally determined in order to obtain a cell mortality of 50%.

It can be observed from the data obtained after Western Blot that there was no significant change in the level of PDIA3 protein expression in the MCF-7 cells treated with tert-butyl hydroperoxide compared to the control samples.

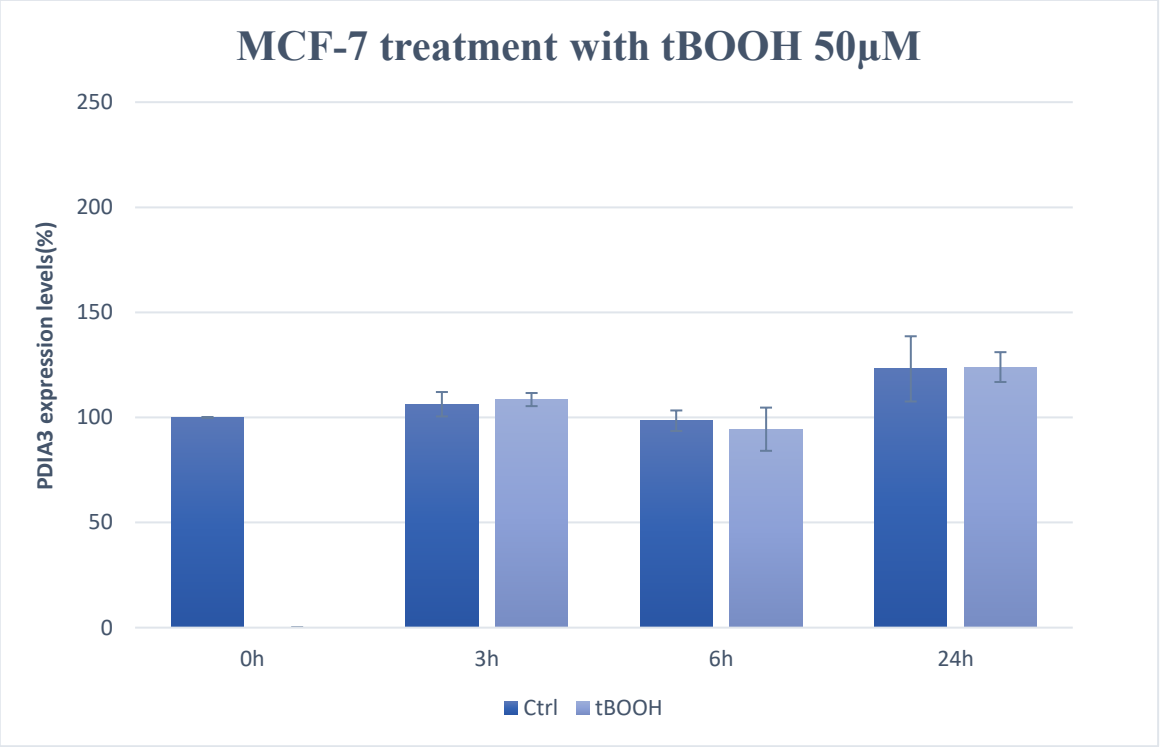

Figure 5: Levels of expression PDIA3 protein in MCF-7 with 50µM tBOOH

The data represent the mean ± S.D. (n=2)

6.3 Co-treatment with tert-butyl hydroperoxide and 17ß-estradiol

The two cell lines MDA-MB 468 and MCF-7 differ by the presence of the estrogen receptor. MDA-MB 468 cells are derived from ERec-negative breast cancer and MCF-7 are

cells derived from ERec-positive breast carcinoma. Because of this fact, a co-treatment with tert-butyl hydroperoxide and 17β-estradiol was performed at a concentration of 20 nM for both cell lines.

6.3.1 Expression of PDIA3 protein in the MDA-MB 468 cell line after co-treatment with tert-butyl hydroperoxide and beta estradiol.

After obtaining the values of the Western Blot method, we can say following:

- in the control group treated with solely estradiol, the levels of PDIA3 protein expression increased after 3 hours and subsequently after 6 and 24 hours they remained the same.
- in the group co-treated with tert-butyl hydroperoxide, the levels of PDIA3 protein increased after 3 and 24 hours, but after 6 hours the levels of this protein decreased.
- it should also be noted that when comparing controls and treated samples, protein levels in the treated cells were elevated after 3 and 24 hours, whereas after 6 hours we observed higher levels in control cells stimulated only with estradiol.

