Nuclear Activation of proteasome in oxidative stress and aging

Thesis presented in fulfillment of
the thesis requirement for the degree of
Doctor of Philosophy in Natural Sciences (Dr. rer. Nat.)

Faculty of Natural Sciences
Universitat Hohenheim

Institut für Biologische Chemie und Ernährungswissenschaft
Lehrstuhl Biofunktionalität und Sicherheit von Lebensmitteln
Prof. Dr. med. Tilman Grune

Presented by
Betul Catalgol

Place of Birth: Turkey

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Index

Acknowledgement **1**

Index **2**

1. Introduction **5**

1.1 Oxidative stress in the nucleus 5

1.2 Nuclear proteasomal degradation 7

1.3 Poly(ADP)ribose polymerase-1 (PARP-1) and poly(ADP-ribosyl)ation reactions 8

1.4 Histones and their post-translational modifications 12

1.5 The proteasome-PARP-1 interaction 14

1.6 Goals 16

2. Materials and Methods **18**

2.1 Chemicals 18

2.2 Experiments in cell culture 19

2.2.1 Cell line propagation 19

2.2.1.1 Cell treatments with inhibitors and H_2O_2 19

2.2.1.2 Isolation of nucleus 20

2.2.1.3 Protein amount measurements 21

2.2.2 MTT viability test 22

2.2.3 Proteasome activity analysis 22

2.2.4 Immunoblot analysis 24

2.2.5 qPCR analysis 24

2.2.6 Protein carbonyl measurement in cell lysates 25

2.2.7 Comet assay 27

2.2.8 8-OHdG analysis 29

2.2.9 PARP activity measurement 30

2.3 In vitro experiments 31
2.3.1 Histone oxidation 31
2.3.2 Protein carbonyl measurement in isolated histones 32
2.3.2.1 ELISA 32
2.3.2.2 Western blot 32
2.3.3 Fluorescamine assay 33
2.3.4 Measurement of poly(ADP-ribosyl)ation of histones 34
2.3.4.1 Liquid scintillation counting 34
2.3.4.2 Western blot 34
2.4 Aging effects on the model 35
2.4.1 Cell culture 35
2.4.2 Immunoblot analysis 35
2.4.3 PARP activity measurement 36
2.4.4 Proteasome activity measurement 36
2.4.5 Poly(ADP-ribosyl)ation of proteasome 37

3. Results 38
3.1 Results in cell culture 38
3.1.1 MTT viability test 38
3.1.2 Proteasome activity 39
3.1.3 Immunoblot analysis 40
3.1.4 qPCR analysis 40
3.1.5 Protein carbonyl measurement in cell lysates 41
3.1.6 Comet assay and PARP activity 42
3.1.7 8-OhdG amounts 44
3.2 Results in isolated histones 44

3.2.1 Fluorescamine assay in isolated histones....44
3.2.2 Measurement of poly(ADP-ribosyl)ation of histones....45
3.2.3 Protein carbonyl measurement in isolated histones....45
3.3 Aging effects on the model....46
3.3.1 Immunoblot analysis....47
3.3.2 PARP activity changes in young and senescent fibroblasts....47
3.3.3 Proteasome activity changes in young and senescent fibroblasts....48
3.3.4 Poly(ADP-ribosyl)ation of proteasome in young and senescent fibroblasts....48

4. Discussion....50
4.1 Activation of the proteasome by PARP-1....50
4.2 Role of the proteasome in chromatin repair....53
4.3 The protesome-PARP-activation in the senescence process....55
4.4 Outlook into future research....57

5. Conclusion....59
Literature list....61
Figure list....73
Table list....74
Abbreviation list....75
Summary....78
Zusammenfassung....80
Supplement
1.Declaration / Erklärung....82
2.Curriculum Vitae and Publications....83

1 Introduction

1.1 Oxidative stress in the nucleus

Free radicals are atoms or molecules that contain unpaired electrons in their outer orbitals and this feature makes them to take place in oxidation reactions easily. Free radicals include several reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive species (RS), generated by diverse mechanisms, cause oxidative modifications of cellular components. The most prominent feature of RS is their high reactivity with biomolecules, causing their denaturation and inactivation. Several cellular systems exist to minimize oxidizing effects of RS are called antioxidant systems. Oxidative stress is referred to as an imbalance between the RS generation and the corresponding antioxidant defenses. Oxidative stress can produce injury by multiple pathways that overlap and interact in complex ways [1-3].

Nucleus is one of the targets of oxidative stress. Nuclear membranes act as a barrier to high molecular weight macromolecules and complexes. On the one hand, most oxidants and reactive species generated in the cytosol are able to reach the nucleus and chromatin through diffusion. On the other hand the nucleus can be a direct target of hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) formed by ionizing radiation or singlet oxygen ($^1O_2$) formation by ultraviolet radiation [4].

In human beings, tumor cells are frequently subjected to oxidation because of antitumor chemotherapy. A number of antitumor drugs act via the oxidation of nuclear material in the tumor cell. It is therefore important to know if tumor cells can effectively and precisely cope not only with oxidatively induced DNA damage, but also with nuclear protein oxidation (5). Free radical production following antitumor chemotherapy may be caused by

intratumor drug metabolism or redox cycling reactions [6]. Ultimately, the desirable DNA damage that chemotherapy causes in tumor cells is both the consequence of direct reaction of cytotoxic drugs with DNA, e.g., alkylating drugs [7], and strand breaks or nucleobase oxidation, caused by radical-producing redox cycling in close proximity to the chromatin, e.g., anthracyclines [5,6]. Thus the antioxidant defense and repair capacity of tumor cells may reduce the efficiency of antitumor chemotherapy and may be a major cause of chemotherapy resistance. Adaptation to oxidative stress appears to be one element in the development of long-term resistance to many chemotherapeutic drugs and the mechanisms of inducible tumor resistance to oxidation are of obvious importance [8].

In the nucleus different structures can be targets of oxidative damage. Radical attack to DNA can cause structural alterations such as base pair mutations, abasic sites, strand breaks, rearrangements, deletions, insertions and sequence amplification [9]. Numerous studies including nuclear proteins and isolated amino acids demonstrated that proteins are also susceptible to ROS attack [10]. Oxidative damage on proteins leads to oxidation of side chains in amino acid residues, the formation of protein-protein covalent and non-covalent cross-links and protein fragmentation due to oxidation of the peptide backbone [11].

A limited number of studies refer to modifications of nucleoproteins upon ROS attack. Oxidative lesions in free DNA were shown to be higher than in chromatin indicating that nucleosomal histone proteins protect DNA from oxidative damage [12,13]. Thus association of DNA with histones in chromatin is generally considered to have beneficial effects for the maintenance of DNA integrity [14]. Also multiple pathways and repair systems repair in vivo oxidative DNA damage. For instance strand breaks are annealed and modified bases are removed by a variety of enzymes [15].

1.2 Nuclear proteasomal degradation

After oxidation of proteins, living cells try to rescue defect polypeptides and restore their function. Mammalian cells exhibit only limited direct repair mechanisms and most oxidized proteins undergo selective proteolysis. The degradation of proteins is a physiological process required to maintain normal cellular function. Therefore, cells have developed highly regulated intracellular proteolytic systems responsible for the removal of such non-functional proteins before they start to aggregate. Mammalian cells contain several pathways for general protein breakdown, comprising membrane proteases, lysosomal cathepsins, calcium-activated calpains, caspases, mitochondrial proteases and the proteasomal system [16-19]. Besides all proteolytic systems, the major proteolytic system responsible for the removal of oxidized cytosolic and nuclear proteins is the proteasomal system [20].

The proteasome is a large multicatalytic protease that exists in all eukaryotic cells and it is responsible for the major part of intracellular proteolysis events [21,22]. Within the proteasomal system for cytosol and nucleus, the 20S proteasome is the core particle of the proteasome and degrades proteins in an ATP and ubiquitin independent manner. The 20S core proteasome is regulated or activated by a number of regulators, including the so called 19S regulator and 11S regulator. It is generally believed that the 20S proteasome core complex is sufficient for the degradation of oxidized proteins [23-25]. The main accepted proteasomal cleavage of the proteins is on the carboxyl side of basic, hydrophobic and acidic amino acids (trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide-hydrolase activity).

Tumor cells seem to have much higher proteasome activity than do nonmalignant cells and most of the extra proteasome appears to be localized in the nucleus [26]. Following

oxidation reactions, selective degradation of oxidatively damaged histones might be a necessary cellular function for maintaining chromatin integrity. If true, a detailed understanding of such mechanisms would be important in antitumor chemotherapy and radiation therapy [8].

1.3 Poly(ADP-ribose)polymerase-1 (PARP-1) and poly(ADP-ribosyl)ation reactions

Poly(ADP-ribosyl)ation is a severe post-translational modification of glutamate, aspartate and lysine residues of nuclear proteins (acceptor proteins) and represents an immediate eukaryotic cellular response to DNA damage as induced by ionizing radiation, alkylating agents and oxidants. Poly(ADP-ribosyl)ation is catalysed by poly(ADP-ribose) polymerases (PARPs). PARPs use the ADP-ribosyl moiety of NAD^+ to covalently modify acceptor proteins in successive transfer cycles thus creating linear and/or branched chains of poly(ADP-ribose) (pADPr). In the presence of DNA strand breaks, PARP activity and the levels of ADPr polymers can be increased by 10-500 fold [27-29], while celular NAD^+ levels are correspondingly reduced [30]. pADPr, is a homopolymer of ADP-ribose (ADPr) units linked by glycosidic bonds [31,32].

The polymer is most probably attached onto proteins via the γ-carboxy groups of glutamic acid residues [33,34]. The polymers of ADPr are degraded rapidly by poly(ADP-ribose) glycohydrolase (PARG) in vivo, which accounts for their transient nature in living cells [28]. PARG splits the unique ribose-ribose linkages between ADP-ribosyl units of the

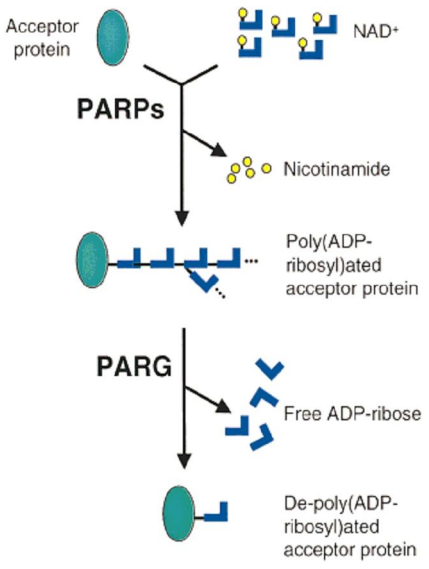


Fig.1.1 PARP and PARG in the poly (ADP-ribosyl)ation reactions [35]

polymer, thus creating free ADP-ribose (Fig.1.1) [35]. In addition, branched and short polymers are degraded more slowly than long and linear polymers [36,37]. The endoglycosylase activity of PARG is physiologically important, because it is responsible for the generation of protein-free ADPr polymers that can interact with histones and other nuclear proteins [38].

Today seven PARP-like enzymes are known with predominantly nuclear localisation. Among these 116 kDa PARP-1 is by far the best studied and most abundant member of the PARP protein family and appears to be the major poly(ADP-ribosyl)ating enzyme in higher eukaryotes after DNA damage [39]. It is not yet known whether other enzymes have the ability to catalyse all the reactions necessary to produce branched pADPr polymer, or whether they can only synthesize linear polymers [40]. The human gene encoding the PARP-1 is termed ADPRT. The ADPRT gene is constitutively expressed at a level depending on the type of tissue or cell [41].

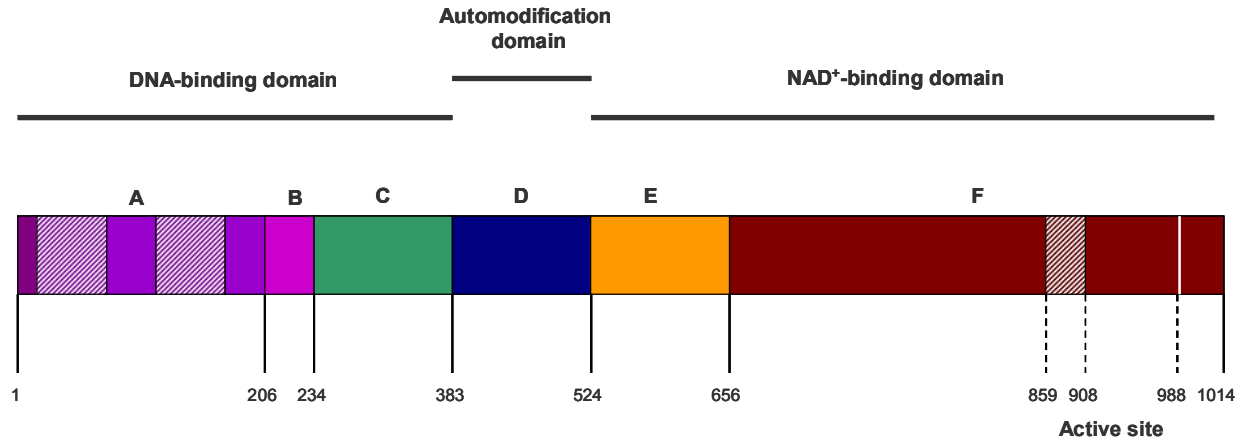


Fig.1.2 Domain structure of PARP-1. Three major proteolytic domains can be broken down into modules (A-F). Numbers refer to amino acid positions [Modified from 35]

PARP-1 is a highly conserved enzyme that has been detected in most eukaryotes and displays a characteristic three-domain structure (Fig.1.2). Its aminoterminal DNA binding domain binds to single- or double-strand breaks with high affinity, and induces immediate activation of the catalytic centre in the carboxyterminal NAD^+-binding domain. The crystal structure of NAD^+-binding domain shows homology with bacterial toxins that act as mono-ADP-ribosyl transferases [42]. The enzyme requires the functional DNA-binding domain to perform efficient poly(ADP-ribosyl)ation [43,44]. Automodification domain is basic and it contains the majority of the 15 glutamic acid residues that would be involved in PARP automodification [45,46]. This domain can not serve as an acceptor for ADPr chains unless it is associated with the catalytic and DNA binding domain of the enzyme [47]. Several proteins have been shown to interact with PARP through its automodification domain. These include especially human ubiquitin conjugating enzyme (hUBC9) and histones [48,49]. Also PARP-1 is a metalloenzyme that binds zinc molecules specifically [50].

