Differentiation-associated Downregulation of Poly(ADP-Ribose) Polymerase-1 (PARP-1) Expression in Skeletal Muscle Increases Its Resistance to Oxidative Stress

Abstract of the PhD thesis

Gabor Olah

Doctoral School of Sport Sciences
University of Physical Education

Consultants: Dr. Zsolt Radak, PhD, DSc
Dr. Csaba Szabo, MD, PhD, DSc

Official reviewers: Dr. Attila Bacsi, PhD
Dr. Erika Koltai PhD

Head of the Final Examination Committee:
Dr. Gabor Pavlik, MD, PhD, DSc

Members of the Final Examination Committee:
Dr. Marta Szmodis, PhD
Dr. Attila Bacsi, PhD

Galveston, TX, USA

2016

INTRODUCTION

PARP-1, Poly(ADP-ribose) polymerase 1

PARP-1, Poly(ADP-ribose) polymerase 1, is the major isoform of the poly (ADP-ribose) polymerase family, consisting of 17 proteins in humans and 16 in mice. It is a constitutive nuclear and mitochondrial protein with well-recognized roles in various essential cellular functions, such as deoxynucleic acid (DNA) repair, signal transduction, different cell death pathways, and a variety of pathophysiological conditions including burn, sepsis, diabetes and cancer. Most of the functions mediated by PARP are related to oxidative stress. Activation of PARP-1 in response to oxidative stress catalyzes the covalent attachment of the poly (ADP-ribose) (PAR) groups on itself and other acceptor proteins, utilizing nicotinamide adenine dinucleotide ($NAD^{+)}$ as a substrate. Overactivation of PARP-1 depletes intracellular NAD^{+}, influencing mitochondrial electron transport, cellular adenosine triphosphate (ATP) generation and, if persistent, can result in necrotic cell death.

Other PARPs

Based on the comparative analysis of their catalytic domain homology, 17 mammalian proteins were named PARPs (Ame et al, 2004).

PARP-2 was discovered as a residual PARP activity in embryonic fibroblast derived from PARP-1 deficient mice (Shieh et al., 1998; Ame et al., 1999). It is a nuclear enzyme, its crystal structure is very similar to the PARP-1. PARP-2 deficient mice show a delay in DNA-strand breaks resealing similar to that observed in PARP-1 deficient cells. (de Murcia et al. 2003; Schreiber, 2002).

PARP-3 is a mono-ADP ribosylase, a core component of the centrosome located at the daughter centriole throughout the cell cycle (Augustin et al., 2003). PARP-3 interacts with PARP-1 suggesting a link between the DNA-damage surveillance network and the mitotic fidelity checkpoint.

VPARP (PARP-4), the largest of the PARP family possessing a mono-ADP-ribosylation feature. It is a part of the Vault particles, which are ribonucleoprotein

(RNP) complexes containing small RNAs and proteins. It also localizes in the nuclei, is associated with the mitotic spindle.

PARP5a or Tankyrase-1 was named by Smith and colleagues as TRF-interacting, ankyrin-related ADP-ribose polymerase (Smith et al., 1998). It has a nuclear origin, but also can be found in the cytosol and its activity is modified by mitogen-activated protein kinases (MAPK).

TiPARP or PARP-7 was found when analyzing the expression patterns of messenger RNAs (mRNAs) after 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment. It is a mono-ADP-ribosylase and co-localized in the nucleus with AHR (aryl hydrocarbon receptor), a transcription factor activated by synthetic hydrocarbons and some endogenous ligands.

Other members of the PARP family not yet mentioned above can be categorized as CCCH-type PARPs, Macro PARPs and other PARPs. The group of CCCH-type PARPs contains PARP-12 and -13, and the already reviewed TiPARP above. They share a common CCCH-like zing finger, WWE (Tryptophan: W; Glutamate: E amino acids) and the catalytic domain. Macro domain PARPs or BAL PARPs, PARP-9,-14,-15 (BAL 1, 2, 3), belong to this group. Macrodomains are highly conserved domains throughout the animal kingdoms, they have the capability of binding PAR. PARP-9 was described using a differential display method from diffuse large B-cell lymphoma (DLB-CL) patients. PARP-14 has a role in focal adhesion turnover, and is localized at the end of actin stress fibers (Vyas et al., 2013).

