Role of poly(ADP-ribose) polymerase-1 (PARP-1) in the regulation of transcription

by

Attila Brunyánszki

Supervisor: Dr. Péter Bai
1. Table of Contents

1. TABLE OF CONTENTS ............................................................................................................. 1
2. ABBREVIATIONS ................................................................................................................ 3
3. INTRODUCTION .................................................................................................................. 5
3.1. Enzymatic properties of poly(ADP-ribose) polymerase-1 ........................................ 5
3.1.1. Reactive species evoked oxidative stress: activators of PARP-1 ......................... 7
3.1.2. Endogenous sources of reactive species ................................................................. 8
3.1.3. The harmful effects of reactive species ................................................................. 13
3.2. Biological functions of PARP-1 .................................................................................. 15
3.2.1. Role of PARP-1 in the regulation of transcription ................................................ 15
3.2.1.1. Transcriptional events in inflammation mediated by PARP-1 .................... 20
3.2.1.2. PARP-1 in the transcriptional control of oxidative energy metabolism ......... 22
4. LITERARY OVERVIEW AND AIMS .............................................................................. 25
5. MATERIALS AND METHODS ......................................................................................... 26
5.1. Methods ....................................................................................................................... 26
5.1.1. In vivo studies ........................................................................................................ 26
5.1.2. Preparation of MEF cells ...................................................................................... 26
5.1.3. Histology and microscopy ..................................................................................... 27
5.1.4. Myeloperoxidase activity assay ........................................................................... 27
5.1.5. MMP activity assay .............................................................................................. 28
5.1.6. 8-oxo-2′-deoxyguanosine (8-OHdG) determination ........................................ 28
5.1.7. Protein carbonyl assay .......................................................................................... 29
5.1.8. Lipid peroxidation assay ...................................................................................... 30
5.1.9. Total RNA preparation, reverse transcription and qPCR .................................. 31
5.1.10. Transcription factor transactivation studies ..................................................... 33
5.1.11. mtDNA analysis ................................................................................................. 34
5.1.12. Statistical analysis .............................................................................................. 34
6. RESULTS ......................................................................................................................... 35
6.1. Role of PARP-1 in oxazolone-induced contact hypersensitivity ............................ 35
6.1.1. Characterization of the oxazolone-induced contact hypersensitivity reaction in PARP-1−/− mice ................................................................. 35
6.1.2. Lack of oxidative/nitrosative stress in PARP-1−/− mice ........................................ 38
6.1.3. PARP-1 mediated gene expression in oxazolone-induced contact hypersensitivity ........... 42
6.2 Transcriptional changes affected by the PARP-1 SIRT1 interdependence ........... 44
6.2.1. Brown adipose tissue and muscle, but not liver from PARP-1−/− mice have higher mitochondrial content. ................................................................. 44

6.2.2. Reduced PARP-1 activity in murine embryonic fibroblasts promotes oxidative metabolism.... 47

6.2.3. Pharmacological inhibition of PARP-1 activity enhances oxidative metabolism via SIRT1..... 48

7. DISCUSSION ........................................................................................................................................... 49

7.1. Role of PARP-1 in oxazolone-induced contact hypersensitivity ......................... 49

7.2. Transcriptional effects of the PARP-1 SIRT1 interplay................................. 53

8. SUMMARY ............................................................................................................................................. 57

9. ÖSSZEFOGLALÁS .................................................................................................................................. 58

10. REFERENCES ....................................................................................................................................... 59

11. KEYWORDS .......................................................................................................................................... 75

12. ACKNOWLEDGEMENT ....................................................................................................................... 76

13. APPENDIX ............................................................................................................................................. 77
2. Abbreviations