More than 30 nuclear substrates of PARPs have been identified in vivo and in vitro. Most of the physiological substrates of poly(ADP-ribosyl)ation reactions are nuclear proteins (Tab.1.1). In intact cells, PARP-1 itself is the major acceptor protein, as it catalyses its own automodification to complete its shuttling off DNA strand breaks [51].

Importantly, since poly(ADP-ribose) is turned over rapidly due to efficient degradation by PARG, the existence of poly(ADP-ribose) in intact cells is not permanent and occurs following the DNA strand breaks (Fig.1.3). The total dependence of poly(ADP-ribosyl)ation on DNA strand breaks strongly suggests that this posttranslational modification is involved in the metabolism of nucleic acids [40] identified as protective with regard to cell survival and maintenance of genomic stability of cells under genotoxic

stress. PARP-1 has also been implicated in the regulation of transcription and proteasomal function, playing a crucial role in protein turnover [52,53].

Potential Function	Acceptor
Modulation of chromatin structure	Histones PARP Topoisomerases
DNA synthesis	DNA ligases DNA polymerases Topoisomerase II
DNA repair	PARP DNA ligases DNA polymerases Histones
Transcription	RNA polymerases Fos p53
Cell cycle	Fos p53

Tab. 1.1 Substrates of poly(ADP-ribosyl)ation reactions with their proven functions [modified from 40].

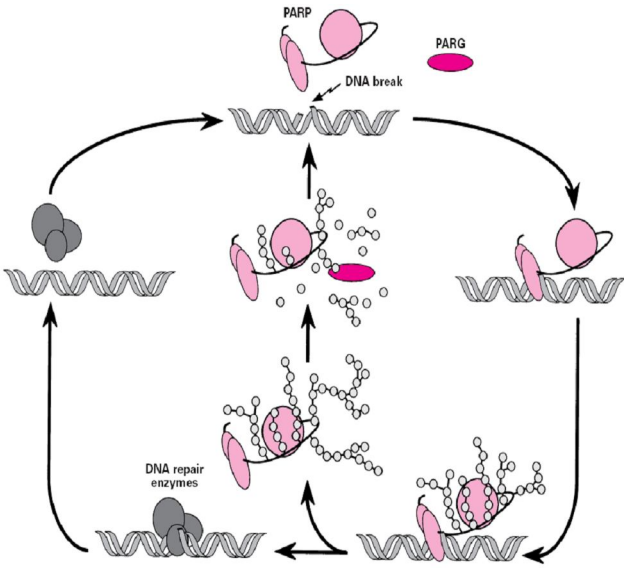


Fig.1.3 Automodification of PARP and repair of DNA strand breaks. The beads on PARP represent pADPr [40]

Non-covalent interactions between free pADPr and proteins are of importance. Indeed, it has been demonstrated that interactions between the polymer and nuclear proteins such as p53 and histones are very stable, and could modify the functional properties of these and other proteins in living cells [54-56].

1.4 Histones and their post-translational modifications

Histones are the chief protein components of chromatin. They act as spools around which DNA winds, and they play a role in gene regulation. Without histones, the unwound DNA in chromosomes would be very long. There are six classes of histones organized into two classes which are core histones (H2A, H2B, H3, H4) and linker histones (H1, H5) [57].

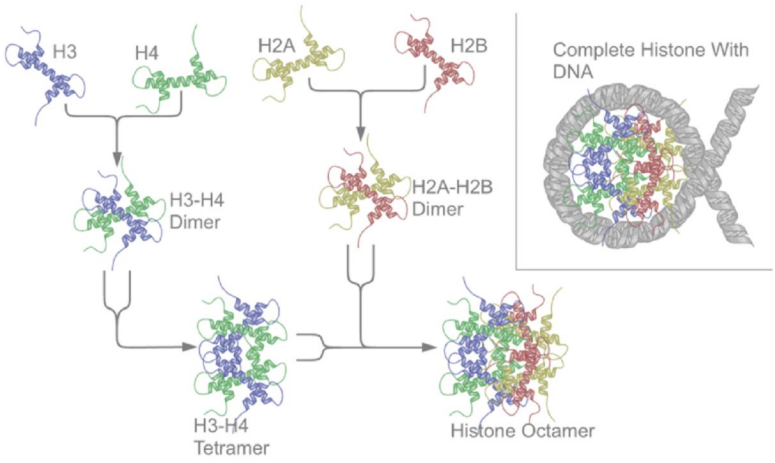


Fig.1.4 Assembly of the core histones into the nucleosome [58].

Two of each of the core histones assemble to form one octameric nucleosome core particle. The linker histone H1 binds the nucleosome and the entry and exit sites of the DNA, thus locking the DNA into place and allowing the formation of higher order structure. The most basic such formation is the 10 nm fiber or beads on a string conformation. This involves the wrapping of DNA around nucleosomes with approximately 50 base pairs of DNA

spaced between each nucleosome (also referred to as linker DNA). The assembled histones and DNA is called chromatin [58].

Histones are subject to post-translational modifications by enzymes primarily on their N-terminal tails, but also in their globular domains. Such modifications include methylation, citrullination, acetylation, phosphorylation, sumoylation, ubiquitination, and ADP-ribosylation. These modifications affect their function of gene regulation. Furthermore, histone modifications display alterations during development, the cell cycle, the particular chromatin fraction within the same cell type or the variant form within the same histone fraction [59]. Poly(ADP-ribosyl)ation is the most dramatic post-translational modification of histones in nuclei as well as in nucleosomes [60,61]. The onset of poly(ADPribosyl)ation may be accompanied by automodification of PARP-1 and probably also by modification of histone proteins. Through these heteromodifications as well as via non-covalent binding of histones to the automodified PARP-1 a large and increasingly negatively charged, histone–pADPR–PARP-1 complex may be formed at the site of the DNA lesion, which eventually dissociates from the DNA. It was shown that, histones H1 and core histones are the main histones poly(ADP-ribosyl)ated in vivo during DNA repair [62,63]. It has been also shown that histones can bind branched polymers of ADPr in a non-covalent manner, with the following relative affinity: H1>H2B>H2A>H3>H4 [64]. The kinetics of DNA repair are greatly influenced by the presence of histones on damaged DNA. The well established poly(ADP-ribosyl)ation of histones in response to DNA damage strongly suggests that PARP plays an important role in DNA repair when DNA is structured in chromatin [65,66]. Other studies have shown that maximal enzyme activity for PARP was associated with trinucleosomes and tetranucleosomes and histone requires NAD^+ concentrations higher then 100 μM and 1 mM to be poly(ADP-ribosyl)ated . By the

way histones stimulate PARP activity up to 20-fold. They act as allosteric activators of the enzyme by decreasing the K_m of PARP for NAD^+ and by increasing its *V*max [67-69].

Additionally, nucleosomal histones are known to protect DNA from free radical-mediated damage [70] and are susceptible to oxidative damage in vitro [71]. It is therefore necessary to efficiently degrade oxidatively damaged histones in order to maintain genomic integrity. Histone proteins are not synthesized only during a limited phase of the cell cycle, but some are produced in a constitutive manner, even in nonproliferating cells. Due to the long lifespan and low turnover rate of histones, proteolytic reactions are required to be highly selective and well regulated [72].

Core histones demonstrate remarkable sequence conservation, whereas the linker histones, especially the histone H1 protein family, diverge significantly in sequence and structure [73]. Histone H1 is considered to exhibit a specific role in the transcriptional regulation of the expression of particular genes and might be required more for the assembly of specific regulatory nucleoprotein complexes than for simply maintaining the nucleosome structure of chromatin [74,75]. Thus, in contrast to the core histones, functional impairment of the histone H1 protein family by oxidative damage might severely disturb the transcriptional regulation of specific genes and therefore essential cellular functions. The higher susceptibility of histone H1 to the proteasome both in vitro [71] and in living cells might represent an evolutionary advantage by enabling a rapid reconstitution of disturbed transcription regulation after acute or chronic oxidative challenge.

1.5 The proteasome-PARP-1 interaction

In the nucleus of mammalian cells, the rapid activation of the 20S proteasome activity in response to oxidative stress is accompanied by and depends on poly(ADP-ribosyl)ation [8,

76-79]. In vitro studies revealed that the 20S proteasome interacts with pADPR or poly(ADP-ribosyl)ated PARP-1, leading to the specific stimulation of its proteolytic activity [80]. This interaction however, was restricted to long pADPR polymers. Furthermore no covalent attachment of the poly(ADP-ribose) polymer to the 20S proteasome has been demonstrated until now. Therefore, interaction of PARP-1 with the 20S proteasome requires previous automodification by poly-(ADP-ribosyl)ation, and association of the resulting poly-(ADP-ribosyl)ated PARP-1 with the 20S proteasome is supposed to occur via non-covalent, but specific binding to pADPR. Subsequent studies using different experimental approaches and several cellular models support the notion that PARP-1 specifically activates the nuclear proteasome in response to ROS attack [76-78].

As mentioned above there exist several kinds of PARP enzymes. However, due to the clear effects of PARP-1 in in vitro experiments and due to the co-immunoprecipitation experiments, it is suggested that PARP-1 is involved in the proteasome activation.

Simultaneously, activation of the nuclear proteasome through non-covalent interaction with the automodified PARP-1 results in its enhanced proteolytic activity in close vicinity to the poly(ADP-ribosyl)ation-driven histone shuttling event. In addition, heteromodified histones might also lead to activation of the proteasome. The proteasome, in turn, would rapidly and selectively degrade the oxidatively damaged histones whose proteolytic susceptibility is increased due to their oxidative modifications. Furthermore, it was hypothesised that oxidatively damaged histones may be excluded from the histone shuttling complex, thus being degraded either in DNA bound or soluble form [77]. At this time, the damaged DNA should be essentially liberated from associated proteins, thus being accessible for the DNA repair machinery as pictured in the PARP-1-BER models. In this context, removal of damaged histones from the DNA by the proteasome might even

facilitate DNA repair by supporting PARP-1-mediated chromatin decondensation or repair complex formation at the damaged sites. Following DNA repair processes, poly(ADP-ribose) glycohydrolase (PARG), might strip automodified PARP-1 of its pADPR polymer, thereby liberating the histone proteins, which could then reassemble into a native chromatin structure [78,79].

1.6 Goals

Since several lines of evidence exist for a role of the PARP and proteasome interaction in pathophysiolgy, I decided to further clarify the role of this interaction in oxidatively stressed cells. For this purpose the following questions were addressed:

1) Characterize the PARP-proteasome-interaction in HT22 cells

- To investigate the role of selective proteasomal degradation of oxidized proteins in the nucleus of HT22 cells
- To characterize the role of PARP in the activation of proteasomal degradation.
- To correlate the mRNA expression of PARP-1 with increased oxidative damage and proteasomal degradation.

2) Establish the role of PARP-proteasome-interaction in the 'chromatin-repair'

- To demonstrate the role of PARP-1-proteasome-interaction in the removal of oxidized proteins
- To investigate the role of proteasome-mediated breakdown of oxidized proteins in DNA repair following oxidative damage in HT22 cells measured by single strand breaks and 8OhdG amount

3) Characterize the role of histone oxidation in the PAR-histone shuttling

- To see the protein carbonyl formation in isolated histones

- To see the difference in the proteasomal degradation of oxidized histones with and without poly(ADP-ribosyl)ation.

- To observe the poly(ADP-ribosyl)ation of histones following H_2O_2 treatment.

4.) Modulation of the PARP-proteasome interaction in the senescence process

- To see the PARP-1 and proteasome protein expressions in young, middle aged and senescent fibroblasts

- To see the PARP and proteasome activity changes in oxidatively damaged young, middle aged and senescent cells.

- To prove the main role of PARP in proteasome activation in young and senescent cells.

2 Materials and Methods

2.1 Chemicals

Antibodies:

Anti-Dinitrophenyl (DNP)-rabbit-IgG antiserum (Sigma-Aldrich D9656)

Monoclonal Anti-Rabbit IgG (γ-chain specific), Peroxidase Clone RG-96 (Sigma-Aldrich A1949)

20S proteasome 'core' subunits, rabbit polyclonal antibody (Biomol PW8155)

Anti-poly(ADP-ribose), mouse monoclonal antibody (Biomol SA-216)

PARP antibody, rabbit polyclonal (Cell Signaling 9542)

Histone H4 antibody (Cell Signaling 2592)

Goat Anti-Rabbit IgG, H & L Chain Specific Peroxidase Conjugate (Calbiochem 401315)

Rabbit Anti-Mouse IgG, H & L Chain Specific Peroxidase Conjugate (Calbiochem 402335)

Chemicals:

20xLumiGLO Reagent and 20x Peroxide (Cell Signaling 7003)

PageRuler Unstained Protein Ladder (Fermentas SM0661)

iScript cDNA Synthesis Kit (Biorad 170-8890)

Qiagen DNeasy Blood Tissue Kit, Cat no. 69504

IQ SYBR Green Supermix (Biorad 170-8882)

Highly Sensitive 8-OhdG Check ELISA kit (JaICA, KOG-HS10E)

Bio Rad Protein Assay (Bradford) (Biorad 500-0006)

Bio Rad, DC Protein Assay, Reagent A (Biorad 500-0113)

Bio Rad, DC Protein Assay, Reagent S (Biorad 500-0115)

Bio Rad, DC Protein Assay, Reagent B (Biorad 500-0114)

[carbonyl-14C]Nicotinamide adenine dinucleotide, ammonium salt (GE Healthcare CFA372)

PARP-1 human recombinant, expressed in E.coli (Sigma-Aldrich P0996)

Nonidet P-40, Octylphenolpoly(ethyleneglycolether) (Roche 11 332 473 001)

All the other chemicals used were obtained from Sigma.