Other PARPs include PARP-6, PARP-8, PARP-10, PARP-11, PARP-16. Their function is still relatively unknown. PARP-6 is a mono-ADP-ribosyl-transferase found in colorectal cancer specimens. PARP-10 inhibits c-myc, a transcriptional regulator deregulated in cancers, and has a role in cell proliferation (Yu et al. 2005). PARP-16's crystal structure was revealed by Karlberg (Karlberg et al., 2012) and showed auto-mono-ADP-ribosylating activity.

Based on the domain structure of the PARP family, different interactions are possible in different cell compartments. Moreover, PARP-1 interacts with PARP-2 or PARP-3 and Tankyrase 1 and 2 (Cook et al., 2002), widening the possibilities of their biological functions.

Metabolism of poly(ADP-ribose)

Poly(ADP-ribose), pADPr Synthesis

Poly(ADP-ribosylation) is a covalent, reversible modification by PARPs attaching poly-ADP-ribose on a variety of target proteins, using NAD^+ as the substrate. In 1963, historically speaking, Chambon published that there is an enzymatic activity responsible for the synthesis of polymers (PAR) that requires NAD^+ (Chambon et al., 1963). That was the pioneer work that sparked interest from different laboratories and outgrew a competitive field of research.

In resting cells, the acceptor proteins are mono- or oligo--ADP-ribosylated, while in stress conditions, such as under nuclear genotoxic stimuli, proteins are poly-ADP-ribosylated.

There are numbers of proteins as targets for PARylation, such as RNA/DNA polymerases, Topoisomerase II., p53, PCNA just to name a few. Adding ADP ribose units to a protein changes the protein net charge, which results in modified function. Usually this prevents the interaction of a DNA-binding capability (D'Amours et al. 1999).

Poly(ADP-Ribose) Catabolism: Poly(ADP-Ribose) glycohydrolase and others degrades PAR

Mono-and poly-ADP-ribose units can be removed by PAR-removing enzymes. A prediction was made that the lifetime of the PAR polymer is less than 1 minute, which indicates a strictly controlled regulation of the synthesis and degradation (Virag and Szabo, 2002). The principal member of the enzymes is Poly(ADP-Ribose) glycohydrolase **(**PARG), inactivating the gene causes lethality in early embryonic age mice (Koh et al., 2004). Using the lentiviral system elegantly presented by Erdelyi et al., it has been shown that PARG serves as an apoptosis to necrosis switch during severe oxidative stress. (Erdelyi et al., 2009).

Biological function of PARP-1

Role of PARP-1 in the differentiation and gene expression

Differentiation is the process by which a cell changes from one cell type to another to fulfill specific tasks, so they gain new functions and lose others. For example, myosatellite cells or satellite cells are precursors of skeletal muscle cells and upon activation re-enter the cell cycle, start proliferation and differentiate to myotubes.

PARP-1 has been shown to function in various aspects of the transcription process through a variety of mechanisms, including roles as a modulator of chromatin, a coregulator for DNA-binding transcription factors, and a regulator of DNA methylation. Chromatin is a protein–DNA complex, the structural base of information coded, that comprises genomic DNA, core histones (i.e., H2A, H2B, H3, and H4), linker histones (e.g., H1), and other chromatin-associated proteins. One possible way PARP-1 regulates chromatin structure and transcription is the PARylation of proteins bind to chromatin and the auto-PARylation of PARP-1, which affects the structure of chromatin. Histones are targets for PARP1 binding in vitro, and they control the enzymatic activity of PARP1 (Pinnola et al., 2007; Kotova et al., 2011).

Role of PARP-1 in DNA repair

PARP-1 is a key mechanism in maintaining the stability and integrity of the cell DNA. There are constant intracellular (normal metabolism byproducts) and extracellular damaging sources (radiation, chemical agents, heat shock etc.), which might modulate the chemical status of a cell effecting its DNA. These could subsequently cause mutations, chromosomal aberrations, or cell death. PARP-1 inhibition sensitizes cells to genotoxic stimuli (Küpper et al., 1995; Ding and Smulson, 1994). PARP $^{-/-}$ mice are viable and fertile with normal phenotype, but are highly sensitive to ionizing radiation and alkylating agents (de Murcia et al., 1997).

The base excision repair/single-strand break repair process (BER/SSBR)

DNA single-strand break repair is a primary cellular pathway for repairing damaged bases or single-strand breaks. Investigating wild type and PARP-1 deficient cell extracts gave insight into the involvement of PARP-1 in repair. PARP-1 interacted with DNA polymerase β giving a striking consequence. Cell extract from PARP-1 deficient cells failed in the Long Patch Repair and showed moderate effect in Short Patch Repair (Dantzer et al., 2000).