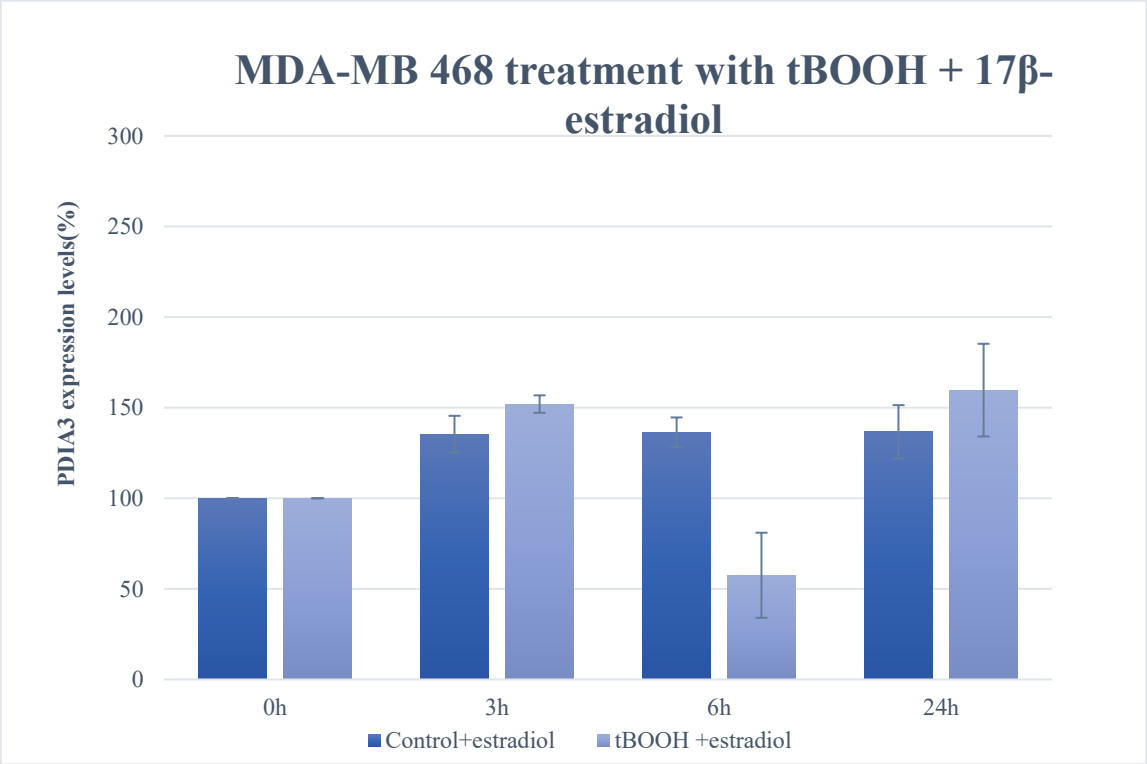

Figure 6: Expression levels of PDIA3 protein in 468 MDA-MB cells co-treated with 75μM tBOOH and 20nM 17β-estradiol. The data represent the mean ± S.D. (n=2)

6.3.2 Expression of PDIA3 protein in MCF-7 cells after co-treatment with tBOOH and 17ß-estradiol by Western Blot

After obtaining the results of the Western Blot method, we can say following:

- in the group of control samples treated with estrogen, there is a progressive elevation in protein expression at 3h, 6h and 24h.
- in the group treated with tBOOH there is no significant variation of PDIA3 expression observed.
- instead, when comparing the control and the treated cells, it is noted that there is a more intensive expression of the protein PDIA3 in the cells not treated with tBOOH, in particular after 6h and 24h.