2.2 Experiments in cell culture

2.2.1 Cell line propagation

HT22 cells (mouse hippocampal neuronal cells) were cultured at 37 °C and 5% CO_2 under standard conditions. A high glucose DMEM medium containing an additional 0.35% glucose was used supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomysin and 1% glutamine. Cells initially were seeded at a density of 0.2 $x10^6$ cells/ml and then grown for three days to a density of $3x10^6$ cells/ml.

2.2.1.1 Cell treatments with inhibitors and H_2O_2

Firstly, cells were treated with different concentrations of hydrogen peroxide (H_2O_2) for various time periods (Tab.2.1). The concentrations and time periods were chosen according to the MTT viability test results (Fig.3.1). Used concentrations and time periods provided viability higher than 75%.

H_2O_2 solutions were prepared as:

1M stock solution: 114 µl 30% H_2O_2 + 886 µl PBS with Ca & Mg

0.25 mM: 7.5 µl stock solution / 30 ml PBS with Ca & Mg

0.50 mM: 15 µl stock solution / 30 ml PBS with Ca & Mg

0.75 mM: 22.5 µl stock solution / 30 ml PBS with Ca & Mg

1.00 mM: 30 µl stock solution / 30 ml PBS with Ca & Mg

H_2O_2 concentrations (mM) for 30 min	0.00 – 0.25 – 0.50 – 0.75 – 1.00
Time durations (min) with 1.00 mM H_2O_2	0 – 10 – 20 – 30 – 40 – 50 – 60

Tab.2.1 H_2O_2 concentrations and time durations for the incubation of HT22 cells

Incubations with several inhibitors were done as indicated in the Tab.2.2. All incubations were carried in 3 ml PBS with Ca & Mg.

Chemical	Function	Concentration (Stock solution)	End concentration	Duration of pretreatment
3-ABA	PARP-1 inhibition	0.5 M in PBS	5 mM	30 min
PJ-34	PARP-1 inhibition	4 mM in PBS	40 µM	30 min
LC	20S proteasome inhibition	1328 µM in PBS	20 µM	30 min

Tab.2.2 Inhibitors used in PARP-1 and 20S proteasome inhibition with HT22 cells.

2.2.1.2 Isolation of nucleus

Following the incubations, the cells were washed once with ice-cold PBS, scraped with ice- cold PBS into an eppendorf tube and centrifuged at 2000 rpm for 5 min at 4°C. The supernatants were removed and lysis buffer (Tab.2.3) was added with the final volume equivalent to five times the pellet volume, incubated on ice for 10 min. 10% NP-40 was added in the amount necessary to obtain a final concentration of 1%, mixed by pipetting, and centrifuged at 15000 rpm for 1 min at 4°C. Supernatants were removed and discarded. The same volume of extraction buffer (Tab.2.4) as lysis buffer was added and incubated on

ice for 20 min with vigorous vortexing every 5 min, centrifuged at 15000 rpm for 10 min at 4°C and the supernatants were used for the assays [81].

Lysis buffer
10 mM HEPES, pH 7.8
10 mM KCl
2 mM $MgCl_2$
0.1 mM EDTA

Tab. 2.3 Lysis buffer used in the nuclear extraction of HT22 cells

Extraction buffer
50 mM HEPES, pH 7.8
50 mM KCl
300 mM NaCl
0.1 mM EDTA
10% Glycerol

Tab. 2.4 Extraction buffer used in the nuclear extraction of HT22 cells

2.2.1.3 Protein amount measurements

Bradford Assay

In 96-well plate, 4 µl of standard or sample solutions were incubated for 5 min with 200 µl of Bio-Rad reagent and absorbance was measured using 590 nm filter (Reference filter; 750 nm) with UV/Vis-Multiwell reader. The amounts of proteins were calculated according to the standad curve as mg protein/ml [82].

Lowry Assay

Reagent A, S and B were obtained from Bio-Rad. Reagent A' was prepared freshly by shaking. Reagent A and S as 1/50 (v/v). In 96-well plate, 5 µl of standard or sample solutions were incubated with 25 µl of Reagent A' and 200 µl of Reagent B for 15 min and absorbance was measured using 750 nm filter (Reference filter; 750 nm) with UV/Vis-

Multiwell reader. The amounts of proteins were calculated according to the standad curve as mg protein/ml [83].

2.2.2 MTT viability test

MTT test was performed to determine treatment parameters in HT22 cells following the conditions showed in Tab.2.1. The concentration higher than 1.00 mM of H_2O_2 for 30 min and the duration longer than 60 min with 1.00 mM H_2O_2 caused a cell death higher than 75%. In the viability test, $2x10^6$ cells were used in 60 mm petri dishes. Following the indicated treatments, incubation solutions (generally in PBS) were discarded. 100 µl of MTT solution was mixed with 3 ml DMEM and incubated for 2 h at 37 °C and 5% CO_2. Following this incubation period, medium was discarded and 1 ml of solubilizing reagent was added, incubated for 5 min and the absorbance of 50 µl of supernatant were measured in the 96-well plate using 590 nm (Reference filter; 660 nm) with UV/Vis-Multiwell reader. Calculations were done related to te absorbance of control samples which was equaled to 100%.

MTT (Thiazolyl blue tetrazolium bromide)	10 mg MTT / 1 ml PBS
Solubilizing reagent	10 g SDS / 99.4 ml DMSO + 0.6 ml acetic acid

Tab. 2.5 Solutions for MTT viability assay

2.2.3 Proteasome activity analysis

Following the treatments of HT22 cells in Tab 2.1 indicated conditions, nuclear extracts were obtained as described in 2.2.1.2 and these extracts were used in proteasome activity analysis. The design of one well in 96-well plate was done as shown in Tab. 2.6. The reaction was carried out at 37 °C for 30 min. As a result of this reaction, chymotrypsin-like activity of 20S proteasome was determined. The fluorescence of the liberated MCA was

monitored at 365 nm excitation and 460 nm emission wavelengths. The amount of substrate degradation is calculated using the calibration curve prepared with free MCA standards as nmol MCA / mg protein x min.

Content	Amounts
Nuclear extract	10 µl
Proteolysis buffer	33.3 µl
DTT	0.2 µl
H_20	45.5 µl
2-Deoxy-D-glucose	9 µl
Hexokinase	12 µl
Suc-LLVY-MCA	10 µl

Tab. 2.6 One well design for the proteasome activity analysis

Proteolysis buffer	0.45 M Tris, 90 mM KCl, 15 mM $Mg(CH_3COO)_2 \cdot 4 H_2O$, 15 mM $MgCl_2$ x $6H_2O$ (pH 8.2)
DTT (Dithiothreoitol) (1M)	0.309 g DTT / 2 ml ice-cold H_2O
Suc-LLVY-MCA (2 mM)	10 mg / 6.55 ml DMSO
2-Deoxyglucose (200 mM)	0.33 g / 5 ml H_2O
Hexokinase	1 mg / 1 ml H_2O
MCA standards	1.stock (100 mM): 88 mg / 5 ml DMSO 2.stock (100 µM): 10 µl 1.stock + 9.99 ml dilution buffer (1/3 diluted proteolysis buffer containing 10% DMSO) Standards: 10 µM: 100 µl 2.stock + 900 µl dilution buffer 5 µM: 50 µl 2.stock + 950 µl dilution buffer 2 µM: 20 µl 2.stock + 980 µl dilution buffer 1 µM: 10 µl 2.stock + 990 µl dilution buffer 0.5 µM: 5 µl 2.stock + 995 µl dilution buffer

Tab. 2.7 Solutions for proteasome activity analysis

2.2.4 Immunoblot analysis

Following the treatments of HT22 cells with 1.00 mM H_2O_2 in the 0-30-50 min durations, nuclear extracts were obtained as described in 2.2.1.2 and whole cell lysates were obtained at 4 ºC using 10mM Tris HCl pH 7.5 buffer containing 1 mM Pefabloc, 0.9% NP-40, 0.1% SDS. Nuclear extracts and whole cell lysates were blotted separately. The protein concentrations of the nuclear extracts were determined according to Bradford [82] while the protein concentrations of whole cell lysates were determined according to Lowry [83]. 30 µg of total protein in reducing Laemmli-buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerol, 0.03% bromophenol blue) were denatured at 95 °C for 5 min and applied to SDS-PAGE of 12% (w/v) acrylamide for proteasome and of 7.5% (w/v) acrylamide for PARP-1, followed by electrophoresis and blotting onto nitrocellulose membrane according to standard procedures. Immunodetections were performed with the following antibodies: rabbit polyclonal anti-PARP (Cell Signaling) at 1:1000 dilution, rabbit polyclonal anti-20S proteasome 'core' subunits at (Biomol) 1:1000 dilution. After exposure to peroxidase-coupled secondary antibodies (Calbiochem), membranes were developed using Lumi-Light western blotting substrate (Cell Signaling).

2.2.5 qPCR analysis

Following the treatments of HT22 cells with 1.00 mM H_2O_2 for the time periods indicated in Tab. 2.1, cells were lysed and total RNA was isolated using the Qiagen RNeasy Mini Kit RNA isolation was carried out according to the manufacturer's protocol as follow: $5x10^6$ cells were disrupted by adding 350 µl buffer RLT. Cell lysates were collected with a cell scraper and mixed by pipetting. 350 µl of 70% ethanol was added to the lysate and mixed well by pipetting. The samples were pipetted into a RNeasy spin column, centrifuged for 15s at 14000 rpm. Flow through was discarded, 700 µl of buffer RW1 was pipetted into the column and centrifuged for 15s at 14000 rpm to wash. Flow through was

discarded, 500 µl of buffer RPE was pipetted into the column and centrifuged for 15s at 14000 rpm to wash. Flow through was discarded, 500 µl of buffer RPE was pipetted into the column and centrifuged for 3 min at 14000 rpm to wash. Spin column was transferred in a new collection tube, 50 µl of RNase-free water was pipetted directly onto the membrane, centrifuged for 1 min at 14000 rpm to elute the RNA. The amount and purity of the extracted RNA were determined via spectrophotometry in a Smartspec 3000 (BioRad). An iScript cDNA synthesis kit and 1 µg of RNA were used for cDNA synthesis. Quantitative real-time PCR was performed using a BioRad iCycler 3.0 and the BioRad IQ Sybr Green reaction mixture. The sequences of primers used in this work were as follows: mouse PARP-1 left: TGAAAGGGACGAACTCCTATT, mouse PARP-1 right: GGCATCTGCTCAAGTTTGT-TA.

2.2.6 Protein carbonyl measurement in cell lysates

Protein carbonyl amounts were measured in the cell lysates 0.5-2-3 h after 1.00 mM H_2O_2 treatments of HT22 cells for 30 min. These post incubations following H_2O_2 treatment of the cells were carried out in DMEM medium. This experiment was to determine the recovery from carbonyl formation by the time and the effects of 3-ABA and LC on this recovery. Carbonyls were determined in cell lysates (4 mg/ml in lysis buffer) by an ELISA as introduced by Buss et al. [84]. According to the protein amount used, different concentrations of BSA standard solutions were prepared for standard curve. The onset of the experiment was as follow:

15 µl of samples or standards + 45 µl of fresh DNP solution

Incubated for 45 min at room temperature in the dark with continuous shaking

↓

5 µl of the incubation solution + 1 ml coating buffer

↓

3x200 µl were loaded into 96-well Nunc Immuno Plate Maxisorb
Incubated overnight at 4 °C

↓

Samples were discarded which are not absorbed onto the plate

Plates were washed with PBS to remove excess DNP

↓

250 µl of blocking solution for 1.5 h at room temperature in the dark

↓

200 µl anti-DNP antibody for 1 h at 37 °C

↓

Washed three times with PBS containing 0.1% Tween 20

↓

200 µl anti-rabbit-IgG-POD-antibody for 1 h at room temperature in the dark

↓

Washed three times with PBS containing 0.1% Tween 20

↓

200 µl substrate solution for 15 min at 37 °C

↓

The reaction was stopped with 100 µl of 2.5 M H_2SO_4

↓

Absorbance was measured at 492 nm wavelength using a microplate reader (BioTek Instruments EL 340).

Lysis buffer	200 µl of 500 µM HEPES + 40 µl of 250 mM DTT + 9.76 ml H_2O
DNP buffer	6 M Guanidinehydrocloride + 0.5 M KH_2PO_4 pH 2.5
DNP solution	10 mM = 2 mg/ml in DNP buffer
Coating buffer	0.71 g Na_2HPO_4 + 0.6 g NaH_2PO_4 + 4.1 g NaCl in 500 ml H_2O (pH 7.0)
Blocking solution	10 ml 11 mg/ml BSA + 90 ml PBS containing 0.1% Tween 20
Developer	0.71 g Na_2HPO_4 + 0.5 g citric acid in 100 ml H_2O
Anti-DNP-antibody (rabbit) (Sigma D-9656)	1:1000 dilution in blocking solution
Anti-rabbit-IgG-POD-antibody (Sigma A-1949, monoclonal)	1:10000 dilution in blocking solution
Substrate solution (60 mg tablet o-phenylenediamine, Sigma P-1063)	60 mg / 5 ml in developer ⟶ 1 ml + 19 ml developer + 8 µl H_2O_2
Oxidized BSA	50 mg/ml in 50 mM hypochloric acid for 2 h at room temperature incubation

Tab. 2.8 Solutions for protein carbonyl measurement by ELISA

2.2.7 Comet Assay

HT22 cells were seeded in 12-well plate as 50000 cells/well. Confluent cells were used in the experiment. Object slides were covered with normal melting agarose and kept until use. Low melting agarose was boiled in microwave until dissolved, aliquoted in 2 ml eppendorf tubes and kept on the 40 °C heating block. Following the incubations, cells were trypsinized and 400 µl of 1% LMA added onto the wells. 70 µl of the suspension pipetted onto the covered object slides and covered each with cover glass and kept at 4 °C for 15-20 min. Slides were incubated in the Triton-X 100 solution for 1h at 4°C and in the

electrophorese buffer for 40 min at 4°C. Electrophoresis was run 25V, 300 mA for 30 min. Slides were washed three times with neutralizing buffer, fixed for 15 min with fixative and comets were determined with fluorescence microscopy.