1. 4. 2. 2. Double-strand breaks (DSBs) repair by HR and NHEJ

Double-strand breaks (DSB) have the most serious consequences. DSB can be provoked by ionizing radiation, anti-cancer drugs or even by reactive oxygen/nitrogen species. Nature evolved two mechanisms that step in when this type of damage develops, homologous recombination (HR) and non-homologous end joining (NHEJ). HR takes place when the NHEJ pathways are suppressed; HR requires PARP-1 (Hochegger et al., 2006). NHEJ can be divided into classical-NHEJ and alternative-NHEJ, depending on the active participation of KU70-KU80. When C-NHEJ fails, A-NHEJ (A-EJ) takes over the repair. There is a competition between PARP-1 and KU proteins for the DNA. If KU is not available, PARP-1 is recruited and plays an active role in the A-NHEJ.

PARP-1 in nucleotide excision repair (NER)

The main repair mechanism in prokaryotes and eukaryotes against UV-induced DNA lesions and damages is the nucleotide excision repair (NER). Mutations in the repair machinery members are associated with xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Robu and colleagues showed that PARP-1 works collaboratively with DDB2 (DNA-binding protein 2) and XPC (xeroderma pigmentosum group C) at UV-damaged lesions (Robu et al., 2013; Luijsterburg et al., 2012; Pines et al., 2012).

PARP-1 in mitochondria

Although the scientific field is divided regarding the presence of mitochondrial PARP-1 (mtPARP-1) there are bodies of evidence that support this idea and the role of PARP-1 in mitochondrial DNA repair, bioenergetics, and mitochondrial cell death signaling (Masmoudi et al., 1988; Du et al., 2003; Rossi et al., 2009; Brunyanszki et al., 2016). Depletion of PARP-1 by siRNA in A549 lung epithelial cells or using tissue from PARP-1 KO mice showed enhanced mitochondrial biogenesis and mitochondrial repair (Szczesny et al., 2014).

1. 4. 4. PARP-1 in Cell Death

PARP-1 has been implicated in different modes of cell death, apoptosis and necrosis, parthanatos and autophagy (Galluzzi et al., 2012). DNA strand breaks activate PARP-1 which plays in the repair process. The persistent activation of the enzyme producing excessive poly(ADP-ribose) account for the rapid cell death due to decreased NAD^+ pool and ATP synthesis.

Apoptosis is a tightly regulated process is accompanied by cell shrinkage, chromatin condensation and DNA fragmentation, blebbing of cell membrane, cellular organelles degradation. The apoptotic process can be divided by two subgroups: a, extrinsic initiated by extracellular ligands and b, intrinsic activated by intracellular stressors, DNA damage, oncogenic factors converging on mitochondria. One of the hallmark of apoptosis is the generation of the cleaved PARP-1 by caspase-3 and caspase-7. These two caspases cleave PARP-1 for a 24 kDa N-terminal DNA-binding domain (DBD), which still maintain a strong DNA binding capability, and a 89 kDa C-terminal catalytic fragment which loses its catalytic activity upon cleavage (Tewari et al., 1995).

Necrosis or necroptosis as a new nomenclature was implemented based on the fact that it is a well-regulated process which was considered before as a passive, unregulated way of dying. PARP-1 overactivation-induced necrosis was implicated in several pathophysiological conditions. High concentration of reactive oxygen and nitrogen species (ROS/RNS) caused overactivation of PARP-1.

Parthanatos, which refers to PAR and the Greek word of death, is a recognized cell

death. It is a PARP-1 activation driven upon nuclear damage through AIF translocation from mitochondria to nucleus showing phosphatidylserine externalization, loss of mitochondrial membrane potential, chromatin condensation, shrinkage of the cell typically seen also in apoptosis, but the distinction of loss of cell membrane integrity, the lack of dependence on caspase activation and on energy with the appearance of large 50kb DNA fragments. The translocation of AIF was abolished in PARP-1-KO fibroblasts.

Autophagy is thought to be a self-defense mechanism helping cells with the maintenance of energy homeostasis degrading damaged compounds. There are different inducers such as oxidative stress, starvation, DNA-damage. PARP-1, as discussed above, is activated during oxidative stress and DNA damage which depending on the intensity of the stimuli could also activate the autophagy machinery.