3-AB 3-amino-benzamide
8-OHdG 8-oxo-2'-deoxyguanosine
ACC acetyl-CoA carboxylase
ACO acyl-CoA oxidase
AP-1 activator protein-1
ARH3 ADP-ribosyl hydrolase-3
ATF-2 activating transcription factor
ATP5g1 ATP synthase lipid-binding protein
BAT brown adipose tissue
BCL6 B-cell lymphoma 6 protein
B-Myb Myb-related protein B
BRCT breast cancer associated protein C-terminus
CHS contact hypersensitivity reaction
COX17 cytochrome c oxidase or Complex IV
CPT1 carnitine palmitoyltransferase I
CXCL1 chemokine (CXC motif) ligand 1 (CXCL1)
Cyt C cytochrome c
DAPI 4,6-diamidino-2-phenylindole
Dio iodothyronine deiodinase
DTT dithiothreitol
eNOS endothelial nitric oxide synthase
ERR estrogen-related receptor
E-selectin endothelial selectin
FOXO forkhead box O
G6Pase glucose-6-phosphatase
GK glucokinase
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNE trans-4-hydroxy-2-nonenal
I-CAM intercellular cell adhesion molecule
IL-1β interleukin-1β
iNOS inducible nitric oxide synthase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-CAM</td>
<td>leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>MCAD</td>
<td>medium chain acetyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MHC</td>
<td>myosine heavy chain</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>Oct-1</td>
<td>organic cation transporter member 1</td>
</tr>
<tr>
<td>OXA</td>
<td>oxazolone</td>
</tr>
<tr>
<td>PAR</td>
<td>poly(ADP-ribose)</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenol pyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PPAR gamma coactivator 1-α</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Sp1</td>
<td>stimulating protein-1</td>
</tr>
<tr>
<td>SIRT</td>
<td>siruin</td>
</tr>
<tr>
<td>TFF-1</td>
<td>Trefoil factor 1</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Trop</td>
<td>troponin</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>V-CAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
3. Introduction

3.1. Enzymatic properties of poly(ADP-ribose) polymerase-1

Poly(ADP-ribose) polymerase-1 (PARP-1) belongs to the poly(ADP-ribose) polymerase (PARP) superfamily. This superfamily consists of 17 members sharing a similar catalytic domain (Ame et al., 2004). PARP-1, the founding member of the superfamily, is a multidomain protein comprising a DNA binding domain at the N-terminus, an automodification domain, functioning as a target for direct covalent auto-modification (D'Amours et al., 1999) and a catalytic domain at the carboxyl terminus that polymerizes ADP-ribose units (Fig. 1). The first two zinc finger motifs at the N-terminus facilitate tight binding of PARP-1 to DNA that promotes the activation of the catalytic domain at the C-terminus. The third zinc finger also plays an important role in the inter-domain interactions and is vital for PARP-1 enzymatic action (Langelier et al., 2008). The automodification domain contains a BRCT motif that is involved in protein-protein interactions, which promote the recruitment of DNA repair enzymes to the site of DNA damage (El-Khamisy et al., 2003; Masson et al., 1998). The catalytic domain contains the NAD⁺ acceptor sites and critical residues involved in the initiation, elongation and branching of poly(ADP-ribose) polymers (PAR).

![Figure 1. Domain architecture of human poly(ADP-ribose) polymerase-1.](image)

NLS: nuclear localization signal; ZBD3: third zinc-binding domain; BRCT: BRCA1 C-terminus-like (BRCT) motif. Reproduced from (Kraus, 2008).

The catalytic function of PARP1 relates to its role as DNA-damage sensor and signalling molecule. The zinc fingers of PARP-1 recognize single- and double-stranded DNA breaks and PARP-1 subsequently forms homodimers and catalyses covalent binding of ADP-ribose units from donor NAD⁺-molecules to a variety of target proteins resulting in linear or branched polymers of PAR as large as 200 units (D'Amours et al.,
This covalent modification takes place at glutamate and less commonly at aspartate, or lysine residues of target molecules (D’Amours et al., 1999; Rolli et al., 1997).

**Figure 2. Biochemistry of poly(ADP-ribosylation).**

PARP hydrolyse NAD$^+$ and catalyze the successive addition of ADP-ribose units mainly to glutamate residues of either acceptor proteins (heteromodification) or themselves (automodification). PAR is heterogeneous in size and complexity, as indicated by the $x$, $y$ and $z$ labels that represent values from 0 to >200. PARG and ARH3 can both hydrolyse PAR at the indicated positions; activators and outcomes of PAR synthesis are indicated. Ade: adenine; Nam: nicotinamide; PAR: poly(ADP-ribose); PARP: poly(ADP-ribose) polymerase; Rib: ribose; ARH3: ADP-ribosyl hydrolase-3; PARG: poly(ADP-ribose) glycohydrolase. Reproduced from (Hakme et al., 2008b).