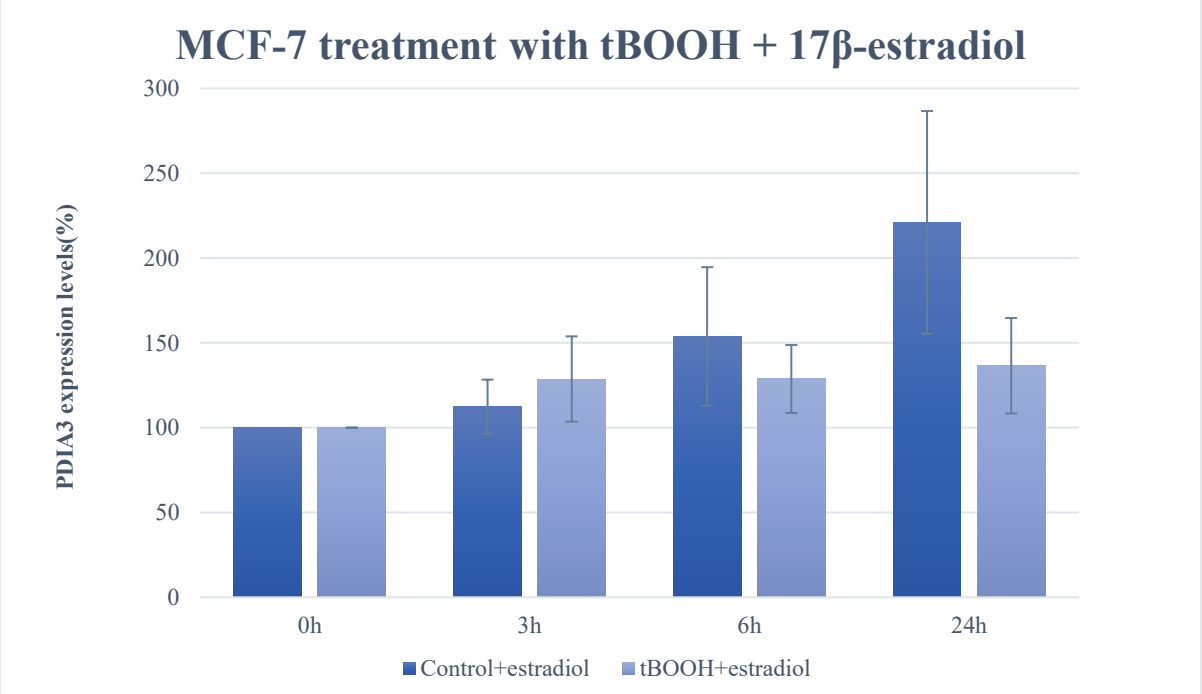

Figure 7: Expression levels of PDIA3 protein in 468 MDA-MB cells co-treated with 75µM tBOOH and 20nM 17β-estradiol. The data represent the mean ± S.D. (n=2)

6.4 The effect of estradiol on cell lines MDA-MB 468 and MCF-7 by Western Blot method

6.4.1 Levels of expression of PDIA3 protein in the MDA-MB 468 cell line after treatment with 17β-estradiol

What can be observed in Figure 8 is the fact that in the non-stimulated cells with 17β-estradiol there is a gradual increase in PDIA3 expression after 3, 6 and 24h. Instead, in the cells stimulated with estrogen, an increase in the levels of the protein PDIA3 is observed, but they remain unchanged after 3, 6 and 24h.

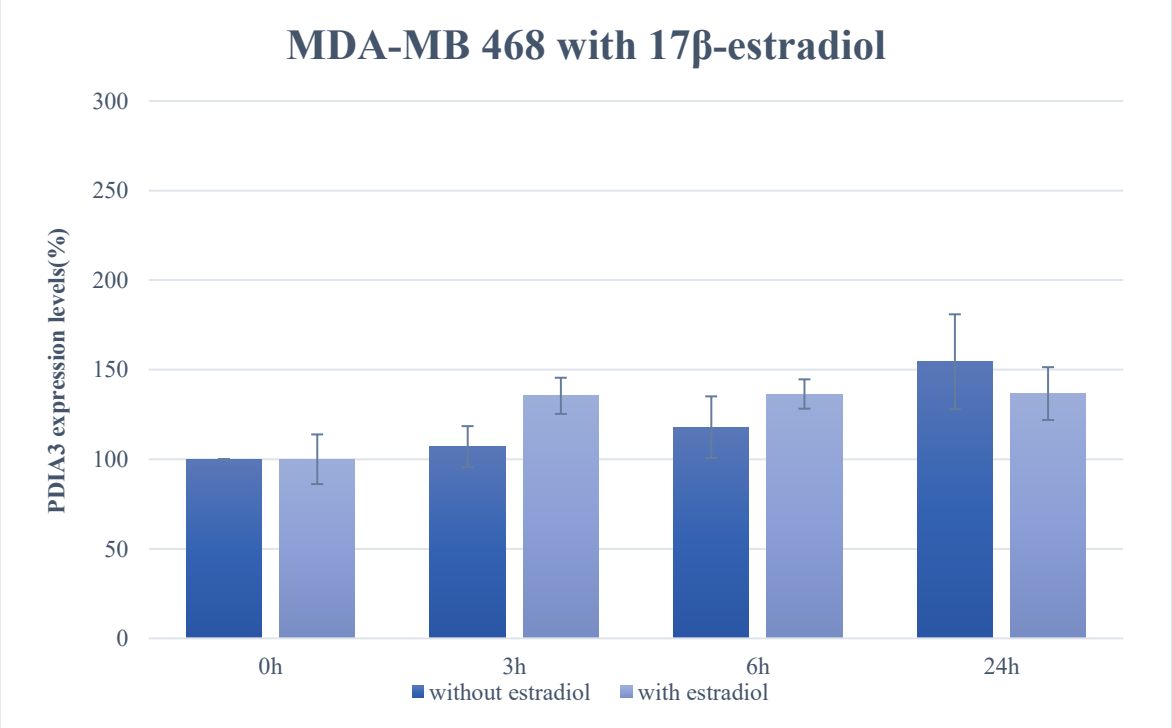

Figure 8: Comparation of PDIA3 expression levels in stimulated and unstimulated MDA-MB 468 cells with 17β-estradiol