Treatment design

Dose dependent DNA damage: 0.1-0.2-0.3-0.4-0.5 mM H_2O_2 in PBS with Ca&Mg for 30 min.

Recovery following H_2O_2 treatment: Incubation with 0.4 mM H_2O_2 in PBS with Ca&Mg for 30 min ⟶ Incubation in medium for 0-1-2-3-4-6-24 hours.

Effects of LC, 3-ABA and PJ-34 on the recovery: 30 min incubation with 20 µM LC, 5 mM 3-ABA, 40 µM PJ-34 in PBS with Ca&Mg (for control samples, incubation with PBS with Ca&Mg) ⟶ Incubation with 0.4 mM H_2O_2 in PBS with Ca&Mg for 30 min ⟶ Incubation in medium for 3 hours.

Normal melting (NMA) and low-melting agarose (LMA)	1% in PBS
PBS	8 g NaCl + 0.2 g KCl + 0.2 g KH_2PO_4 + 1.44 g Na_2HPO_4 in 1000 ml H_2O (pH 7.4).
Lysis solution	146 g NaCl + 37 g Na_2EDTA + 1.2 g Tris Base in 1000 ml H_2O (pH 10).
Triton-X 100 solution	1% in lysis solution (prepared fresh)
Electrophoresis solution	12.1 g NaOH + 0.37 g Na_2EDTA in 1000 ml H_2O
Neutralizing solution	48.5 g Tris base in 1000 ml H_2O (pH 7.5)
Fixative	100% methanol

Tab. 2.9 Solutions for COMET assay

2.2.8 8-OHdG analysis

Treatment design

20 µM LC and 0.4 mM H_2O_2 at the end concentrations were used in the incubations. Subconfluent HT22 cells in petri dishes;

-30 min in PBS with Ca&Mg + 30 min in PBS with Ca&Mg ⟶ The cells were scraped

-30 min in PBS with Ca&Mg + 30 min in PBS with Ca&Mg + 3h in medium ⟶ The cells were scraped

-30 min in PBS with Ca&Mg + 30 min H_2O_2 ⟶ The cells were scraped

-30 min in PBS with Ca&Mg + 30 min H_2O_2 + 3h in medium ⟶ The cells were scraped

-30 min in LC + 30 min H_2O_2 ⟶ The cells were scraped

-30 min in LC + 30 min H_2O_2 + 3h in medium ⟶ The cells were scraped

DNA extraction was done according to the procedure described in Qiagen DNA extraction kit with spin-column protocol as follow: $5x10^6$ cells were scraped and centrifuged for 5 min at 300xg. The pellet was resuspended in 200 µl PBS + 20 µl proteinase K. 200 µl buffer AL was added, mixed throughly by vortexing and incubated at 56 °C for 10 min. 200 µl ethanol was added to the sample and mixed throughly by vortexing. The mixture was pipetted into the Dneasy Mini spin column placed in a 2 ml collection tube, centrifuged at 8000 rpm for 1 min, the flow through and collection tube were discarded. Dneasy Mini spin column was placed in a new collection tube, 500 µl buffer AW1 was added, centrifuged for 1 min at 8000 rpm, flow through and collection tube were discarded. Dneasy Mini spin column was placed in a new collection tube, 500 µl buffer AW2 was added, centrifuged for 1 min at 14000 rpm to dry the Dneasy membrane, flow through and collection tube were discarded. Dneasy Mini spin column was placed in a new collection

tube, 200 µl buffer AE was pipetted directly onto the Dneasy membrane, incubated at room temperature for 1 min, centrifuged for 1 min at 8000 rpm to elute the DNA. For 8-OHdG analysis, highly sensitive 8-OHdG Check ELISA kit was used that was obtained from JaICA (Japan Institute Control of Aging) and 50 µg of DNA was used in the assay. 50µl standards and samples were added into 96-well plate and incubated overnight at 4 °C with 50 µl of primary antibody which is a monoclonal antibody specific for 8-OHdG. The contents were poured off. Following washing steps, 100 µl of secondary antibody which is HRP-conjugated were added into each wells and incubated for 1h at room temperature. Following washing steps, 100 µl of 3,3',5,5'-tetramethylbenzidine + hydrogen peroxide/citrate chromatic substrate solution was added into wells and incubated for 15 min at room temperature. 100 µl of 1 M phosphoric acid reaction terminating solution was added and the absorbance was measured at 450 nm wavelength using a microplate reader (BioTek Instruments EL 340). In the principle of the assay, the special plate was precoated with 8-OHdG. Therefore higher concentrations of 8-OHdG in the sample solution led to reduced binding of the antibody to the 8-OHdG on the plate and decreases the absorbance at the end. 8-OHdG amounts were calculated as ng/mg DNA according to the standard curve.

2.2.9 PARP activity measurement

Cellular PARP activity was detected using cellular ELISA method [85]. Cells were seeded in 96-well plates pretreated with 20 µM LC, 5 mM 3-ABA and 40 µM PJ-34 and stimulated with 0.4 mM H_2O_2. After treatment for 30 min at 37^oC, plates were rinsed with 200µl PBS. Cells were fixed in 10% ice-cold TCA (100 µl/well) at -20^oC for 10 minutes, then dehidrated by successive 10 min washes in 100µl prechilled 70%, 96% and 100% ethanol at -20^oC. After 3x5 min rinses with PBS, endogenous peroxidase activity was blocked by 15 min incubation in 0.5% hydrogen peroxide/methanol. After washing with

PBS, plates were blocked in 5% bovine serum albumin (BSA) in PBS-Triton X-100 for 60 min at 37°C. BSA solution was then aspirated and cells were incubated with 10H monoclonal anti-poly(ADP-ribose) antibody diluted 1:5000 in 1% BSA/PBS- Triton X-100 for 2 h at 37°C. After three washes with PBS-Triton X-100, samples were incubated with peroxidase conjugated anti-mouse IgG (Calbiochem) diluted 1:300 in 1% BSA/PBS-Triton X-100 for 90 min at 37°C. Cells were then washed three times with PBS Triton X-100 and reaction was developed with Amplex Red substrate. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelength with a microplate reader.

2.3 In vitro experiments

2.3.1 Histone oxidation

Histone stock solutions were prepared in different concentrations as follow according to the experiment:

Protein carbonyl ELISA: 4.4 mg histone / ml PBS ⟶ 4 mg/ml end concentration

Protein carbonyl western blot: 9.7 mg histone / ml PBS ⟶ 8.8 mg/ml end concentration

Fluorescamine: 2.2 mg histone / ml PBS ⟶ 2 mg/ml end concentration

PAR of histones: 9.7 mg histone / ml PBS ⟶ 8.8 mg/ml end concentration

The histone solutions were dialyzed against PBS 2x3 h and overnight before and after oxidation. Oxidation process was carried out for 2h at 25 °C with 500 rpm shaking. 330 mM stock H_2O_2 solution was used to prepare 0-1-5-10-15-20-30 mM end concentrations.

2.3.2 Protein carbonyl measurement in isolated histones

2.3.2.1 ELISA

0-5-10-15-20-30 mM oxidized histones were used in the experiment. The experimental procedure was carried out same as described in 2.2.6 without any lysis procedure.

2.3.2.2 Western blot

50 µl of histone samples oxidized with 0-10-20 mM H_2O_2 mixed with 25 µl nonoxidized nuclear extract (prepared as described in 2.2.1.2), 50 µl 2 mM NAD^+ (Sigma N8410) solution and 125 µl reaction buffer including 300 mM sucrose and 10 mM HEPES. The reaction mixture was incubated for 15 min at 37°C, centrifuged 720xg for 5 min. 45 µl of supernatants mixed with 15 µl reducing Laemmli-buffer prepared as described in 2.2.4 and were denatured at 95 °C for 5 min and applied to SDS-PAGE of 10% (w/v) acrylamide, followed by electrophoresis and blotting onto nitrocellulose membrane according to standard procedures.

Following the electroblotting step, the membrane were equilibrated in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) containing 20% methanol for 5 min, washed in 2N HCl for 5 min, incubated with 10 mM DNPH solution for 5 min, washed 3x5 min in 2N HCl and washed 5x5 min in 50% methanol. DNPH treated membrane was blocked with 5% non-fat dry milk in TBST (TBS contains Tween 20) for 1h at room temperature with constant agitation. Blocked membrane was washed 3x5 min with TBST and incubated with rabbit anti-DNP antibody freshly diluted 1:25000 in 5% non-fat dry milk/TBST for 1h at room temperature with constant agitation. Blotted membrane was washed 3x5 min with TBST and incubated with anti-rabbit-IgG-POD-antibody freshly diluted 1:5000 in 5% non-fat dry milk/TBST for 1h at room temperature with constant agitation. The blotted membrane was

washed 5x5 min with TBST. Membrane was developed using Lumi-Light western blotting substrate.

2.3.3 Fluorescamine Assay

In the principle of this assay, fluorescamine reacts quantitatively with primary amines, forming a fluorescent product. The concentration of primary amines in TCA-soluble fractions reflects the rate of proteolysis of the substrate protein by the proteasome. In the experiment, 50 µl of histone samples oxidized with 0-1-5-10-15-20-30 mM H_2O_2 mixed with 25 µl nonoxidized nuclear extract (prepared as described in 2.2.1.2), 50 µl 2 mM NAD^+ solution (for the samples without poly(ADP-ribosyl)ation H_2O was added instead of NAD^+) and 125 µl reaction buffer including 300 mM sucrose and 10 mM HEPES. The reaction mixture was incubated for 15 min at 37°C, centrifuged 720xg for 5 min. After centrifugation, 20.2 µl supernatant + 20 µl of 0.7 mg/ml isolated proteasome (provided from P.Voss) + 84.8 µl reaction buffer were mixed and incubated for 2h at 37°C with 350 rpm shaking. 50 µl incubation solution + 450 µl ice-cold 10% TCA were incubated on ice for 30 min and centrifuged 3000xg for 10 min at 4°C. 250 µl fluorescamine solution (9 mg in 30 ml acetone) were added in 5 min intervals into the 125 µl supernatant + 625 µl 1M HEPES mixture. Following 5 min incubation of each samples in dark, fluorescence was measured at 390 nm excitation / 475 nm emission wavelength with a microplate reader. Glycine was used as a standard to determine amino acid liberation by proteolysis. Proteolysis rates were calculated as the difference between sample values and blank values.

2.3.4 Measurement of poly(ADP-ribosyl)ation of histones

2.3.4.1 Liquid scintillation counting

17 µl of histone samples oxidized with 0-1-5-10-15-20-30 mM H_2O_2 were mixed with 167.5 µl reaction mixture, 0.5 µl PARP-1 enzyme, 10 µl DNA and 5 µl ^{14}C-NAD^+ , incubated for 10 min at 37°C. The reaction was stopped by addition of 0.8 ml of ice-cold 20% TCA. After standing on ice for 30 min, samples were centrifuged 14000xg for 10 min. Onto the pellet, 200 µl of solvable was added and following the solubilizing, radioactivity of samples were counted in scintillation cocktail.

Reaction mixture	100 mM Tris-HCl + 10 mM $MgCl_2$ + 5 mM DTT
PARP-1 ready enzyme (human expressed in E.coli, Sigma P0396)	12 µg / 20 µl
DNA (from calf thymus, sigma D1501)	3.25 mg / 5 ml H_2O ⟶ Sonicated 10 x 20s
[carbonyl-^{14}C]Nicotinamide adenine dinucleotide (GE Healthcare, CFA372)	370 kBq, 10 µCi
ULTIMA GOLD Scintillation cocktail Perkin Elmer 6013327	
SOLVABLE Perkin Elmer 6NE9100	

Tab. 2.10 Solutions for poly(ADP-ribosyl)ation of histones by liquid scintillation counting

2.3.4.2 Western blot

50 µl of histone samples oxidized with 0-10-20 mM H_2O_2 mixed with 25 µl nonoxidized nuclear extract (prepared as described in 2.2.1.2), 50 µl 2 mM NAD^+ (Sigma N8410) solution and 125 µl reaction buffer including 300 mM sucrose and 10 mM HEPES. The reaction mixture was incubated for 15 min at 37°C, centrifuged 720xg for 5 min. 45 µl of

supernatants mixed with 15 µl reducing Laemmli-buffer [0.25 M Tris pH 6.8, 8% SDS, 40% glycerol, 0.03% bromophenol blue] and were denatured at 95 °C for 5 min and applied to SDS-PAGE of 7.5% (w/v) acrylamide, followed by electrophoresis and blotting onto nitrocellulose membrane according to standard procedures. Immunodetection was performed with the anti-poly(ADP-ribose) mouse monoclonal (Biomol) at 1:2000 dilution. After exposure to peroxidase-coupled secondary antibodies (Calbiochem), membranes were developed using Lumi-Light western blotting substrate (Cell Signaling).

2.4 Aging effects on the model

2.4.1 Cell culture

Human foreskin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml and 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Experiments were performed with cells of different population doubling numbers as 19+/-4, 36+/-4, 56+/-3.