Under physiological conditions enzymatic activity of PARP-1 is very low, but it is stimulated dramatically in the presence of a variety of allosteric activators such as single- or double-strand breaks, crossovers, cruciforms, supercoils, some specific double-stranded sequences (Rolli et al., 2000), nucleosomes, and a variety of protein-binding partners (D’Amours et al., 1999; Kim et al., 2004; Kun et al., 2002; Oei and Shi, 2001). Moreover, a DNA damage independent PARP activation pathway has also been reported (Homburg et al., 2000). The best-characterized activator of PARP-1 is DNA damage induced by free radicals that exert several effects in cells. Enhanced oxidative stress-provoked excessive PARP-1 activation may deplete cellular NAD$^+$/ATP stores blocking apoptosis and resulting in necrosis (Virag and Szabo, 2002). Moreover, reactive species can play a role in a series of other cell process such as cell signalling, genome integrity or transcription. To understand the role of PARP-1 in these processes, hereby we will overview the origin of reactive species.
3.1.1. Reactive species evoked oxidative stress: activators of PARP-1

Oxidative stress is an imbalance evoked by the excessive formation of ROS (Reactive Oxygen Species, ROS) and/or RNS (Reactive Nitrogen Species) and limited antioxidant defenses (Turrens, 2003). Molecules or elements bearing an unpaired electron are defined as free radicals, in chemistry. Free radicals are often regarded as reactive species formed in vivo in biology, of which numerous bear unpaired electrons (hence fall into the chemical category of true free radicals, e.g. superoxide anion). However, some reactive species possess reactive character although lack unpaired electrons (e.g. peroxynitrite). Free radicals and their derivatives can be classified as reactive oxygen species and reactive nitrogen species depending on the central atom. Various derivatives are formed at the same time and at the same place under oxidative stress, developing their biological impact on the cells together. Hence, in biology different free radicals and their derivatives are referred as reactive species representing their joint participation in specific biological process. The most frequent reactive species are summarized in Table 1.

<table>
<thead>
<tr>
<th>Radicals</th>
<th>Name</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxygen (bi-radical)</td>
<td>**O₂</td>
</tr>
<tr>
<td>“true” free radicals</td>
<td>Superoxide anion</td>
<td>*O₂⁻</td>
</tr>
<tr>
<td></td>
<td>Hydroxyl radical</td>
<td>*OH</td>
</tr>
<tr>
<td></td>
<td>Peroxyl radical</td>
<td>ROO*</td>
</tr>
<tr>
<td></td>
<td>Alkoxy radical</td>
<td>RO*</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide</td>
<td>NO*</td>
</tr>
<tr>
<td>radicals without unpaired electrons</td>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
</tr>
<tr>
<td></td>
<td>Organic peroxide</td>
<td>ROOH</td>
</tr>
<tr>
<td></td>
<td>Hypochlorous acid</td>
<td>HOCl</td>
</tr>
<tr>
<td></td>
<td>Ozone</td>
<td>O₃</td>
</tr>
<tr>
<td></td>
<td>Aldehydes</td>
<td>HCOR</td>
</tr>
<tr>
<td></td>
<td>Peroxynitrite</td>
<td>ONOO⁻</td>
</tr>
</tbody>
</table>

Table 1. List of important reactive species in biological systems.
The most reactive among these species are the hydroxyl radicals, hydrogen peroxide and peroxynitrite. Hydroxyl radicals are short lived (Halliwell and Gutteridge, 1999). Hydroxyl radical is a powerful oxidizing agent that can react at a high rate with most organic and inorganic molecules in the cell. In particular, hydrogen peroxide is not radical by definition but causes damage to the cell at a relatively low concentration (10 µM). Hydrogen peroxide is freely dissolved in aqueous solution and can easily penetrate biological membranes (Halliwell and Gutteridge, 1999). This is in contrast to superoxide radicals that are considered relatively stable and have constant, relatively low reaction rates with biological components. Superoxide can react with NO forming peroxynitrite:

\[ \cdot \text{O}_2^- + \text{NO}^* \rightarrow \text{ONOO}^- \]

Peroxynitrite (ONOO⁻) is a powerful oxidizing agent (Beckman and Koppenol, 1996) that - upon protonation - decomposes to hydroxyl radical and nitrogen dioxide. It causes the depletion of sulphydryl (SH) groups and oxidation of a wide variety of molecules causing damage (Halliwell and Gutteridge, 1999).

### 3.1.2. Endogenous sources of reactive species

Cells are exposed to a large variety of ROS and RNS from both exogenous and endogenous sources. Although the exogenous exposure to ROS can also be significant, endogenous sources is much more important because it can be produced continuously during the life span of every cell (Kohen, 1999). Hereby we concentrate on the endogenous sources of reactive species.