Role of PARP-1 in pathophysiology

An unbalanced cell cycle regulation, cell proliferation or DNA-repair are hallmarks of tumorigenesis. Deletion of one PARP-1, -2, -3 enzymes or pharmacological inhibition under non-stress condition does not lead to major physiological issues or subsequently cancer. But when they are challenged instability occurs. PARP-1$^{-/-}$ mice challenged with whole body γ-irradiation or N-methyl-N-nitroso urea (MNU) showed genomic instability. Cells derived from PARP-1$^{-/-}$ mice also displayed high sensitivity to MNU. (de Murcia et al., 1997, Rouleau et al., 2007).

Reactive oxygen/nitrogen species (ROS/RNS) damage the cells and the components of it such as proteins, lipids, nucleic acids. Depending on the severity of the oxidative stress signal, it might induce apoptosis, necrosis or autophagy. This is associated with a wide spectrum of diseases including cardiovascular, neurological, immunological and diabetic complications (for review, see Virag and Szabo 2002). PARP-1 also has a role in energy metabolism serving as a metabolic regulator. Muscles from PARP-1$^{-/-}$ mice showed higher mitochondrial activity, in connection with SIRT-1 (another NAD^+ consuming enzyme), improved glucose removal and insulin sensitivity (Bai and Canto 2012). Long term pharmacological inhibition of PARP improves fitness in mice by increasing the number of mitochondrial complexes and enhancing mitochondrial

respiratory capacity (Pirinen et al., 2012).

Oxidative stress, as a trigger of PARP activation

As it was discussed earlier, the best characterized activator of PARP-1 is DNA-damage induced by free radicals. Prolonged ROS/RNS (Reactive oxygen/nitrogen species) production results in an excessive PARP-1 activation reducing the cell NAD^+ and ATP stores, blocking apoptosis and resulting necrosis.

Reactive oxygen and nitrogen species are known to contribute to wide variety of diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders, or aging. As a nomenclature point of view, reactive species, either ROS or RNS, are a collective terms and can be divided into two subgroups, free radicals and non-radicals. ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, $^1O_2$, H_2O_2). In other words, *all oxygen radicals are ROS, but not all ROS are oxygen radicals*. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as non-radicals such as HNO_2 and N_2O_4. We can distinguish endogenous and exogenous source of reactive species. The endogenous sources are mitochondria, peroxisomes, phagolysosomes and NO producing enzymes, NO synthases.

The main producers of the reactive species are mitochondria which are continuously working energy producing organelles in cells and are responsible for the terminal oxidation and oxidative phosphorylation. In its electron (e^-) transport chain (ETC), e^- is transferred in the inner mitochondrial membrane and the proton (H^+) gradient generated used for ATP synthesis. In this system, electrons can prematurely reduce oxygen in Complex I., II. and Complex III producing superoxide. Peroxisomes are multifunctional organelles in eukaryotic cells oxidizing fatty acids by the α- and β-oxidation, participating ether-phospholipid biosynthesis, amino acid catabolism, polyamine oxidation. There are different peroxisomal oxidases as part of the metabolic pathways that produce ROS/RNS. Phagolysosomes are organelles which actually use reactive species to destroy invaders. It is a single-membrane phagosome fused with a lysosome.

They could be found in certain white blood cells such as granulo-, mono- and lymphocytes (Forman and Torres 2001).

The effect of the oxidative/nitrosative stress in the context of PARP

In general, ROS and RNS are capable of damaging polyunsaturated fatty acids, proteins, nucleic acids altering their function leading to dysfunction.

Peroxidation of lipids can alter the membrane assembly causing fluidity and permeability changes. The byproduct is 4-hydroxy-2-nonenal (HNE) and acrolein (Niki, 2009).

Proteins also undergo oxidation upon RNS/ROS stimuli. Tyrosine residues are altered by nitration resulting nitrotyrosine which goes hand in hand with tertiary structure changes and loss of function in enzymatic activity. The products of the oxidation are aldehydes, keto- and carbonyl compounds of the proteins.

The other very important molecule, which suffers reactive species-caused damage, is the DNA. In the human body tens of thousands of DNA damage take place a day, which corrected by specific DNA repair mechanisms such as base excision repair or nucleotide excision repair. Whether the repair is successful or not and depending on the rate of the oxidative damage, cells might survive or undergo apoptosis or necrosis in connection with PARP-1 activation (Virag and Szabo, 2002).

We have to keep in mind that the sensitivity to oxidative stress is different between nuclear and mitochondrial DNA, for instance while a given concentration of oxidant (glucose-oxidase treatment) give a rise of DNA damage in a concentration dependent manner in mitochondria, the same amount of oxidant does not generate damage in the nuclear genetic material (Szczesny et al, 2013).