2.4.2 Immunoblot analysis

Nuclear extracts were prepared as described in 2.2.1.2 from different aged fibroblasts as PD 19+/-4, PD 36+/-4, PD 56+/-3. 20 µg of total protein in reducing Laemmli-buffer were denatured at 95 °C for 5 min and applied to SDS-PAGE of 12% (w/v) acrylamide for proteasome and of 7.5% (w/v) acrylamide for PARP-1, followed by electrophoresis and blotting onto nitrocellulose membrane according to standard procedures. Immunodetections were performed with the following antibodies: rabbit polyclonal anti-PARP (Cell Signaling) at 1:1000 dilution, rabbit polyclonal anti-20S proteasome 'core' subunits (Biomol) at 1:1000 dilution, rabbit polyclonal anti-histone H4 (Cell Signaling) at

1:1000 dilution. After exposure to peroxidase-coupled secondary antibodies (Calbiochem), membranes were developed using Lumi-Light western blotting substrate (Cell Signaling).

2.4.3 PARP activity measurement

Different aged fibroblasts with PD 19+/-4, PD 36+/-4, PD 56+/-3 were seeded in 96-well plates and fixed in 10% ice-cold TCA (100 µl/well) at -20°C for 10 minutes, then dehidrated by successive 10 min washes in 100µl prechilled 70%, 96% and 100% ethanol at -20°C. After 3x5 min rinses with PBS, endogenous peroxidase activity was blocked by 15 min incubation in 0.5% hydrogen peroxide/methanol. After washing with PBS, plates were blocked in 5% BSA in PBS-Triton X-100 for 60 min at 37°C. BSA solution was then aspirated and cells were incubated with monoclonal anti-poly(ADP-ribose) antibody diluted 1:5000 in 1% BSA/PBS- Triton X-100 for 2 h at 37°C. After three washes with PBS-Triton X-100, samples were incubated with peroxidase conjugated anti-mouse IgG (Calbiochem) diluted 1:300 in 1% BSA/PBS- Triton X-100 for 90 min at 37°C. Cells were then washed three times with PBS- Triton X-100 and reaction was developed with Amplex Red substrate. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelength with a microplate reader.

2.4.4 Proteasome activity measurement

Different aged fibroblasts with PD 19+/-4, PD 36+/-4, PD 56+/-3 were treated with 0.5 mM H_2O_2 for 0-5-15-30 min. Nuclear extracts were prepared as described in 2.2.1.2. Proteasome activity was analyzed in these supernatants as described in 2.2.3.

2.4.5 Poly(ADP-ribosyl)ation of proteasome

Nuclear extracts were prepared as described in 2.2.1.2 from different aged fibroblasts as PD 19+/-4 and PD 56+/-3. 50 µl of isolated proteasome mixed with 25 µl nonoxidized nuclear extract, 50 µl 2 mM NAD^+ solution and 125 µl reaction buffer including 300 mM sucrose and 10 mM HEPES. For some samples active PARP-1 was added instead of proteasome. The reaction mixture was incubated for 15 min at 37°C, centrifuged 720xg for 5 min. Proteasome activity was analyzed in these supernatants as described in 2.2.3.

3 Results

3.1 Results in cell culture

3.1.1 MTT viability test

To determine the concentrations and durations of H_2O_2 treatments in the experiments with HT22 cells, first a viability assay was performed. According to the results shown in Fig.3.1, 0.00-1.00 mM hydrogen peroxide with 30min duration (Fig.3.1.A) and 0-60 min durations with 1.00 mM concentration (Fig.3.1.B) did not cause any significant cell death which are not less than 75%.

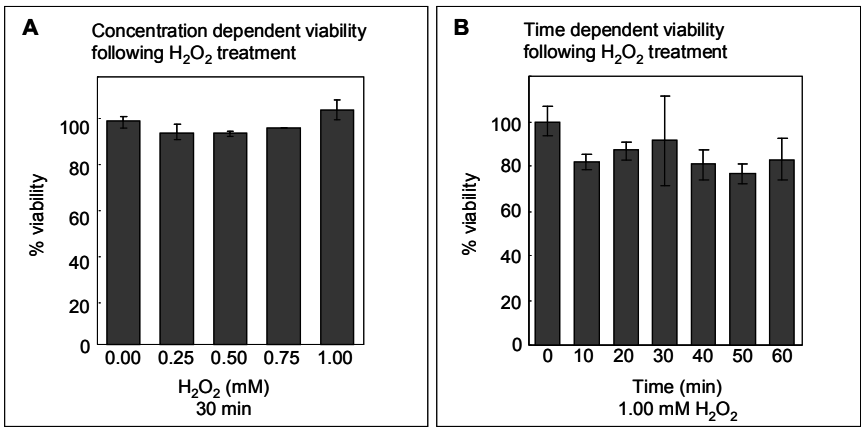


Fig. 3.1 Viability in different concentrations of H_2O_2 (A) and different durations of incubations (B). Incubation time for H_2O_2 in (A) 30 min, incubation concentration in (B) 1.00 mM H_2O_2. Controls were incubated only with PBS (+Ca&Mg) and shown as 100% viable. (n=3, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ±SEM).

3.1.2 Proteasome activity

Proteasomal activity in HT22 cells was measured following H_2O_2 treatment of HT22 cells. Proteasomal degradation of the model proteasome peptide substrate suc-LLVY-MCA increased in a time and concentration dependent fashion .

Because it is known that the nuclear proteasome can be stimulated by interaction with the PARP-1 in vitro, in lysates of hydrogen peroxide-treated isolated nuclei and in living cells [76-79], we used the specific PARP-1 inhibitors 3-ABA and PJ-34 and tested the effect of PARP-1 inhibition on proteasomal degradation in HT22 cells. 3-ABA and PJ-34 inhibited the induction of proteasome activation and these results proved the role of PARP-1 in the activation of proteasome.

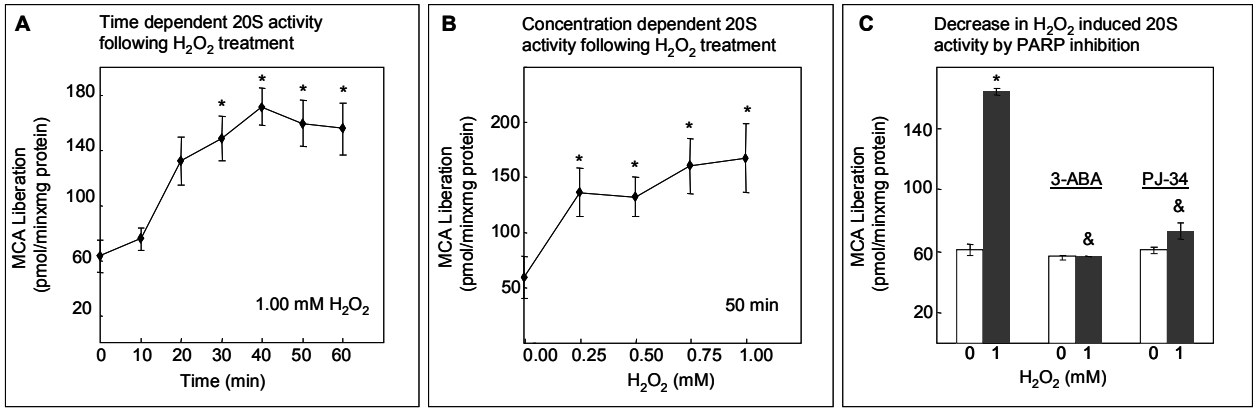


Fig. 3.2 20S proteasome activity in different concentrations of H_2O_2 (A), different durations of incubations (B) and the effects of PARP-1 inhibitors 3-ABA (0.5 M) and PJ-34 (40 μM) in the proteolytic activation of proteasome (C). Incubation concentration in (A) 1.00 mM H_2O_2, incubation time for H_2O_2 in (B and C) 30 min. Controls were incubated only with PBS (+Ca&Mg). (n=3; *$p<0.05$ vs treated, $^{\&}p<0.05$ vs treated without inhibitors. ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ±SEM).

3.1.3 Immunoblot analysis

Whole cell lysates and nuclear extracts of HT22 cells were analyzed for PARP-1 and 20S 'core' proteasome protein expressions following H_2O_2 treatments. As seen in Fig.3.3 there was no change in protein expressions of PARP-1 and 20S proteasome both in whole cell lysates and nuclear extracts. This explains that the proteasome was activated only enzymatically without any change in the protein amount and PARP-1 protein amount was also not affected following oxidative damage in the cells.

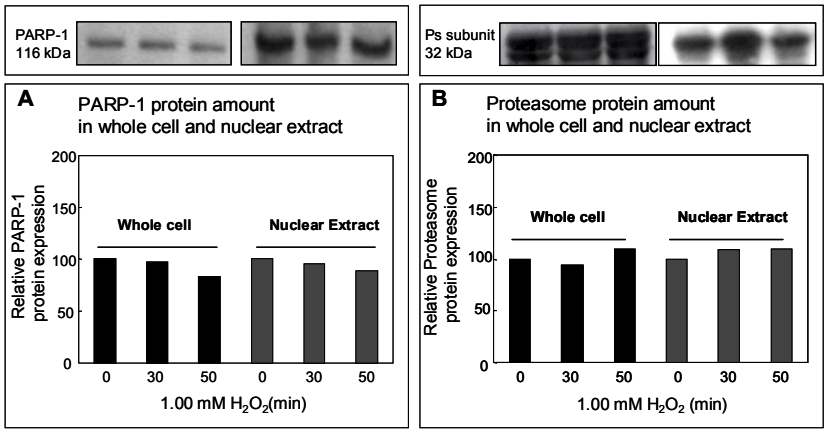
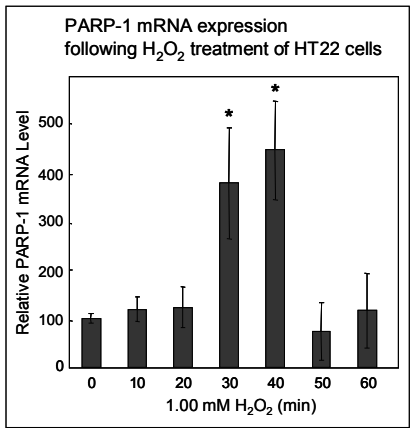


Fig. 3.3 Protein expressions in different time periods of 1mM H_2O_2 in whole lysates and nuclear extracts of HT22 cells. PARP-1 protein amounts (A), 20S proteasome subunit protein amount (B). Controls were incubated only with PBS (+Ca&Mg). 30 µg proteins were electrophoresed (SDS-PAGE gel) and immunoblotted with rabbit polyclonal anti-PARP1 antibody and rabbit polyclonal anti-20S proteasome-core subunits antibody.

3.1.4 qPCR analysis

Following no significant change in protein amount of PARP-1, PARP-1 mRNA expressions were checked with quantitative PCR. Results showed that mRNA expression was increased in 30 and 40 min of 1.00 H_2O_2 treatment.

Fig. 3.4 The dose dependence of PARP-1 mRNA expressions following 1.00 mM H2O2 treatment. (n=3; *p<0.05 vs untreated, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM)



3.1.5 Protein carbonyls in cell lysates

As an indicator of oxidized proteins the amount of protein-bound carbonyls were measured following H_2O_2 treatment according to Buss et al. [84]. The use of protein carbonyl groups as a biomarker of oxidative stress is widely accepted due to relative early formation and the stability of carbonylated proteins [86]. The protein carbonyl content of cells was enhanced with H_2O_2 treatment as seen at 0 time point in Fig.3.5, and the cells did recover within 3h after H_2O_2 treatment. With LC and 3-ABA pretreatment of the cells, the recovery from carbonyls was decreased. These results can be explained as follow: 1)The proteasome plays a major role in the degradation of oxidatively modified proteins. In this study, the increase in the activation of proteasome was shown in Fig.3.2.A-B following H_2O_2 treatment. This proteasomal activity may cause the recovery from carbonyls and the inhibition of the proteasomal activity by specific 20S proteasome inhibitor LC caused a significant decline in the recovery. 2)As the second mechanism related to the first one is the activation of proteasomal degradation with poly(ADP-ribosyl)ation. Here also PARP-1 enzyme was inhibited by 3-ABA pretreatment and also a significant decline in the recovery

from carbonyls was seen which confirms that the inhibition of PARP-1 also causes a decline in the proteasomal degradation.

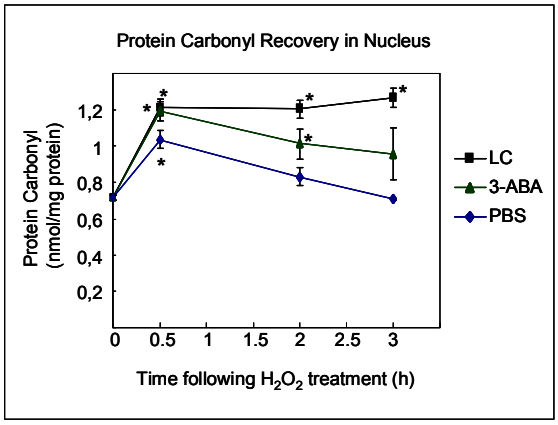


Fig. 3.5 Protein carbonyl recovery in different time points after H2O2 treatment and the effects of lactacystin (LC) and 3-aminobenzamide (3-ABA) pretreatments on this recovery. (n=3; *p<0.05 vs time point 0, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM)

3.1.6 Comet assay and PARP activity

Comet assay is a special analysis to determine the DNA damage especially DNA strand breaks. This test was performed to measure the oxidative DNA damage. In Fig.3.6.A, an increase in the oxidative DNA damage was shown as concentration dependent of H_2O_2. As shown in carbonyl recovery, there is also another mechanism to ensure the recovery from DNA damage and single strand breaks. In Fig.3.6.B there is a recovery from single strand breaks caused by H_2O_2. I thought that this recovery may be arised from both proteasomal degradation and PARP-1 activity. To confirm this, the cells were pretreated with LC, 3-ABA and PJ-34 and all of them caused a slow down of DNA repair (Fig.3.6.C), which means proteasome and PARP inhibition caused a decline in the recovery. Since it is known that PARP is activated following DNA damage especially by single strand breaks, I also checked PARP activitation following H_2O_2 treatment and the effects of LC, 3-ABA and PJ-34 on this activation. In Fig.3.6.D one can see that PARP was activated following H_2O_2 treatment. LC pretreatment did not affect this PARP activation confirming that LC inhibits only proteasomal activity. On the contrary, 3-ABA and PJ-34 strong inhibitors of PARP-1 caused a decline in PARP activity.