**Mitochondria** The most prominent ROS producing organelle is the mitochondrion. Mitochondria are working continuously under the whole life span of a cell and represent the place of terminal oxidation and oxidative phosphorylation. Electrons are transferred from reducing equivalents (such as NADH and FADH₂) to molecular oxygen leading to the build up of a proton gradient across the inner mitochondrial membrane that is then used for ATP synthesis in terminal oxidation. Importantly, electrons may leak from the respiratory chain at complex I, complex III and reduce oxygen to superoxide (Fig. 3) instead of serving as substrates for the complete reduction of molecular oxygen to water as catalyzed by cytochrome c oxidase (complex IV).
Figure 3. Generation of superoxide at the respiratory chain of the inner mitochondrial membrane.
Superoxide is formed by reduction of molecular oxygen by electrons leaking from complex I (NADH: ubiquinone oxidoreductase, also known as NADH dehydrogenase) as well as complex III (ubiquinol: cytochrome c oxidoreductase). Q: ubiquinone; Cyt c: cytochrome c. Reproduced from (Jacob and Winyard, 2009).

It has been estimated that up to 1% of all oxygen molecules used in respiration is only partially reduced and thus yield superoxide (Powers and Jackson, 2008). The respiratory complex I and complex III can leak electrons generating superoxide, but only complex III is capable of leaking electrons to the intermembrane space. As a result of this process, several different oxygen derivatives are formed (e.g. H₂O₂) (Ames BN, 1995; Bacon and Britton, 1989).

**Peroxisomes** Peroxisomes also possess ROS generating abilities (Schrader and Fahimi, 2006). The main metabolic processes contributing to the generation of H₂O₂ in peroxisomes are the β-oxidation of fatty acids and the enzymatic reactions of the flavin oxidases (Fig. 4.). Furthermore, transition metal ion complexes (e.g. iron and copper) are abundant in peroxisomes. Under certain conditions, these metal ions might be released (e.g. by xenobiotics) and catalyze the formation of *OH in the Fenton reaction:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}$$
The occurrence of reactive species leads to lipid peroxidation, damage of the peroxisomal membrane and loss of peroxisomal functions (Bacon and Britton, 1989; Yokota et al., 2001).

**Figure 4. Schematic overview of peroxisomal enzymes that produce or degrade ROS.**

H$_2$O$_2$ is produced by peroxisomal oxidases (e.g., acyl-CoA oxidase that is involved in the β-oxidation of fatty acids). H$_2$O$_2$ is decomposed by catalase and glutathione-peroxidase (GPx) or converted to hydroxyl radicals (*OH). Hydroperoxides formed in this process can be decomposed by catalase and glutathione-peroxidase. Superoxide anion (*O$_2^-$) are generated by peroxisomal oxidases (for example, xanthine oxidase (XOx)) Nitric oxide synthase (NOS) catalyses the oxidation of L-arginine (L-Arg) to nitric oxide (NO). H$_2$O$_2$ and NO can penetrate the peroxisomal membrane and act in cellular signalling. Protein Mpv17 (Mpv17) and Mpv17-like protein (M-LP) regulate the peroxisomal ROS production. SOD: superoxide dismutase. Reproduced from (Schrader and Fahimi, 2006).
**Phagolysosomes** In phagolysosomes, exogenous invaders (e.g., invading bacteria) are destroyed by reactive species. Phagolysosomes are mainly present in certain white blood cells (granulocytes, monocytes, and lymphocytes) (Forman and Torres, 2001; Yokota et al., 2001) and major producers of endogenous ROS during inflammatory processes (Ginsburg, 1998; Ginsburg and Kohen, 1995). Following stimulation, these cells undergo a respiratory burst (Babior et al., 2002). The consequence of the respiratory burst is that a number of reactive oxygen species are produced, which kill the engulfed bacteria. NADPH serves as a donor of electrons to an activated enzymatic complex in the plasma membrane. This NADPH-oxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. Following dismutation, the production of H$_2$O$_2$ leads to the formation of *OH by the metal-mediated, Haber-Weiss reaction (Fig. 5A). The enzymatic activity of myeloperoxidase (the most abundantly in neutrophils) (Hawkins et al., 2001; Male et al., 2006; Rodrigues et al., 2002) leads to the production of HOCl by interaction between hydrogen peroxides and chlorides (Fig. 5B) (Hawkins et al., 2001).