Endogenous antioxidant systems

In order to eliminate or neutralize the ROS/RNS products, the living cells are endowed with antioxidant systems and molecules. Also it is important to note the other source of antioxidant, which come from nutritional sources. There are enzymes such as catalase (CAT), glutathione-peroxidase (GPX) and superoxide dismutase (SOD) which facilitate

the detoxification of the ROS/RNS species. The SOD converts superoxide to hydrogen peroxide while the CAT and GPX transform hydrogen peroxide to water. Two type of SOD can be distinguished, Cu- and Zn-SOD (SOD1) which are from cytoplasmic origin, and manganese-SOD (SOD2) which is found in mitochondria (McCord and Fridovich, 1968, Marklund, 1990). CAT and GPX are found in mitochondria and cytosol.

The non-enzymatic system includes vitamins such as Vitamin C and Vitamin E, or belongs to the carotenoids which originated from plants.

The biology of skeletal muscle

The most fundamental action in life is movement. Moving the whole body, or just part of it, digestion in the intestinal system as it transits nutrient, the movement of the eye, just to name a few are essential for life. To accomplish these complex function, nature evolved muscle which are a very complicated system, but share a common function: contraction based on the molecular mechanism of action of actin and myosin.

The muscles can be divided as striated and smooth. Within the striated group there are two types: skeletal and cardiac. The prior is classified by two regular phenotypes: fast twitch and slow twitch.

Molecular machinery of the contractile force

The vertebrate muscle has a striated appearance and consists of multinucleated cells. Within the cells there are parallel structures called myofibrils. The longitudinal section of the myofibril shows a well-characterized unit called sarcomere and has a repeats in every 2.3 μm along the fibril axis. The underlying structure was revealed by early pioneering work in the 50's and 60's and opened the model for the sliding filament hypothesis of muscle. Two filamentous formations was distinguished: the thick which mainly consists of myosin and the thin filaments which is composed of actin, tropomyosin and troponin complex. In the 1940's Albert Szent-Györgyi showed that at the molecular level, the contraction is based on the interplay of myosin, actin and ATP and it requires Mg^{2+} (1942).

Origin of skeletal muscle

Skeletal muscle originates from the mesoderm which gives rise to somite, a complex transient embryonic structure. A cross-talk has to be taken place between the somite and adjacent structure to form muscle

The cellular basis of myogenesis in adults

In the early 1960's Alexander Mauro made discovery for existence of additional proliferative cells in the skeletal muscle of the tibialis anticus of the frog (Mauro, 1961). They were called satellite cells by A. Mauro which responsible for maintaining and repair of damaged myofibers (Hawke and Garry, 2001). Upon stimulation (exercise, damage), activating different signaling pathways (TWEAK, NF-κB), they proliferate/differentiate to myoblasts then fusing into multinucleated myofibers.

Skeletal muscle cells as a model system

The commonly used model for myogenesis, satellite cell function and morphology is the use of C2C12, primary line of murine myoblasts, isolated in the late 70's, which easily differentiate to myotubes under certain condition (Yaffe and Saxel, 1977). They behavior is very similar of satellite cells and can be used as a model system for such.

AIMS

In differentiating muscle, from myoblasts to myotubes, we showed a marked reduction of PARP-1 expression toward the differentiated myotubes.
Since PARP-1 is activated by oxidative stimuli by ROS/RNS raises some questions whether or not:

- the decreased PARP-1 in myotubes means a possible advantage for the differentiated cells compared against oxidative stress
- the mitochondrial function altered and has beneficial effect comparing undifferentiated and differentiated cells
- pharmacological inhibition of PARP-1 mimic the advantage of the down-regulated features
- the inhibition of PARP could be beneficial in pathological condition

MATERIALS AND METHODS

Reagents. Unless otherwise indicated, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and from Life Technologies (Carlsbad, CA, USA).

Cell culture. The murine C2C12 (Catalog# ATCC® CRL-1772™), rat L6 (Catalog#ATCC® CRL1458™) skeletal muscle cell lines and the human monocyte histiocytic lymphoma cell line, U937 (ATCC® CRL-1593.2™) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Preparation of whole-cell extracts and Western blots. Whole-cell extracts were prepared using NP-40 lysis buffer (20 mM Tris–HCl, pH 8.8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 12 mM Na-deoxycholate). Protein concentration was determined with Pierce BCA Protein Assay Reagent (Thermo Scientific). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose (Bio-Rad) membrane.

MTT viability assay. The MTT assay was performed as described earlier **(**Gerö et al., 2013**)**.