The data represent the mean ± S.D. (n=2)

6.4.2 Expression levels of PDIA3 in MDA-MB 468 cells stimulated or not with 17ß-estradiol and subsequently treated with tBOOH

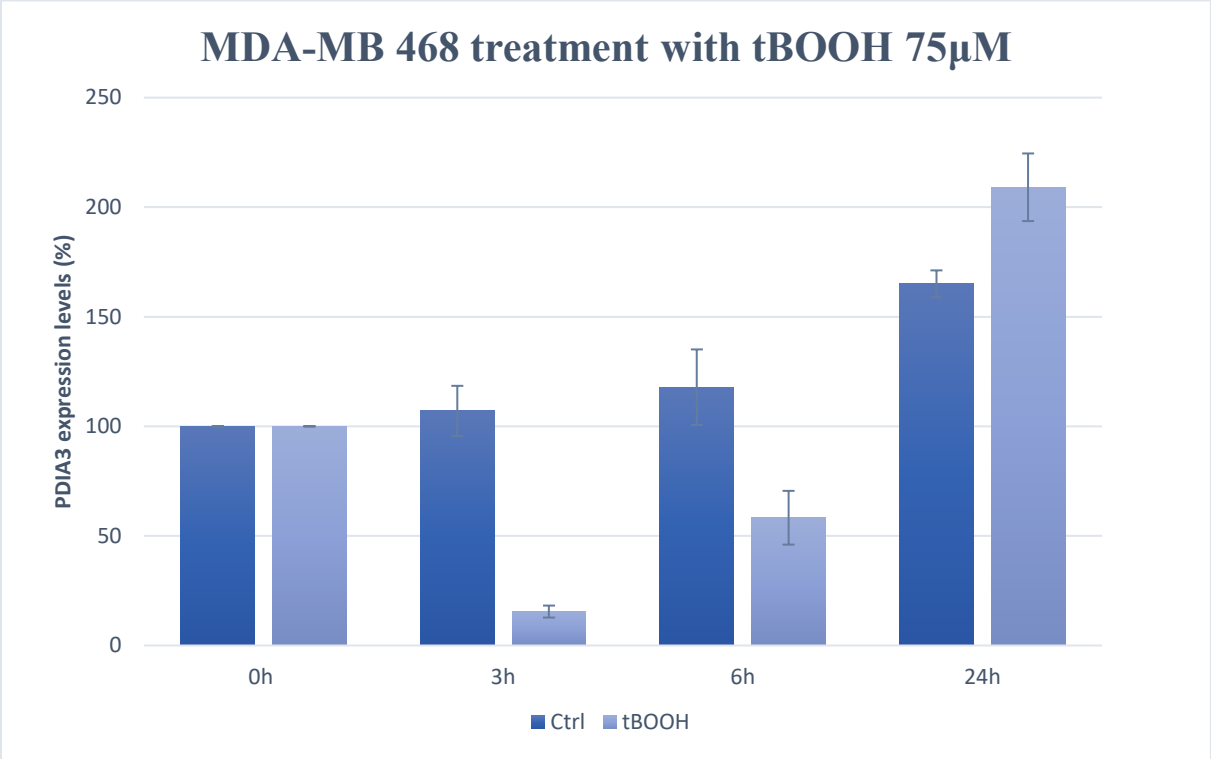

Figure 9: Levels of expression PDIA3 protein in MDA-MB 468 with 75µM tBOOH

The data represent the mean ± S.D. (n=2)