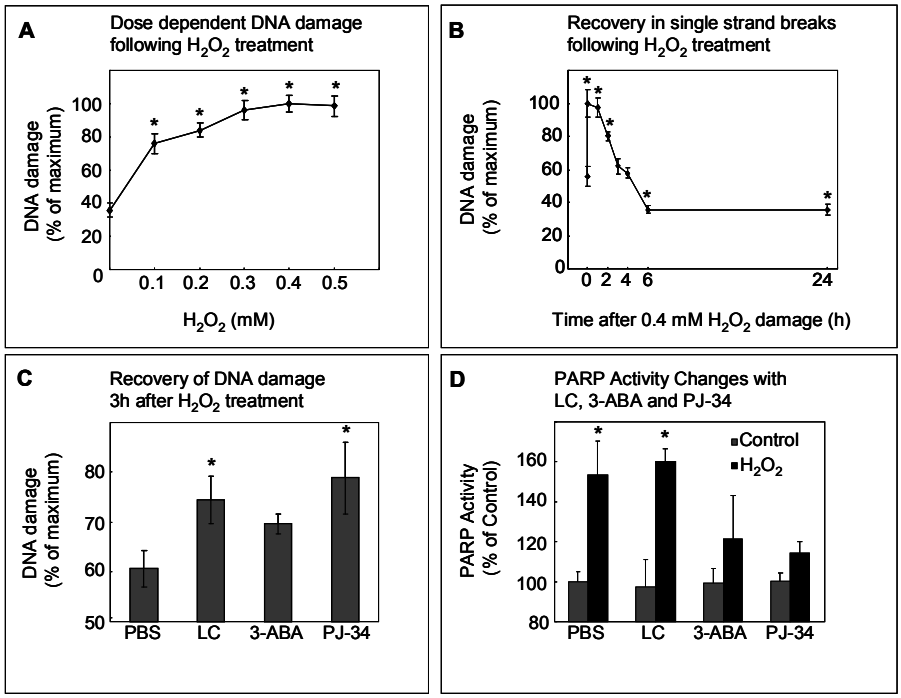


Fig. 3.6 Comet assay and PARP cELISA analysis following H_2O_2 treatment in HT22 cells. Dose dependent DNA damage following H_2O_2 treatment. HT22 cells were treated with different concentrations of H_2O_2 for 30 min (n=3; *p<0.05 vs untreated, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (A). Recovery in single strand breaks in different time points after H_2O_2 treatment (n=3; *p<0.05 vs time point 0, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (B). Recovery of DNA damage 3 h after H_2O_2 treatment and the effects of proteasome inhibitor lactacystin (LC), and PARP-1 inhibitors 3-aminobenzamide (3-ABA) and PJ-34 on this recovery. Inhibitor pretreatments and following H_2O_2 treatment were done for 30 min (n=3; *p<0.05 vs control which was pretreated with PBS+Ca&Mg, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (C). PARP activity changes following H_2O_2 treatment of HT22 cells and the effects of proteasome inhibitor lactacystin (LC), and PARP-1 inhibitors 3-aminobenzamide (3-ABA) and PJ-34 on PARP activity. Inhibitor pretreatments and following H_2O_2 treatment were done for 30 min (n=3; *p<0.05 vs control which was not treated with H_2O_2, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (D).

3.1.7 8-OHdG amounts

8-Hydroxy-2'-deoxyguanosine (8-OHdG), one of the most abundant oxidative DNA adducts, is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals. Fig.3.7 shows that 0.4 mM H_2O_2 treatment caused an increase in the 8-OHdG formation and this effect was declined with 3h incubation in medium showing the recovery. LC as proteasome inhibitor caused a decline in this 8-OHdG recovery which was also seen in Fig.3.6.C with single strand breaks.

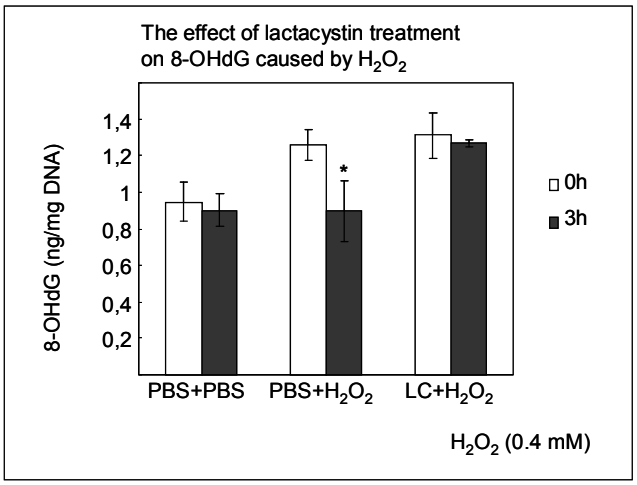


Fig. 3.7 The recovery in 8-OHdG formation 3h after H2O2 treatment of HT22 cells and the effect of lactacystin (LC) on this recovery. LC pretreatment and following H2O2 treatment were done for 30 min (n=3; *P<0.05 vs time point 0, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM)

3.2 Results in isolated histones

3.2.1 Fluorescamine Assay in isolated histones

Histones are the most abundant nuclear proteins, and therefore the turnover of individual histones were measured. Because the proteasome is well known to be responsible for the degradation of oxidatively damaged proteins [23,24], we investigated the proteasomal contribution to the total histone degradation in the next set of experiments. After H_2O_2

treatment of HT22 cells, proteasomal degradation of oxidized histones increased significantly until 10 mM concentration and then decreased with the increased concentration. After reaching an optimum degree of oxidative damage at 10 mM, further damage decreased the proteolytic susceptibility of histone. With poly(ADP-ribosyl)ation of oxidized histones there was no change in the proteasomal degradation of histones (Fig.3.8.A).

3.2.2 Measurement of poly(ADP-ribosyl)ation of histones

As mentioned above, poly(ADP-ribosyl)ation is known to activate proteasome activity. On the other side histone-pADPr-PARP-1 complex is thought to be formed at the site of DNA lesion. Here poly(ADP-ribosyl)ation of unfractionated histones, provided from Sigma, was tested following oxidation. This can bring the idea into the mind that this complex formation may cause decrase in the histone degradation. As seen in Fig.3.8.A the proteasomal degradation of histones increased as H_2O_2 concentration dependent and with PAR of histones this degradation was declined to the basal level. Following this result we checked PAR of histones in the same conditions by scintillation counting and western blot and saw a decline in the PAR of histones as H_2O_2 concentration dependent. This decrease in the PAR of histones may be inducing the proteasomal degradation selectively for oxidized proteins.

3.2.3 Protein carbonyl measurement in isolated histones

Histones (provided from Sigma) were analyzed following the derivatization by DNPH for the detection of oxidative modifications by ELISA and western blot. As demonstrated in Fig.3.8.C, there was not such a high increase in the protein carbonyl formation with active proteasome in the nuclear extracts.

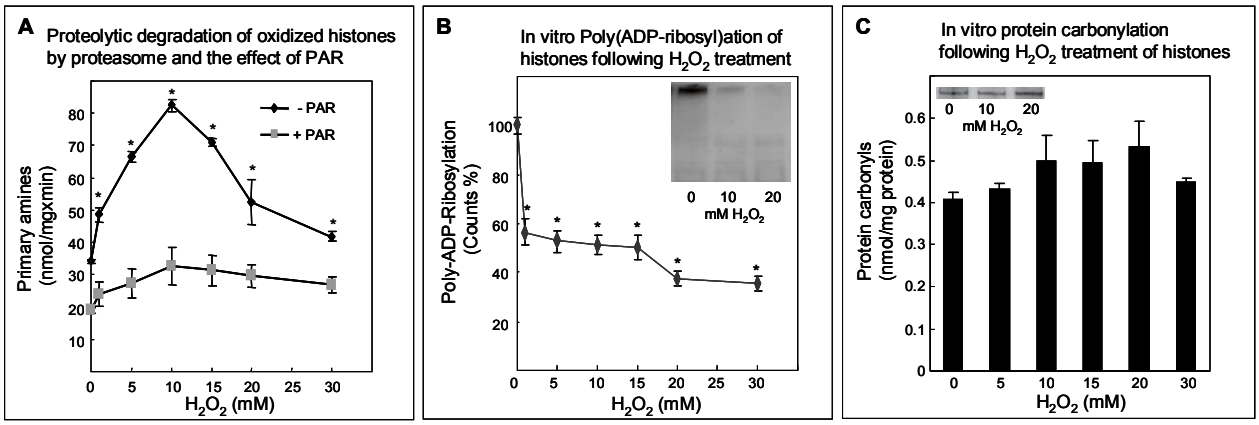


Fig. 3.8 Results in isolated histones. Histones were treated with different concentrations of H_2O_2 for 30 min. Susceptibility of oxidatively modified histones to proteolytic degradation by proteasome and the effect of poly(ADP-ribosyl)ation on this degradation. Proteolysis experiments were performed with the isolated proteasome from human erythrocytes (0.7 mg/ml), prepared according to Hough et al. [87], the damaged and nondamaged histones with and without poly(ADP-ribosyl)ation. Proteolytic degradation was measured using the fluorescamine assay and proteolysis is expressed as nanomoles of free amines generated per minute per mg of proteasome (n=3; *p<0.05 vs untreated, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (A). Poly(ADP-ribosyl)ation of histones following H_2O_2 treatment. Tested both liquid scintillation counting and western blot (inset), mouse monoclonal anti-poly(ADP-ribose), product size 175-225 kDa (n=3; *p<0.05 vs untreated, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (B). Dose dependent protein carbonyl formation following H_2O_2 treatment. Tested both by ELISA and western blot (inset) (MW of the protein is 150 kDa) rabbit anti-DNP antibody for both procedure (n=3; *p<0.05 vs untreated, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (C).

3.3 Aging effects on the model

This part of the thesis shows the changes in PARP-1 and proteasome in different aged fibroblasts with PD 19+/-4, 36+/-4, 56+/-3. PARP-1 and proteasome as nuclear protective mechanisms are assumed to be effected during senescence process.

I want to thank E. Bakondi for her effort in this part.

3.3.1 Immunoblot analysis

Protein amounts of PARP-1 and 20S 'core' proteasome subunit were tested in different aged fibroblasts. The results shown in Fig.3.9 indicate that aging causes a decrease in PARP-1 protein amount whereas does not change 20S proteasome protein amount. Histone H4 was analyzed to show the equal protein amount in the wells.

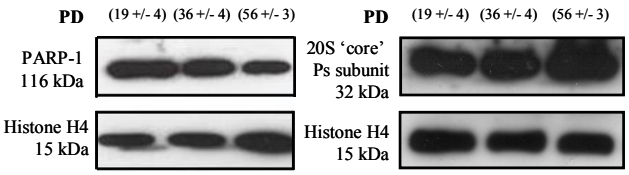
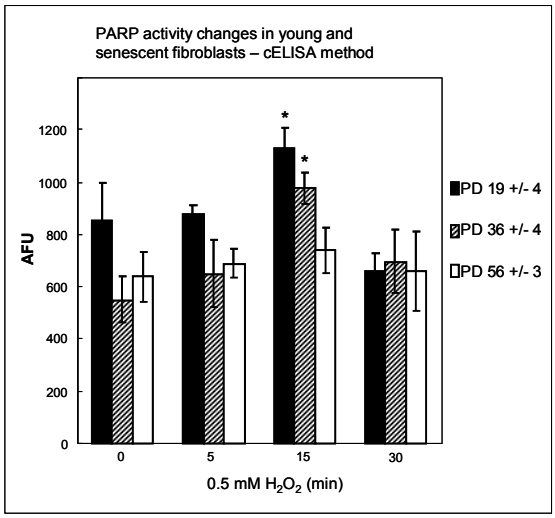


Fig. 3.9 Protein expressions in the nucleus of different aged fibroblasts with population doubling (PD); 19+/-4, 36+/-4, 56+/-3. PARP-1 was detected with rabbit polyclonal anti-PARP1 antibody and proteasome was detected with rabbit polyclonal anti-20S proteasome-core subunits antibody. Histone H4 was tested to show the equal protein amounts in the wells. (Experiments were performed by E. Bakondi).

3.3.2 PARP activity changes in young and senescent fibroblasts

PARP enzymatic activity was analyzed in different aged fibroblasts in 0-5-15-30 min time points following 0.5 mM H_2O_2 treatments. As shown in Fig. 3.10, PARP activity increased 15 min after H_2O_2 treatment in young and middle-aged fibroblasts. This activation in 15 min was not seen in old cells confirming the previous studies that shows the decrease in PARP-1 activity during aging.

Fig. 3.10 PARP activity changes in the nucleus of different aged fibroblasts with population doubling (PD); 19+/-4, 36+/-4, 56+/-3 following 0-5-15-30 min treatments of 0.5 mM H_2O_2. The results are shown as arbitrary fluorescence unit (AFU). (n=3; *p<0.05 vs time point 0, ANOVA, Bonferroni's Multiple Comparison Test. Data are

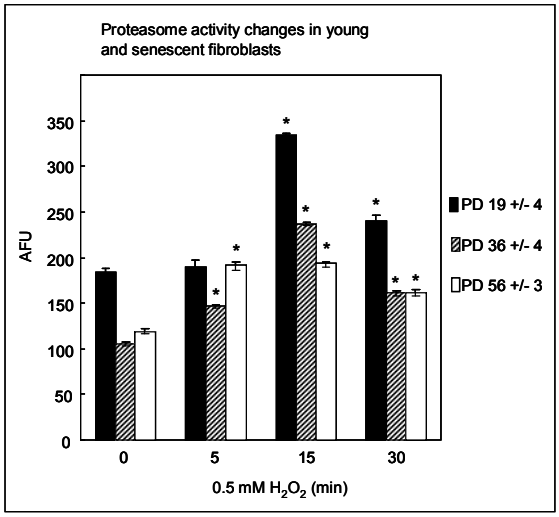


mean ± SEM) (Experiments were performed by E.Bakondi)

3.3.3 Proteasome activity changes in young and senescent fibroblasts

Proteasome activity was analyzed in different aged fibroblasts in 0-5-15-30 min time points following 0.5 mM H_2O_2 treatments. As shown in Fig. 3.11, proteasome activity increased in all cells with H_2O_2 treatment.