![Figure 5](image.png)

**Figure 5.** (A) Oxygen-dependent myeloperoxidase-independent and (B) myeloperoxidase-dependent intracellular killing in neutrophil granulocytes. (A) The activated NADPH oxidase uses oxygen to oxidize NADPH. The result is the production of superoxide anion. Some of the superoxide anion is converted to H$_2$O$_2$ and singlet oxygen by superoxide dismutase. In addition, superoxide anion can react with H$_2$O$_2$ resulting in the formation of hydroxyl radical and more singlet oxygen. The result of all of these reactions is the production of the toxic oxygen compounds superoxide anion (O$_2^-$), H$_2$O$_2$, singlet oxygen (\(^{1}O_2\)) and hydroxyl radical (*OH). (B) Myeloperoxidase utilizes H$_2$O$_2$ and halide ions (usually Cl$^-$) to produce hypochlorite (OCl$^-$), a highly toxic substance. Some of the hypochlorite can spontaneously break down to yield singlet oxygen (\(^{1}O_2\)). Reproduced from (Male et al., 2006).

**Nitric oxide synthase enzymes** Nitric oxide synthase (NOS) enzymes synthesize NO. NOS enzymes convert L-arginine to NO and L-citrulline via the intermediate N-
hydroxy-L-arginine (James, 1998; Moncada et al., 1991; Nathan and Xie, 1994; Stuehr and Marletta, 1985) (Fig. 6). There are three NOS enzymes. Neuronal NOS (nNOS, NOS-1) was originally characterized in neurons and it is expressed in the central and peripheral neuronal tissue (Ignarro, 2000). Endothelial NOS (eNOS, NOS-3) was originally characterized in endothelial cells, it produces NO in blood vessels and regulates vascular function (Ignarro, 2000). The third type of NOS, described in the immune and cardiovascular system, is the inducible form of NOS (iNOS or NOS-2) (Ignarro, 2000). Activity of nNOS and eNOS is activated in response to a calcium signal, while iNOS activity is regulated at the level of expression. Expression of iNOS is induced by several agents including bacterial lipopolysaccharide or cytokines such as interferon-γ (IFNγ), IL-1 or tumor necrosis factor-α (TNFα). In contrast to nNOS and eNOS, iNOS generates high concentrations of NO and this level of synthesis is sustained for hours or days, depending on how long the enzyme is present in the cells or tissue. iNOS has an eminent role in inflammation-related “respiratory burst” (James, 1998; Moncada et al., 1991).

**Figure 6. General reaction mechanism and cofactors of NOS enzymes.**
Electrons are donated by NADPH to the reductase domain of the enzyme and transferred via FAD and FMN redox carriers to the oxygenase domain. There they interact with the hem iron and a cofactor (tetrahydrobiopterin BH₄) at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as end products. Electron flow through the reductase domain requires the binding of calmodulin to the reductase domain. Reproduced from (Alderton et al., 2001).
3.1.3. The harmful effects of reactive species

Reactive species can damage vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids.

Lipid peroxidation influences membrane phospholipids containing polyunsaturated fatty acids. Peroxidation of lipids can disturb membrane assembly causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes. The products of this reaction are 4-hydroxy-2-nonenal (HNE) and acrolein (Esterbauer et al., 1991; Niki, 2009; Parola et al., 1999).

Predominantly hydroxyl, alkoxyl, and nitrogen-centred radicals cause protein damage. Proteins can undergo oxidation, damage to specific amino-acid residues (e.g. nitration of tyrosine yielding nitrotyrosine), or tertiary structure degradation and fragmentation. The consequence of protein damage is usually loss of enzymatic activity and altered cellular functions. Protein oxidation products are usually aldehydes, keto and carbonyl compounds (Berlett and Stadtman, 1997; Grune et al., 1997; Levine and Stadtman, 2001).

Reactive species induce modifications of DNA such as modification of nucleotide bases, single- and double strand DNA breaks, loss of purines (apurinic sites), damage to deoxyribose, DNA-protein cross-linkage, and damage to the DNA repair system. Mainly the *OH can attack DNA bases for example at guanine site to yield an oxidation product, 8-hydroxy-2-deoxyguanosine (8-OHdG)(Cadet et al., 2010; Cooke et al., 2003; Poulsen et al., 2000). The oxidative modifications evoked by reactive species are summarized on Fig. 7.
The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components such as lipids, proteins and DNA. These alterations modify many cellular functions. Reproduced from (Kohen and Nyska, 2002).