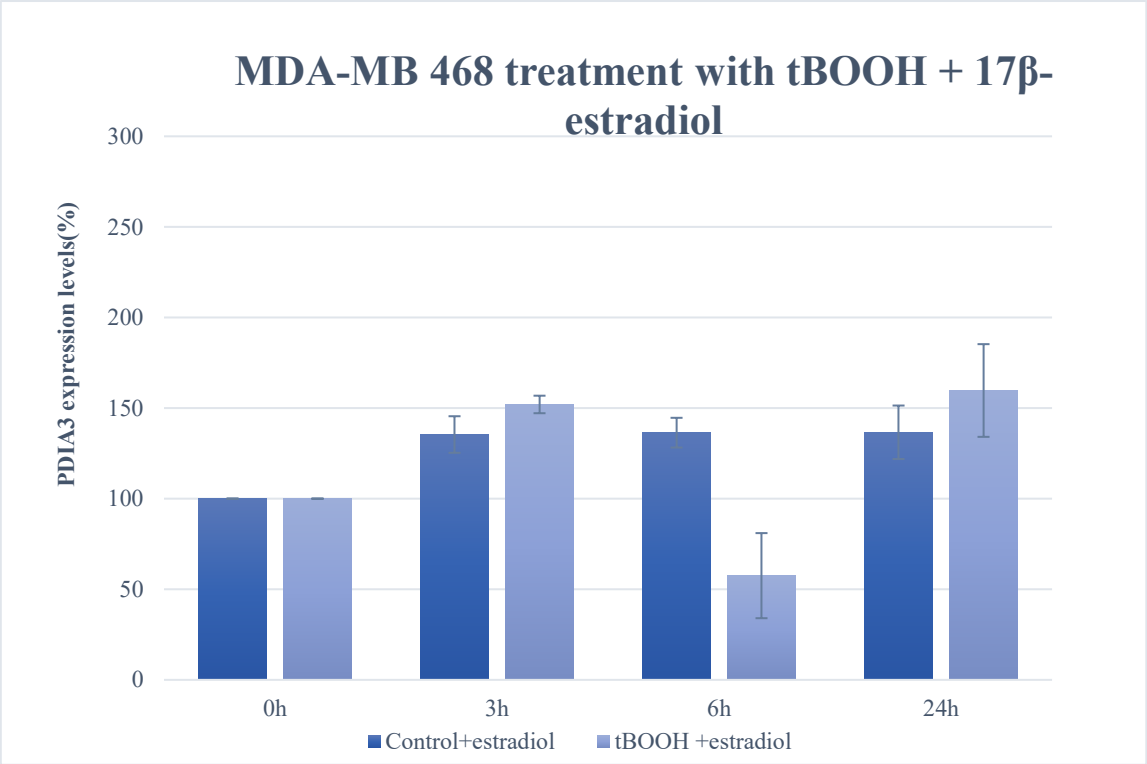

Figure 10: Expression levels of PDIA3 protein in 468 MDA-MB cells co-treated with 75μM tBOOH and 20nM 17β-estradiol. The data represent the mean ± S.D. (n=2)

Both graphs are shown again under each other in order to clearly see the results.

After treatment with solely tert-butyl hydroperoxide in MDA-MB 468 cells (figure 9), a remarkable reduction in protein expression levels is observed after 3 hours of treatment; these levels then increased after 6h to about 60 %, up to a value of about 200 % after 24 hours.

Instead, as a result of co-treatment with tBOOH and estrogen, (figure 10) a reduction in PDIA3 levels is observed only after 6h, but less significantly than for only tBOOH treatment. After 3h and 24h of co-treatment, instead, there is an increase in the PDIA3 protein expression.

What is important to underline is the data that can be obtained from the comparison of the control and the treated sample. In the case of the first cell lines, which were treated only with tBOOH, there is a clear prevalence of the PDIA3 protein expression levels in the control samples compared to the treated ones, with the only exception of 24h. Instead, in the case of co-treatment, this difference is no longer so considerable.

Thus, the absence of estrogen in the first experiment causes this sharp difference in PDIA3 levels between the control and the treated sample, which is not observed in the co-treatment experiment.

6.4.3 Levels of expression of PDIA3 protein in the MDA-7 cell line after treatment with 17β-estradiol

What can be seen in the graph is that in the cells not stimulated with 17ß-estradiol there is no variation in the expression levels of PDIA3 protein after 3h, 6h and 24h. While protein levels are gradually increased in MCF-7 cells stimulated with estrogen after 3h, 6h and 24h.

By comparing the stimulated cells with the non-stimulated cells it can be seen that at 3h the PDIA3 protein levels are the same, while at 6h and 24h, there is a significant increase in its expression levels following stimulation with the hormone.