LDH cytotoxicity assay. Lactate dehydrogenase (LDH) release was measured as described previously (Gerö et al., 2014).

Measurement of NAD^+ levels. Total cellular NAD^+ was determined using NAD^+/NADH Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacture's.

Annexin V-phycoerythrin (Annexin V-PE) -7-aminoactinomycin D (7-AAD) staining for apoptosis/necrosis detection by flow cytometry. Detection of cell death was performed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's recommendations.

Bioenergetic analysis in isolated mitochondria. The XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) was used to measure mitochondrial bioenergetic function. Mitochondria were isolated and extracellular flux analysis was performed as previously described (Frezza et al., 2007; Rogers et al., 2011; Módis et al., 2013).

Mitochondrial membrane potential assay. Changes in mitochondrial membrane potential were monitored with TMRE Membrane Potential Kit from Life Technologies (Carlsbad, California, USA) according to manufacturer's instructions and as previously described (Módis et al., 2013).

Fluorescence microscopy. Myoblasts and myotubes were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked in 0.5% Triton X-100 containing 2.5% horse serum. Primary antibodies were applied overnight at 4ºC. Fluorescence was visualized using a Nikon Eclipse 80i inverted microscope with a Photometric CoolSNAP HQ2 camera and the NIS-Elements BR 3.10 software (Nikon Instruments, Melville, NY, USA).

PARP-1 silencing by small-interfering RNA and bioenergetic analysis in PARP-1 silenced cells. Cells (1×10^{5} /well) were transfected with 40 nM PARP-1 specific siRNA (cat#4390771 s62054, Applied Biosystems/Ambion, Austin, TX, USA) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacture's recommendations. XF24 Extracellular Flux Analyzer was used to measure cellular bioenergetics as described (Módis et al., 2012).

Transient transfection of myoblasts with PARP-1. Myoblasts were transfected on 96-well plates with full-length mouse PARP-1 cDNA inserted into pCMV6-Entry vector (Myc-DDK-tagged) purchased from Origene Technologies (Cat#MR211449) (Rockville, USA, MD) using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

Proximity Ligation Assay (PLA) In situ protein/protein proximity/interaction studies were performed with Duolink *in situ* (Olink Bioscience, Uppsala, Sweden). Images were visualized using a Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS-Elements BR3.10 software.

Collection of muscle biopsy samples from children with severe burn injury. Children aged 0 to 17 years with more than 40% total body surface area burns that would require skin grafting, who arrived at our hospital within 96 hours of injury, were eligible. All subjects received standard burn care as previously described (Herndon et al., 1998). Collection and analysis of the samples occurred with the approval of the institutional IRB committee.

Propranolol treatment. The drug was given in a regimen as previously described (Jeschke et al., 2007), at 4 mg/kg/day by mouth from the time of admission for a period of 10±1 months.

Western blotting for poly(ADP-ribose) (PAR). Muscle samples were homogenized in homogenizing buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton-X-100, 10mM EDTA, Protease Inhibitor Cocktail (Complete Mini by Roche) and was performed as previously described (Tóth-Zsámboki et al., 2006).

Immunohistochemical analysis. Frozen sections of 5 micron thickness were prepared from all biopsy specimens collected. Primary antibodies used in our studies were: 1) PAR (Tulip mouse monoclonal, anti-human 1:10); 2) CD-31 (DAKO mouse monoclonal, anti-human, 1:200); 3) Von Willebrand factor (DAKO mouse monoclonal, anti-human, 1:200); 4) S-100 (DAKO rabbit polyclonal, anti-human, 1:4000).

PAR immunostaining procedure. Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (PAR) biotin solution for 1 hour. Afterwards, slides were incubated in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes.

Double immunostaining procedure. Double immunohistochemical staining was performed according to the following combinations: 1) PAR (FR) and CD-31 (DAB), 2) PAR (FR) and factor VIII-related antigen (DAB), 3) PAR (FR) and S-100 (DAB).

Statistical analysis. Data obtained from C2C12, L6 and U937 cells are shown as means ± SEM and SD. One-way ANOVA was applied for statistical analysis, and Tukey's post-hoc test was used for the determination of significance between individual groups. The value of $p<0.05$ was considered statistically significant. For the analysis of human samples, nonparametric ANOVA test was applied and the Kruskal-Wallis post-hoc test was used. $P < 0.05$ considered as significant.