These reactive species-induced oxidative modifications can also play role in redox signaling. Redox modifications lead to alterations of biomolecules that are the activators of diverse signaling pathways, initiating redox-sensitive regulation and redox-dependent signal transduction. These changes may modulate gene expression through redox-sensitive transcription factors (Haddad, 2002). There are numerous transcription factors that are described as redox-sensitive, such as NF-κB, AP-1. These transcription factors are known to interact with PARP-1, although in most cases the exact molecular background of the interaction is not yet defined. We will outline the consequence of these free radical-evoked processes in the following chapters.
3.2. Biological functions of PARP-1

As mentioned earlier, PARP-1 has diverse biological functions including DNA repair functions such as base excision repair (Dantzer et al., 2000; Dantzer et al., 1999) nucleotide excision repair and contributes to double strand break repair (Audebert et al., 2004; Fisher et al., 2007). PARP-1 also possesses non-repair functions such as regulation of cardiac remodelling (Xiao et al., 2005), vasoconstriction (Albadawi et al., 2006), astrocyte and microglial function (Chiarugi and Moskowitz, 2003), long term memory (Sung and Ambron, 2004), inflammation (Oliver et al., 1999), aging (Burkle et al., 2005), energy metabolism (Asher et al., 2010) and transcription (Kraus and Lis, 2003).

3.2.1. Role of PARP-1 in the regulation of transcription

The regulation of gene expression requires a wide array of protein factors that can modulate chromatin structure, act at enhancers, function as transcriptional coregulators, or regulate insulator function. PARP-1 can regulate transcription as (a) a component of insulator, (b) a component of enhancer/promoter regulatory complexes, (c) a modulator of chromatin structure and (d) a transcriptional coregulator (Kraus, 2008) (Fig. 8.).

PARP-1 has been suggested to act as an insulator (Klenova and Ohlsson, 2005). Insulators are DNA elements that help to organize the genome into discrete regulatory units by limiting the effects of enhancers on promoters or by preventing the spreading of heterochromatin (Wallace and Felsenfeld, 2007). PARP-1-dependent PARylation of CCCTC-binding factor or 11-zinc finger protein (CTCF), an ubiquitous DNA-binding protein that functions at insulator sites have suggested to be crucial for the preservation of insulator function (Yu et al., 2004; Yusufzai et al., 2004) (Fig. 8A).
Figure 8. Modes of transcriptional regulation by PARP-1.
PARP-1 regulates transcription in four modes, as indicated. (A) PARP-1 can function as a component of insulators, (B) can act as an enhancer-binding factor, (C) can modulate chromatin structure and (D) can function as transcriptional coregulator in a manner similar to classical coactivators and corepressors. Reproduced from (Kraus, 2008).

The earliest characterized effects of PARP-1 on the genome were the modulation of chromatin structure and the PARylation of histones (D’Amours et al., 1999; Huletsky et al., 1989; Kraus and Lis, 2003; Malanga and Althaus, 2005; Poirier et al., 1982) (Fig. 8C). PARP-1 binding to nucleosomes in the absence of NAD$^+$ promotes the compaction of nucleosomal arrays into higher order structures (Wacker et al., 2007). In the presence of NAD$^+$, PARP-1 undertakes its own automodification and dispatches from chromatin, leading to decompaction and restoration of transcription in vitro (Kim et al., 2004) (Fig. 8C). PARP-1 can lead to more specific and elaborate changes in chromatin such as the exclusion of H1 from the promoters of some PARP-1 regulated genes (Krishnakumar et al., 2008), possibly by PARylyating nucleosomes or by competing with H1 for binding to nucleosomes (Huletsky et al., 1989). Furthermore, in the case of estrogen-induced transcription of the trefoil factor 1 (TFF1) gene PARP-1 does not only promote the removal of H1, but also increases the levels of a chromatin architectural protein (HMGB1) that enhances transcription (Ju et al., 2006). In addition, PARP-1-dependent PARylation of DEK, an abundant and ubiquitous architectural component of chromatin, promotes the release of DEK from chromatin, the loading of the Mediator coregulatory complex, and the enhancement of transcription (Gamble and Fisher, 2007).
Many of the early studies have described direct effects of PARP-1 on transcriptional regulation. In this action PARP-1 binds to specific DNA sequences or structures in the regulatory regions of genes where PARP-1 acts as a classical enhancer binding factor (Butler and Ordahl, 1999; Huang et al., 2004; Nirodi et al., 2001) (Fig. 8B). In fact, direct binding of PARP-1 to hairpins may underlie an autoregulatory mechanism governing the expression of the PARP-1 gene itself (Soldatenkov et al., 2002). The best examples for such interaction are the promoters of chemokine (CXC motif) ligand 1 (CXCL1) (Amiri et al., 2006) and B-cell lymphoma 6 protein (BCL6) (Ambrose et al., 2007).