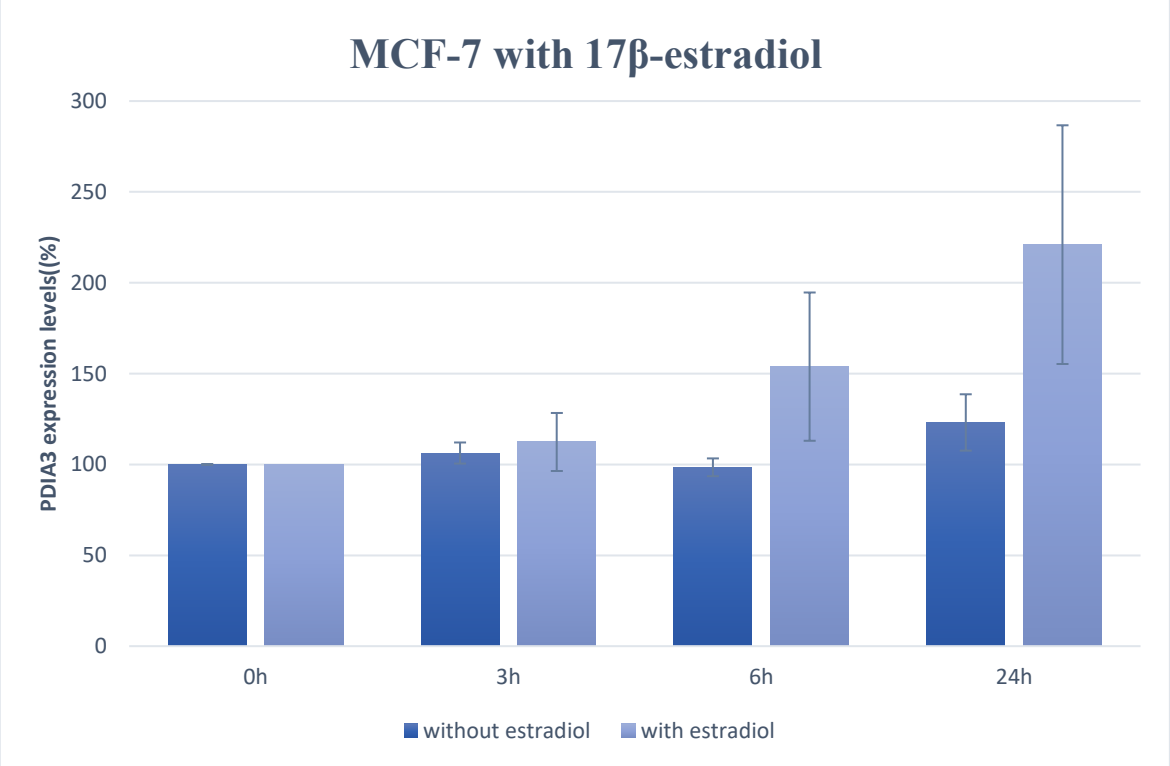

Figure 11: Comparation of PDIA3 expression levels in stimulated and unstimulated MCF-7 cells with 17β-estradiol

The data represent the mean ± S.D. (n=2)

6.4.4 Expression levels of PDIA3 in 468 MCF-7 cells stimulated or not with 17ß-estradiol and subsequently treated with tBOOH

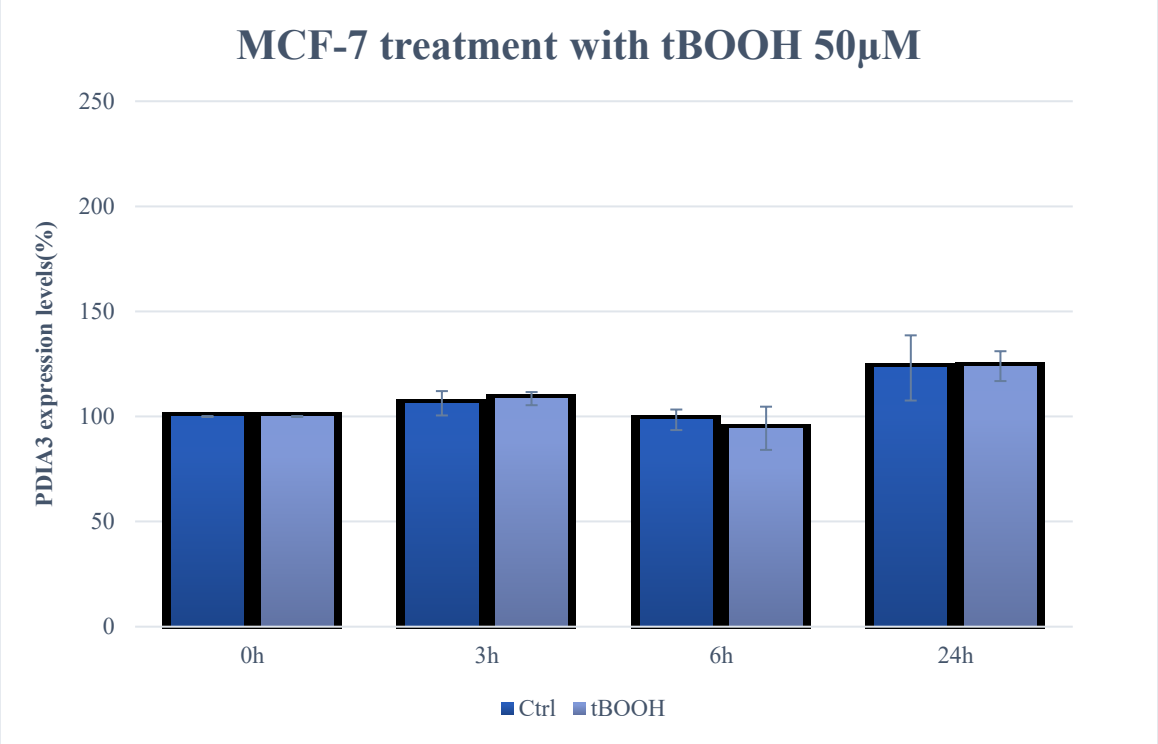

Figure 12: Levels of expression PDIA3 protein in MCF-7 with 50µM tBOOH

The data represent the mean ± S.D. (n=2)