Results

Myoblast differentiation is associated with downregulation of PARP-1 expression

The C2C12 cell line is a well-defined model for skeletal muscle. Proliferating myoblasts differ from terminally differentiated, non-proliferating myotubes in morphology and protein expression profiles. To confirm proper differentiation, we monitored the expression of transcription factor paired-box 7 (Pax7), proliferating cell nuclear antigen (PCNA), and myogenin, which is known to be expressed in differentiated myotubes **(**Wang and Rudnicki 2012**)**. These results confirm that the process of myoblast differentiation is accurately recapitulated. Moreover, we observed a marked decrease in PARP-1 expression in myotubes. To confirm our observation that skeletal muscle cell differentiation is accompanied by reduction in PARP-1 expression, we performed similar Western blot analyses using another well-defined model of skeletal muscle differentiation, namely, rat-derived L6 cells (Hudson et al., 2014). The obtained data clearly indicate that myotubes of L6 cells have reduced expression of PARP-1. Moreover, differentiation of U937 cells, induced by PMA, also showed a reduction in PARP1 expression. As we showed, the level of PARP-1 was not a results of contact inhibition.

Differentiated myotubes develop resistance to oxidative stress

In order to study the effect of PARP-1 inhibition, we first determined the maximum non-toxic concentration of well-known PARP inhibitor, PJ34 and we selected 10μM as the highest, non-toxic concentration to use for subsequent studies.

Consistent with our observations that PARP-1 expression is downregulated in myotubes as compared to myoblasts, H_2O_2 challenge induced a lesser degree of PARP-1 activation in myotubes than in myoblasts, as determined by Western blot analysis of PAR adducts in whole-cell extracts of each cell type.

Next, we compared the changes in the viability of myoblasts and myotubes exposed to various concentrations of H_2O_2 by monitoring the LDH release and the conversion of MTT to formazan, and also quantifying cellular NAD'raml'si citometri'valfff levels. As

expected, increasing concentration of H_2O_2 caused an increase in LDH release in myoblasts. As expected, pre-treatment with the PARP inhibitor, PJ34, significantly reduced H_2O_2-induced LDH release in myoblasts. Similarly, we observed significant reduction of both MTT conversion capability and NAD+ levels in myoblasts exposed to increasing concentrations of H_2O_2, but not in myotubes. Similarly, PJ34 pretreatment attenuated the deleterious effect of H_2O_2 in myoblasts, with only relatively minor effects in myotubes. The cytotoxic effects of glucose oxidase (GOx) were also attenuated in PJ34-treated myoblasts in a concentration-dependent manner. Myotubes were affected only by the highest concentration of GOx. We performed similar sets of experiments in another type of skeletal muscle cell line, namely, rat L6 cells and reached the same conclusions that myotubes were more resistant to the same concentrations of H_2O_2 and GOx.

Our observation that myotubes are resistant to oxidant induced stress was further validated by flow cytometry. Upon oxidative challenge, PARP inhibition with PJ34 reduces cell death primarily by decreasing the portion of necrotic and early apoptotic cell populations suggesting that this process has a significant PARP-1dependent component.

Myotubes preserve mitochondrial functions during oxidative stress

We investigated the differences in major bioenergetics parameters in mitochondria isolated from C2C12 myoblasts and myotubes with Extracellular Flux Analysis. Myotubes were found to have higher ATP turnover (State 3) and maximal respiratory capacity (State 3u) than myoblasts. We also observed a gradual decrease of the mitochondrial membrane potential, as a way to look at mitochondrial function, in response to increasing concentration of H_2O_2 in the myoblasts.

PARP-1 level was found higher in nuclear and mitochondrial fractions of myoblasts as compared to myotubes.

We investigated the effect of transient siRNA-mediated silencing of PARP-1 on the bioenergetic response in C2C12 myoblasts. Silencing PARP-1 expression increases both oxidative phosphorylation and glycolytic activity.

Forced expression of PARP1 increases the oxidant sensitivity of myotubes

PARP1 overexpression was performed in myotubes in order to see whether the increased level of PARP-1 make these cells more sensitive to the oxidative stress and we found that myotubes with forced expression of PARP1 became more sensitive to hydrogen peroxide induced cell injury: they responded with increased LDH release, compared to the sham-transfected myotube controls.

Oxidative stress leads to an early-onset of PARP-1 activation in U937 cells

10 minutes of oxidative stimulus (H_2O_2, 400μM) induced PARP-1 activation (auto-PARylation of PARP-1) in the extranuclear/mitochondrial compartment, but not in the nucleus. At 3-24 hours, in line with other studies, nuclear PARP activation occurred. Other pathophysiologic steps were involved after the early-onset of PARP-1 activation such as mitochondrial DNA damage, cellular oxidant production and compromised cell membrane integrity.