Fig. 3.11 20S proteasome activity in the nucleus of different aged fibroblasts with population doubling (PD); 19+/-4, 36+/-4, 56+/-3 following 0-5-15-30 min treatments of 0.5 mM H2O2. The results are shown as arbitrary fluorescence unit (AFU). (n=3; *P<0.05 vs time point 0, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (Thanks to E.Bakondi)



3.3.4 Poly(ADP-ribosyl)ation of proteasome in young and senescent fibroblasts

Proteasome activity was measured in young and old fibroblasts following different processes. In Fig.3.12.A, young cells had an increase in proteasome activity following poly(ADP-ribosyl)ation whereas old cells did not. In this process, active isolated proteasome was added into the reaction. In Fig.3.12.B, young and old cells both had an increase in proteasome activity following poly(ADP-ribosyl)ation. In this second process, active PARP-1 enzyme was added into the reaction. This shows the limiting role of PARP-1 in the activation in old cells.

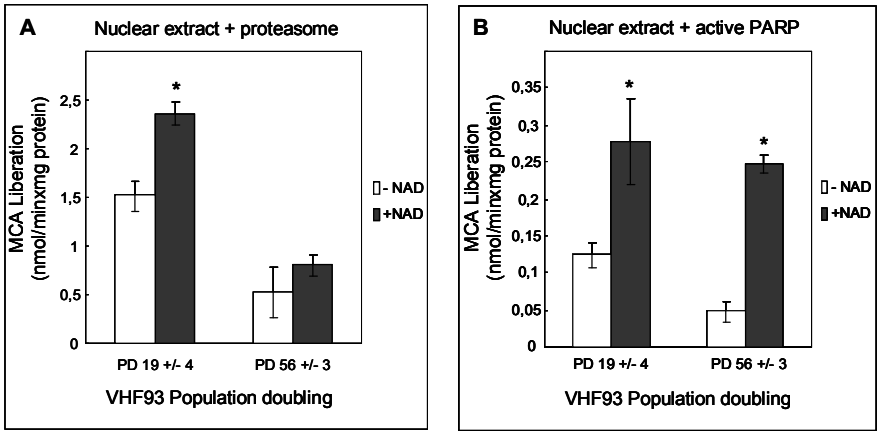


Fig. 3.12 The effect of PARP in the proteasomal activation in young and senescent fibroblasts. Nuclear extraction was done as described in Material and Method. Isolated proteasome was from Dr.Voss that was prepared according to Hough et al. PARP enzyme was obtained from Sigma. Poly(ADP-ribosyl)ation was achieved by the addition of NAD into the reaction. Proteasome was added into the nuclear extracts of young and senescent fibroblasts (n=3; *P<0.05 vs without poly(ADP-ribosyl)ation, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (A). PARP enzyme was added into the nuclear extracts of young and senescent fibroblasts (n=3; *P<0.05 vs without poly(ADP-ribosyl)ation, ANOVA, Bonferroni's Multiple Comparison Test. Data are mean ± SEM) (B).

4 Discussion

4.1 Activation of the protesome by PARP-1

Proteasome as the major proteolytic system responsible for the removal of oxidized proteins may be affected in a different manner during oxidative stress process. It might be activated by moderate oxidative damage parallel to sensitivity of moderately oxidized proteins to proteolytic attack and severely oxidized proteins are often poor substrates and might, however, inhibit the proteasome. In our model, proteasome was activated following H_2O_2 treatment which was in the concentration and duration range (Fig.3.1) without any significant cell death (Fig.3.2). This increase in the proteasome was not because the protein amount of proteasome increased as tested by immunoblotting (Fig.3.3).

The 20S-proteasome is known to interact noncovalently with both poly(ADP-ribose) and automodified PARP-1 [8,80]. Interaction required long ADP-ribose polymers and caused a specific stimulation of the proteasome's peptidase activity. To examine the question if proteasome is activated by PARP, we performed experiments with 3-ABA and PJ-34, selective and strong inhibitors of PARP [88]. Incubation with both of these inhibitors, blocked the degradation of oxidized histones in nuclear lysates (Fig.3.2). 3-ABA and PJ-34 are by far the best characterized and most commonly used inhibitors of PARP. Therefore we used these inhibitors in the bulk of the experiments. These results further support the proposal that PARP may be responsible for the rapid activation of nuclear proteasome during H_2O_2 exposure. In addition there was no change in the protein amount of PARP-1 in whole cell and nucleus following H_2O_2 treatment (Fig.3.3). In contrast PARP-1 mRNA levels increased in 30-40 min of 1.00 mM H_2O_2 treatment (Fig.3.4). This PARP-1 mRNA overexpression was shown to increase in various human malignancies, such as malignant

lymphoma [89], breast carcinoma [90], Ewing's sarcoma [91], hepatocellular carcinoma [92] and endometrial carcinoma [93] as a consequence of oxidative stress.

Also in previous studies, poly(ADP-ribosyl)ation has been shown to be a general nuclear response to oxidative stress [94]. It was demonstrated that DNA-single strand breaks (SSBs), which cause a significant PARP-1 activation after H_2O_2 treatment, are found shortly after H_2O_2 exposure and disappear subsequently due to nuclear repair mechanisms [95,96]. Thus, PARP-1 activation followed the appearance of SSBs and correlated well with the time course of proteasome activation. In this context, it has been demonstrated that the efficient repair of SSBs induced by γ-irradiation and H_2O_2 treatment requires PARP activity in rat germinal cells [97]. Moreover, PARP inhibition resulted in a strong accumulation of DNA damage in human leukaemia cells after adriamycin treatment [78].

Ullrich et al. showed that histone proteins appear to be highly susceptible to oxidant-induced proteolysis in intact cells. They also demonstrated the probable role of nuclear proteasome in histone turnover during oxidant stress with the experiments performed with the proteasome inhibitor lactacystin. Lactacystin completely suppressed the preferential proteolysis of oxidized histones without altering the degradation of nonoxidized histones, in lysates from both H_2O_2 treated nuclei and nontreated nuclei. They examined whether the 20S proteasome itself is a substrate for poly(ADP ribosyl)ation by PARP and showed that H_2O_2 caused a time-dependent increase in nuclear proteasome activity, which was paralelled by an increasing incorporation of radioactivity derived from [^{14}C]-NAD^+ [8].

H_2O_2 is able to induce oxidative DNA damage, including SSBs in the nuclei of HT22 cells leading to the activation of the PARP enzyme (Fig.3.6). Furthermore tumor cells are known to have higher PARP activity and a higher basal poly-ADP ribose content than

normal cells [98], which could explain the strong activation of poly-ADP ribose synthesis in nuclei of HT22 cells after exposure to H_2O_2. Poly(ADP-ribosyl)ation is strongly related to DNA repair. PARP molecules bind tightly to DNA strand breaks and auto-poly(ADP-ribosyl)ation of the protein then effects its release and allows access to lesions for DNA repair enzymes as shown by Ullrich et al. [76,77]. Binding of PARP to strand breaks may represent a signal that switches off DNA replication and transcription to ensure that lesions are not replicated before repair, and a ‘‘nick protection’’ during replication or cellular differentiation also has been proposed. The binding of PARP to DNA lesions and a strong activation after administration of various antitumor drugs has been reported *in vitro*. Increased poly-ADP ribosylation also has been suggested as an adaptive response of tumor cells to long-term antitumor drugs, which may be responsible for the development of chemotherapeutic resistance [5,88,98]. Thus the interaction between PARP and the nuclear proteasome may well play an important role in the secondary antioxidant defenses.

By using the conditions of Banasik et al. [99] for in vitro poly(ADP-ribosyl)ation of proteins, we were indeed able to demonstrate the changes in the susceptibility of isolated histone proteins following PAR. This modification of histones caused a decrease in the proteasomal degradation of histones (Fig.3.8.A). Poly(ADP-ribosyl)ation is the shuttling of histones by detachment and reattachment of histones to chromatin through reversible poly-ADP ribose synthesis and degradation [100].

In this study, we investigated the endogenous degradation of oxidatively damaged histones in HT22 mouse hippocampal cells after oxidative challenge and demonstrated a link to the overall cellular stress response pathways by poly-ADP-ribose-polymerase (PARP). After an oxidative challenge, endogenous nuclear protein degradation, as well as histone degradation, was enhanced.

4.2 Role of the proteasome in chromatin repair

Histones are the main proteins in chromatin structure. In the nuclear oxidative damage (from metabolic free radical formation, ionizing radiation, xenobiotics, chemotherapy) besides the DNA oxidation, protein oxidation also takes an important place. For the protein oxidation, these histone structures are also a target. Due to long lifespan and low turnover rate of histones, proteolytic reactions are required to be highly selective and well regulated. For the turnover of histones, a distinct nuclear proteolytic system is required that brings proteasomal system into mind with the high specificity to degrade oxidized proteins. Degradation of oxidatively damaged histones provides the maintenance of chromatin integrity.

Nucleosomal histones protect DNA from free radical mediated damage [70], and histone detachment and reattachment are closely connected with transcription and replication processes as with DNA repair and therefore requires functionally intact histones. Oxidatively damaged histones are able to cross-link with DNA and would impair the detachment-reassembly process [101]. As mentioned above, automodified PARP-1 activates the proteasome to facilitate selective degradation of oxidatively damaged histones. Heteromodified histones might also lead to the activation of the proteasome [14]. Therefore, I studied the ability of the 20S proteasome to degrade damaged histones as a function of the extent of their oxidation. Data shown in Fig.3.8.A support the concept that mild oxidation exposes hydrophobic residues that render histones susceptible to degradation, whereas more extreme oxidation promotes hydrophobic aggregation, ionic bonds and covalent crosslinks all of which diminish proteolytic susceptibility. This effect has already been described for a number of cytosolic proteins by several groups [23,24,102,103]. In my model system I found a decline in proteolytic degradation of

poly(ADP-ribosyl)ated histones. In the same content Ullrich et al. found a decrease in proteolytic degradation in histones bound to DNA [71].

Protein carbonyl formation following histone oxidation was also tested in this model and was not seen such a high increase in the carbonyl formation [Fig.3.8.C]. This prevention in the carbonyl formation was thought to be as a consequence of the increase in proteolytic degradation. Oxidation of histones was performed for 2h with the nuclear extract, and this duration is thought to be sufficient for oxidized histones to be degraded and prevents the accumulation of carbonylated proteins.

8-OHdG, one of the most abundant oxidative DNA adducts, is used as an indicator of oxidative DNA damage [104]. In the nuclear repair process, following a time point of oxidative damage, damage products are expected to be decreased by several systems. In Fig.3.7 this repair mechanism following 3h after H_2O_2 treatment was confirmed to be proteasome dependent which was effected by selective proteasome inhibitor LC.

Besides all, for the efficiency of chemotherapeutic treatment, closer insights into the cellular defense mechanisms of tumor cells are required. The cytotoxic action of anthracyclines by the generation of free radicals has been demonstrated both in vitro and in vivo [5,105,106]. However, such an antioxidative defense system might also be used by certain leukemia cells to cope with nuclear damages induced by radical-producing compounds. Consequently, these resistant leukemia cells survive chemotherapy and potentially develop a tumor relapse. Previous studies have demonstrated an elevated proteasome activity in leukemia cells and a possible relationship to the proliferative activity [107]. Also PARP enzyme was demonstrated to be highly activated by

chemotherapy treatment [26]. In this context also studies have suggested that PARP inhibition modulates chemotherapy toxicity [108-110].

Each of these functions implies important roles for nuclear proteasome in the chromatin repair activated by PARP enzyme.

4.3 The protesome-PARP-activation in the senescence process

Accumulation of oxidized proteins and a loss of activity of the proteolytic enzymes, including the proteasome, are highlights of age-related changes of cellular metabolism. This accumulation of protein oxidation products might be the result of a reduction of the efficacy of the removal of oxidized proteins or the result of an increased oxidant production. Merker et al. concluded that proteasomal concentration in the nucleus is not changing during the senescence process [111]. The result in Fig.3.9 is consistent with this conclusion and does not show any proteasome amount change in different aged fibroblasts with the PD 19+/-4, 36+/-4, 56+/-3.

The protective functions of PARP-1 discussed above and also its reported role in maintaining telomere length in mice [112] are in line with the previously observed correlation of cellular poly(ADP-ribosyl)ation capacity and life span of mammalian species, whereby the longest-lived species studied (man) displayed 5-fold the level of maximal PARP activity of the shortest-lived (rat), while PARP-1 protein levels in the two species were identical [53]. In analyses of the automodification reaction, human PARP-1 displayed up to 2-fold higher activity. Therefore differences at the level of primary structure of PARP-1 do appear to modulate specific enzyme activity, but this does not fully account for the longevity-related differences in cellular poly(ADP-ribosyl)ation capacity mentioned above. Probably, interaction of PARP-1 with other proteins plays a role.

PARP activity has also been investigated in nuclear fractions from various regions of rat brain as a function of age [113]. In old animals, PARP activity in the hippocampus was found to be lowered by about 50%, but unchanged in cerebral cortex and in cerebellum, compared to adult rats. It was proposed that the lower enzyme activity in aged hippocampus might underlie the higher vulnerability of neurones to various toxic insults in old animals.

An age-dependent decline of cellular poly(ADPribosyl)ation capacity had previously also been found in lymphocytes from rat and man [53], and ADPRT gene expression was recently reported to be downregulated in proliferating fibroblasts from old human donors and from progeria patients compared to normal young donors [114]. Viewed together, various pieces of evidence from a variety of systems and experimental approaches are now converging in support of a close link between poly(ADP-ribosyl)ation and the control of the ageing process.