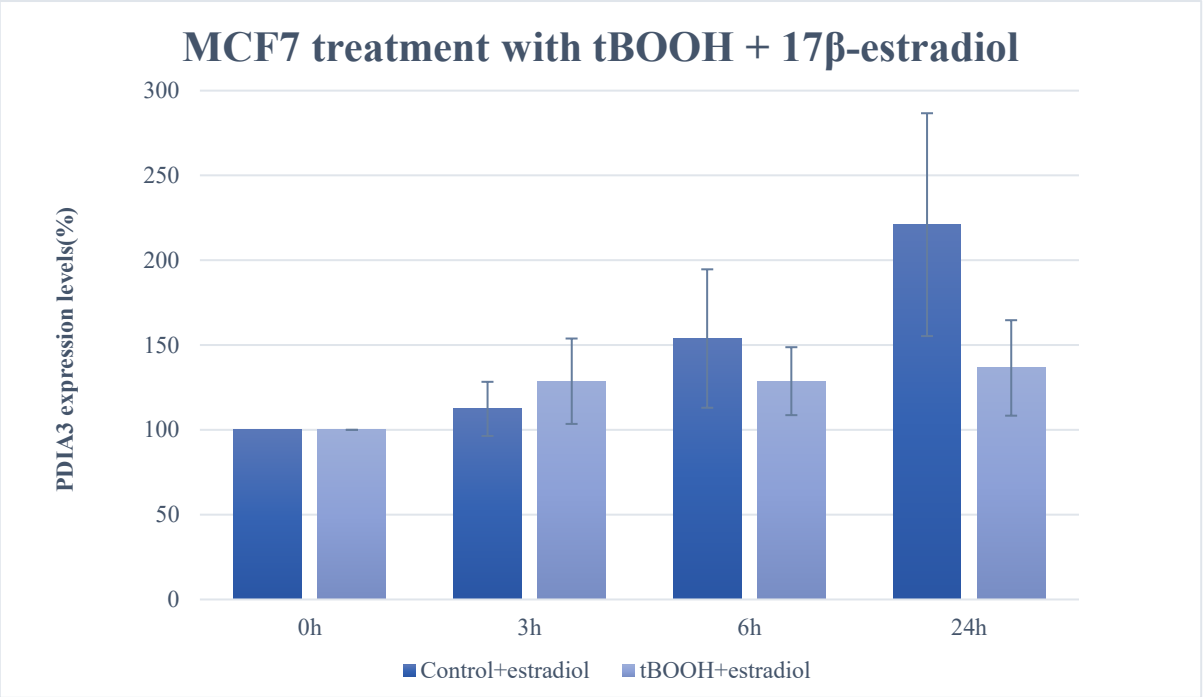

Figure 13: Expression levels of PDIA3 protein in 468 MDA-MB cells co-treated with 75μM tBOOH and 20nM 17β-estradiol. The data represent the mean ± S.D. (n=2)

After the treatment with tert-butyl hydroperoxide on the MCF-7 cell line, no significant variations were observed in PDIA3 expression levels.

Instead, from the results obtained after co-treatment with tBOOH (50μM) and 17ß-estradiol, we can deduce that there is a slight increase in the expression levels of PDIA3 after 3h, 6h and 24h.

What is important to emphasize is that from the data obtained by comparing the control and the treated sample after 6h and 24h of co-treatment, we can see a greater expression of the protein in the control stimulated with estrogen compared to the cells stimulated with the hormone and treated with tBOOH.

Therefore, the presence of estrogen causes a difference in PDIA3 expression levels between the control and the tBOOH treatment, which is not observed in the experiment in which the cells are not previously stimulated with the hormone.

Limitation of this work may be the fact that proliferating cells were used (in the medium was serum) and therefore it can make differences between treated and not treated cells (despite the antiproliferative effect of the tBOOH).

7 Discussion

From the data obtained, it is clear that both cell lines, MDA-MB 468 and MCF-7, respond to stress exposure with tert-butyl hydroperoxide differently in expression of PDIA3 protein. The difference can be seen in MDA-MB 468, where the PDIA3 protein levels rapidly decreased after 3h, at 6h the levels reached 50% of the original concentration. The concentration almost doubled after 24h. Unlike the MCF-7 line, there is no significant difference between the control sample and the stressed sample. MDA-MB 468 and MCF-7 cells differ by the presence of ERec. While MCF-7 cells express these receptors (ERec+) the MDA-MB 468 cell line is ERec-.