β-adrenoceptor signaling is involved in PARP-1 activation during H_2O_2 challenge

Pretreatment of U937 cells with the β-receptor antagonist propranolol (10 μM) prior to 10 minutes of H_2O_2 challenge significantly reduced the increase of PARylation of multiple proteins and the auto-PARylation of PARP-1. The regulation of PARP-1 activation appears to be a general phenomenon since propranolol inhibited the PARylation in C2C12 myoblasts during the H_2O_2 challenge.

The positive effect of PARP inhibition in skeletal muscle biopsies from burn patients

We compared PARP activation in normal skeletal muscles and muscles from different time point after burn injury. Collection of biopsies and analysis of the samples occurred with the approval of the institutional IRB committee. The PARylation mainly affected

PARP-1 at ~120kDa, although other proteins were also modified by the PARylation process.

Immunohistochemical evaluation of PARP activation mostly was observed in the 'Middle' group compared to control group. The signal was primarily seen in the capillary endothelium and co-localized with CD-31 and factor VIII-related antigen. Also, PAR positive staining was observed in mononuclear cells and neuroglial cells confirmed by S-100 staining.

The other goal of the study was to evaluate the effect of propranolol therapy on the PARP activation. The main finding was the reduced PARylation in the 'Middle' and in some degree in the 'Late' group compared to the patients who did not receive propranolol.

Future directions

Observations made in the current study that remain to be investigated include (a) the functional consequence of other PARP isoforms during differentiation, (b) the mechanism through which glycolytic activity increases after PARP-1 silencing in myoblasts, and (c) the reasons why the downregulation of PARP-1 during differentiation is cell-type dependent. It is interesting to note that inhibition of PARP-2 has recently been shown to produce an increase in mitochondrial biogenesis in skeletal muscle (Mohamed et al., 2014). (d) For burn patients it would be also beneficial to explore if incorporating antioxidants or PARP inhibitors as a supplement to exercise program currently used enhances the outcome of therapy and to identify the molecular triggers of PARP activation in burn patients to better address the therapy.

Conclusions

Based on multiple lines of functional data, we concluded that the functional importance of the differentiation associated decrease in PARP-1 in C2C12 muscle cells is that it endows myotubes with increased oxidative stress resistance and bioenergetic function. We realize that the regulation of PARP-1 in myoblasts and myotubes may be a specialized case since PARP-1 has multiple roles in a variety of cell types.

We showed an early PARP-1 activation in a mitochondrial localization and the inhibition of PARylation by the β-adrenoceptor agonist propranolol.

We also provided evidence for PARP activation in muscle tissue of burn patients and were able to demonstrate the protective effect of propranolol. In order to clarify the exact mechanistic role of PARP in human burns, future, interventional studies with PARP inhibitors would be necessary.

- PARP-1 protein expression level is markedly decreased during skeletal muscle cell differentiation in murine and rat cell-based models
- The reduced level of PARP-1 in myotubes results greater oxidative stress resistance than their undifferentiated counterparts.
- PARP-1 participates in the regulation of muscle cell bioenergetics under normal conditions and during oxidative challenge. We showed increased respiratory parameters in myoblasts with siRNA-mediated silencing of PARP-1 suggesting a beneficial effect of the depletion.
- There is an early-onset of PARP-1 activation in the mitochondria in U937 cells and can be inhibited by the β-adrenoceptor agonist propranolol.
- Pharmacological inhibition of PARP-1 has a beneficial effect in burn patients.

List of publication related to the dissertation

Olah G, Szczesny B, Brunyanszki A, Lopez-García IA, Gero D, Radak Z, Szabo C. (2015) Differentiation-associated downregulation of poly(ADP-ribose) polymerase-1 expression in myoblasts serves to increase their resistance to oxidative stress. PloS One, 10: e0134227.

Brunyanszki A, **Olah G**, Coletta C, Szczesny B, Szabo C. (2014) Regulation of mitochondrial poly(ADP-ribose) polymerase activation by the β adrenoceptor / cAMP / protein kinase A axis during oxidative stress. Mol Pharmacol, 4: 450-62.

Olah G, Finnerty CC, Sbrana E, Elijah I, Gero D, Herndon DN, Szabo C. (2011) Increased poly(ADP-ribosyl)ation in skeletal muscle tissue of pediatric patients with severe burn injury: prevention by propranolol treatment. Shock, 36: 18-23.