Chevanne et al. tested PARP-1 and PARP-2 mRNA expressions in B lymphocyte cells from young subjects, old subjects and centenarians. They investigated the relationship between DNA repair capacity and PARP activity in these cell lines. Data showed that cells from centenarians have characteristics in poly(ADP-ribosyl)ation capacity, while in cells from old subjects these phenomena are delayed or decreased. Moreover, cells from old subjects showed a constitutive expression level of both *parp* 1 and *parp* 2 genes reduced by a half, together with a reduced presence of modified PARP-1 and other poly(ADP-ribosyl)ated chromatin proteins in comparison to cells from young subjects and centenarians. They support the hypothesis that this epigenetic modification is an important regulator of the aging process in humans and it appears to be rather preserved in healthy centenarians, the best example of successful aging [115].

In the results of this study, this repair mechanism in senescent cells following oxidative damage was shown to decrease by several experiments. In the immunoblot of PARP-1, a decrease was seen by the increase of the population doublings of the fibroblasts whereas there was no change in the proteasome amount. In Fig.3.10, PARP activity was increased following H_2O_2 treatment in young and middle aged cells and there was no change in PARP activation in senescent cells. Meantime, proteasome activitation increased in all young, middle aged and old cells compared to untreated cells following H_2O_2 treatment. But proteasome activity was found to be less in middle aged and old cells compared to young cells. To confirm all these results, one other experiment was done with young and old nuclear extracts (Fig.3.12). When active proteasome was added into the reaction together with nuclear extracts, proteasome was activated in young cells but not in old cells following poly(ADP-ribosyl)ation and when active PARP was added into the reaction together with nuclear extracts both in young and old cells, there was an increase in the proteasome activities following poly(ADP-ribosyl)ation. This shows that PARP is the limiting factor for the induction of proteasome activity.

4.4 Outlook into future research

In view of the above mentioned prominent role of PARP-1 related activation of proteasomal degradation in tumour cell lines, there is obviously a significant potential of PARP inhibitory substances with appropriate pharmacokinetic profiles as drugs to improve the chemotherapeutic treatment. It will be interesting to see the effects in animal models and clinical studies.

The availability of new tools, such as cell lines deficient in PARPs will contribute greatly to the future understanding of the complex and fascinating functions of poly(ADP-ribosyl)ation in living cells. Using PARP-1 knockout cells, one might expect an

accumulation of oxidative modified proteins and DNA-protein cross links due to reduced proteasome activation and thus a delay in proteolysis of damaged proteins after oxidative stress should be expected.

5 Conclusion

The above discussed experimental findings indicate that the nuclear proteasome selectively degrades oxidatively damaged histones in the nuclei of mammalian cells, where it is activated and regulated by the automodified PARP-1 after oxidative challenge. The interaction of poly-(ADP-ribosyl)ated PARP-1 and the 20S proteasome represents a novel and unique regulatory mechanism that might link together two different nuclear secondary antioxidant defense systems—DNA repair and removal of damaged nucleoproteins, thus allowing restitution of the native chromatin structure and maintenance of genomic integrity under oxidative stress conditions [14].

As previously reported for cytosolic proteins [23,102], oxidative challenge also altered the turnover of endogenous nuclear proteins, demonstrating an enhanced protein degradation in nuclei after hydrogen peroxide treatment. Estimating the importance of this process for normal genome structure and function, tumor cells seem to be well endowed with nuclear proteases, preventing the nucleus from accumulation of erroneously folded proteins.

Because numerous antitumor drugs act via the oxidation of nuclear material in the tumor cell, it is important to know how effectively and precisely tumor cells can cope with nuclear protein oxidation in addition to oxidatively induced DNA damage. Because the proteasomal system as well as the poly(ADP-ribosyl)transferase are involved in the effective and selective removal of oxidized proteins in the nucleus, one may suggest further treatment strategies to effectively interfere with the protein repair and replacement strategies of tumor cells.

As another perspective, besides tumor cells PARP-1 related activation of proteasome was found to be higher in young fibroblasts compared to old ones. This difference brings a new

point to focus in senescence process. Since this repair is less efficient in senescent cells, age related diseases may appear as a consequence and in the clinic this mechanism may be helpful for the efficient treatment.

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Figure list

Fig. 1.1 PARP and PARG in the poly(ADP-ribosyl)ation reactions 8

Fig. 1.2 Domain structure of PARP-1 9

Fig. 1.3 Automodification of PARP and repair of DNA strand breaks 11

Fig. 1.4 Assembly of the core histones into the nucleosome 12

Fig. 3.1 Viability in different concentrations and time durations of H_2O_2 38

Fig. 3.2 20S proteasome activity in HT22 cells 39

Fig. 3.3 Protein expressions of PARP-1 and proteasome 40

Fig. 3.4 PARP-1 mRNA expression 41

Fig. 3.5 Protein carbonyl recovery in HT22 cells 42

Fig. 3.6 COMET assay and PARP cELISA analysis 43

Fig. 3.7 The recovery in 8-OhdG formation 44

Fig. 3.8 Results in isolated histones 46

Fig. 3.9 Protein expressions of PARP-1 and proteasome in different aged fibroblasts 47

Fig. 3.10 PARP activity changes in different aged fibroblasts 47

Fig. 3.11 20S proteasome activity in different aged fibroblasts 48

Fig. 3.12 The effect of PARP in the proteasomal activation in young and senescent cells 49

Table list

Tab. 1.1 Substrates of poly(ADP-ribosyl)ation reactions with their proven functions 11

Tab. 2.1 H_2O_2 concentrations and time durations for the incubation of HT22 cells 20

Tab. 2.2 Inhibitors used in PARP-1 and 20S proteasome inhibition with HT22 cells 20

Tab. 2.3 Lysis buffer used in the nuclear extraction of HT22 cells 21

Tab. 2.4 Extraction buffer used in the nuclear extraction of HT22 cells 21

Tab. 2.5 Solutions for MTT viability assay 22

Tab. 2.6 One well design for the proteasome activity analysis 23

Tab. 2.7 Solutions for proteasome activity analysis 23

Tab. 2.8 Solutions for protein carbonyl measurement by ELISA 27

Tab. 2.9 Solutions for COMET assay 28

Tab. 2.10 Solutions for poly(ADP-ribosyl)ation of histones by liquid scintillation 34

Abbreviation list

3-ABA	3-Aminobenzamide
ADPr	ADP-ribose
AFU	Arbitrary fluorescence unit
ATP	Adenosine triphosphate
BER	Base excision repair
BSA	Bovine serum albumin
Ca & Mg	Calcium&Magnesium
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Fig	Figure
FCS	Fetal calf serum
HEPES	N-2-Hydroxyethylpiperazine-N´-2-ethanesulfonic acid
H_2O_2	Hydrogenperoxide
H_2SO_4	Sulfuric acid
hUBC	Human ubiquitin conjugating enzyme
IgG	Immunoglobulin G
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phophate
LC	Lactacystin
LMA	Low melting agarose

MCA	Methoxycoumarin
$MgCl_2$	Magnesium chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium
NAD	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NMA	Normal melting agarose
NP-40	Nonidet P-40
$^1O_2$	Singlet oxygen
$^{\cdot}OH$	Hydroxyl radical
8-OhdG	8-hydroxy deoxyguanosine
PAGE	Poly acrylamide gel electrophorese
PAR	Poly(ADP-ribosyl)ation
PARG	Poly(ADP-ribose)glycohydrolase
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate buffer saline
PD	Population doubling
PJ-34	N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
SSB	Single strand break

Suc-LLVY-MCA	Succinyl-Leucyl-Leucyl-Valyl-Tyrosyl-4-methylcoumaryl-7-amide
Tab	Table
TCA	Trichloroacetic acid

Summary

Poly(ADP-ribosyl)ation reactions are of interest in recent years and they take place in DNA repair in different processes especially following oxidative nuclear damage. Proteasomal reactions also take place in repair following oxidative nuclear damage with the degradation of oxidized histones.

Antitumor chemotherapy is generally believed to act via the oxidation of nuclear material in the tumor cells. Adaptation to oxidative stress appears to be one element in the development of long-term resistance to many chemotherapeutic drugs. The 20S proteasome has been shown to be largely responsible for the degradation of oxidatively modified proteins in the nucleus. Tumor cells are supposed to have a higher nuclear proteasome activity than do nonmalignant cells. Poly(ADP-ribosyl)ation reactions take place in the tumor cells as a consequence of chemotherapy. Such a reaction might occur with the 20S proteasome –which is known to increase the activity- and also with histones –which is firstly shown to decrease the degradation in this study. After hydrogen peroxide treatment of HT22 cells, degradation of the model peptide substrate suc-LLVY-MCA and degradation of oxidized histones in nuclei increased accompanied by an increase in PARP-1 mRNA expression. In the recovery of the level of protein carbonyls, single strand breaks and 8-OHdG, proteasome and PARP-1 were shown to play a role together. This was tested with inhibitor treatments. The proteasomal activation following poly(ADP-ribosyl)ation of proteasome and the decrease in poly(ADP-ribosyl)ation of histones and increase in the proteasomal degradation of histones following H_2O_2 treatment confirmed our hypothesis.

The second part of the thesis shows the changes in PARP-1 and proteasome in different aged fibroblasts with population doublings 19, 36, and 56. The nuclear protective mechanisms

were shown to be effected during the senescence process. PARP-1 protein amount decreased whereas there was no change in proteasome amount. PARP activation following H_2O_2 treatment increased only in young and middle aged cells. In the nuclear extracts of young and old cells, poly(ADP-ribosyl)ation potentials were tested with NAD^+ addition into the reaction. In addition to that active proteasome and PARP enzymes were added into the reaction and proteasome activity was measured. With active PARP, proteasome activity was increased both in young and old cells whereas there was no increase in old cells without PARP addition. These results show that proteasome activation is mainly limited by PARP activity.

Taken together all results demonstrate the importance of PARP mediated proteasome activation in the repair of oxidatively damaged chromatin.

Zusammenfassung

Poly(ADP-ribosyl)ierungs-Reaktionen sind seit einigen Jahren im Zentrum des wissenschaftlichen Interesses. Sie finden während der DNA-Reparatur, insbesondere nach oxidativer Schädigung des Zellkerns statt. Proteasomale Reaktionen treten ebenfalls nach nukleärem Schaden auf und beinhalten den Abbau oxidierter Histone.

Eine Antitumor-Chemotherapie wirkt in Tumorzellen häufig über die Oxidation nukleärem Materials. Eine Adaptation zu oxidativem Stress ist häufig ein Bestandteil der Entwicklung von Langzeit-Resistenzen gegen viele Chemotherapeutika. Es wurde häufig gezeigt, dass das 20S Proteasom für den Abbau oxidativ modifizierter Proteine im Zellkern verantwortlich ist. Tumorzellen haben eine höhere nukleäre Proteasomaktivität als nichtmaligne Zellen. Poly(ADP-ribosyl)ierungsreaktionen finden im Zellkern als eine Konsequenz der Chemotherapie statt. Solch eine Reaktion erfolgt auch mit dem 20S Proteasom – gefolgt von einer Erhöhung der Aktivität- und auch mit Histonen, mit der Konsequenz eines reduzierten Abbau, wie in dieser Studie erstmals gezeigt. Nach einer Wasserstoffperoxidbelastung von HT22-Zellen steigt der Abbau des Modellpeptides suc-LLVY-MCA und von oxidierten Histonen im Zellkern – begleitet von einem Anstieg der PARP-1 mRNA. Die Wiederherstellung des Spiegels der Proteincarbonyle, der Einzelstrangbrüche und des 8-OHdG setzt eine Interaktion von PARP-1 und des Proteasoms voraus. Dieses wurde mit Inhibitor-Studien untersucht. Die proteasomale Aktivierung nach Poly(ADP-ribosyl)ierung und der Abfall einer Poly(ADP-ribosyl)ierung der Histone sowie ein Anstieg des Histonabbaus nach H_2O_2–induziertem Stress, bestätigten diese Hypothese.

Der zweite Teil der Arbeit beschäftigt sich mit der Veränderung der PARP-1 und des Proteasoms während der Fibroblastenalterung. Es wurden Zellen mit den Populationsverdopplungen 19, 36 und 56 untersucht. Die nukleären Schutzmechanismen waren vom Seneszenzprozess beeinflusst. Der PARP-1-Proteingehalt fiel, während das

Proteasom konstant blieb. Wasserstoffperoxid induzierte eine PARP-Aktivität nur in jungen und mittelalten Zellen. In nukleären Extrakten von jungen und alten Zellen wurde das Poly(ADP-ribosyl)ierungs-Potential nach NAD^+ Zugabe gemessen. Zusätzlich wurden aktives Proteasom und PARP hinzugegeben und die Proteasomaktivität gemessen. Nach Addition von aktiver PARP ist die proteasomale Aktivität in jungen und alten Zellen angestiegen, wobei es zu keinem Anstieg in alten Zellen ohne PARP-Addition gab. Diese Untersuchungen zeigen, dass die proteasomale Aktivierung im wesentlichen durch die PARP-Aktivierung limitiert ist.

Zusammengefasst zeigen meine Resultate die Wichtigkeit der PARP-vermittelten proteasomalen Aktivierung in der Reparatur oxidativ geschädigten Chromatins.

Declaration / Erklärung

I hereby declare that I have made this PhD thesis independently, and used only the specified tools and resources. For all used information the source was indicated.
This work has not been in the same or similar form submitted as thesis in any institution.

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt und nur die angegebenen Quellen und Hilfsmittel benutzt habe und wörtlich oder inhaltlich übernommene Stellen gekennzeichnet wurden.
Diese Arbeit wurde – auch nicht in ähnlicher Form – bei keiner anderen Stelle als Promotion eingereicht.

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6) 15-19 August 2006, Biennial Congress of the International Society for Free Radical Research, Davos, Switzerland, Protective Effects of Vitamin E and Taurin in Methiocarb Induced Oxidative Stress in Rat Liver and Kidney

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