Limitation of this work may be the fact that the experiments were carried out with the proliferating cells (in the medium there were sera), and thus the differences in protein expression observed may be due to different numbers of cells in the individual groups, where plays role the antiproliferative effect of the used substances.

We stimulated both cell lines with estrogen and we expected an effect on MCF-7 cells but not on MDA-MB 468 ones, due to the absence of the ERec. In effect, stimulation with estrogen has a proliferative effect on MCF-7 but not on MDA-MB 468 cells. The results confirmed this hypothesis; in the MCF-7 line, expression of the PDIA3 protein increased after treatment with estradiol. On the contrary, MDA-MB 468 did not alter the expression of the protein, which is explained by the ERec negativity of these cells. The classical pathway activated by the direct interaction of estrogen with ERecs in the cytosol is responsible for the major transcriptional effects of estrogen mediated by ERecα and ERecß. The estrogen-receptor complex translocates into the nucleus where it is able to bind specific DNA sequences, the EREs (estrogen response elements) at the level of estrogen-responsive genes (Hewitt, S.C. *et al*., 2010). An important point to note is that PDI proteins show a structural homology with the binding domain of the α receptor for estrogen (ERec-α) and act as molecular chaperones in hormone-mediated gene regulation. In this case, we assume that after exposing the cells to 17β-estradiol, PDIA3 may help hormonal response with its chaperone and disulfide reductase activities, so an increase in transcription level or reduced degradation of the protein may be expected.

However, when we treated both cell lines with the hormone and stressed with t-BOOH, we saw some differences. In particular MDA-MB 468 cells stressed in absence or presence of the hormone behaved differently. While in the first experiment, after solely peroxide treatment, PDIA3 protein levels drastically decreased after 3 hours and then subsequently increased, co-treatment with estradiol even increased levels of protein at 3 and 24 hours. After 6 hours, the protein level was reduced. A probable explanation of this difference could be a different mechanism of action of the hormone. Instead of binding to its own receptor (ERec), estrogen can bind to other receptors such as to the estrogen growth factor (EGF) receptor. This receptor belongs to the superfamily of receptor tyrosine kinases (RTK), which are characterized by an extracellular ligand binding domain and is overexpressed in MDA-MB 468 cell line. EGF receptor is a tyrosine kinase receptor, which is able to activate numerous signaling cascades, leading to cellular proliferation and inhibition of apoptosis. This process is known as non-genomic effect of the hormone, where it can modulate cellular processes through binding to other cellular receptors different from the natural ones (Gaucci et al., 2013). When we compare these results with the PDIA3 protein expression in MCF-7 cells, we can observe the only significant change with estradiol alone, the explanation is suggested above. When we exposed the cell lines to stress with or without previous estradiol treatment, no changes were observed.

Considering this hypothesis and also previous study on the involvement of PDIA3 in the internalization and recycling of EGF receptor in MDA-MB 468 cell line, we explained the different response in MDA-MB 468 cells subjected to stress in presence or absence of the hormone (Gaucci *et al*., 2013). In the presence of the estadiol, PDIA3 protein may re-localize to sustain the activation of EGF receptor by the hormone and thus make the cell differently sensitive to the stressing condition compared to cells stressed in absence of estrogen. In fact, in MDA-MB 468 cells the hormone pre-teatment changes the response to t-BOOH, shifting the decrease in PDIA3 protein level from 3h to 6h. It seems that in hormone stimulated cells PDIA3 is able to better counteract the negative effect of t-BOOH probably due to its cellular redistribution.

A better response of MDA-MB 468 to the stress induced by t-BOOH may be the result of a higher cytosolic levels of PDIA3 compared to the ER. PDIA3 could be also localized closer to the cell membrane where the initial negative effects of t-BOOH may started. These theories are hypothetical and need to be verified. This effect cannot be observed in MCF-7 cells where the presence or absence of the hormone during the stress does not modify the cell response to the chemically induced stress.

8 Conclusion

In the presented study has been shown that chemically induced stress affects the expression of PDIA3 protein and that this expression varies between MDA-MB 468 and MCF-7 cell lines. It was further found that the cell lines responded to stress differently after previous exposure of cells to 17β-estradiol. It is believed that cells can respond to this fact either genomic via the estrogen receptor or non-genomic via the EGF receptor. The explanation may of course be different. The obtained results extend the information about PDIA3 and their use may contribute to further research that may lead to the treatment of certain diseases. The following research will focus on the mechanism that induces transcription of the PDIA3 protein under stress response. It is believed that this information has contributed to the understanding of the complex issue of this protein.

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