Finally, PARP-1 has been identified as promoter-specific coregulator (either a coactivator or a corepressor) for a number transcription factors, such as NF-κB, nuclear receptors, Myb-related protein B (B-Myb), organic cation transporter member 1 (Oct-1), stimulating protein-1 (Sp1), Nuclear factor of activated T-cells (NFAT) and others (Fig. 8D). PARP-1 can interact with enhancers and promoters of genes by a) direct sequence-specific binding, b) recruitment via DNA binding of transcription factors (e.g., NF-κB), c) direct binding to DNA structures and (d) binding to the dyad axis (Fig 9).

**Figure 9. A Model for Activator/Coactivator Functions of PARP-1 at Enhancers and Promoters**

PARP-1 can interact with enhancers and promoters of genes by (a) direct sequence-specific binding to enhancers, (b) recruitment via DNA binding transcription factors (e.g., NF-κB), and (c) direct binding to DNA structures (e.g., cruciform, curved, supercoiled, and crossover) and (d) to the dyad axis where DNA enters and exits the nucleosome. In many cases, PARP-1 may promote the binding of other factors to DNA, stimulating the formation of enhanceosomes and the activation of transcription in a manner independent of PARP-1 enzymatic activity.
The respective DNA-binding factors recruit PARP-1 to relevant target promoters, where in turn PARP-1 may promote the binding of further factors to DNA, stimulating the formation of enhanceosomes (also indicated as PARP-1 coactivator complex) and the activation of transcription. In some cases, enzymatic activity of PARP-1 is essential to form transcription complex with other transcription factors e.g. SP-1 and NFAT. DNA-binding factors or other components of the coregulatory complex are targets of PARP-1-dependent PARylation. PARP-1 was shown to promote the recruitment of topoisomerase IIβ to hormone-regulated promoters, leading to concomitant promoter DNA cleavage, factor exchange, and transcriptional activation (Ju et al., 2006; Pavri et al., 2005). Recruitment of the PARP-1 coactivator complex also promotes the release of histone H1, the recruitment of HMGB1/2, changes in chromatin architecture, and ultimately increased transcription of the gene (Ju et al., 2006). Together, these studies highlight the diverse mechanisms of PARP-1 coregulator function, which are likely to vary in an activator-and gene-specific manner. The most important PARP-1 associated transcription factors are summarized in Table 2.
<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Characteristics/functions</th>
<th>PARP-1 interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>Inflammation, viral (HIV) activation, NO synthesis, cytokine release</td>
<td>(Oliver et al., 1999)</td>
</tr>
<tr>
<td>AP-1</td>
<td>Cell proliferation, cell adhesion, GST regulation, multidrug resistance, inflammation</td>
<td>(Zingarelli et al., 2004)</td>
</tr>
<tr>
<td>AP-2</td>
<td>Regulator of gene expression during embryogenesis</td>
<td>(Kannan et al., 1999)</td>
</tr>
<tr>
<td>Sp-1</td>
<td>HIV, herpes simplex activation, myocyte differentiation, VEGF, hsp70, and HGH gene regulation</td>
<td>(Zaniolo et al., 2005)</td>
</tr>
<tr>
<td>Oct-1</td>
<td>B cell–specific expression of immunoglobulins</td>
<td>(Tantin et al., 2005)</td>
</tr>
<tr>
<td>B-MYB</td>
<td>Cell cycle regulation (required for S-phase entry), apoptosis, cancer</td>
<td>(Li and McDonnell, 2002)</td>
</tr>
<tr>
<td>TEF-1</td>
<td>High levels of gene expression in skeletal and cardiac muscles, transcriptional regulation of SV40 and papillomavirus, co-factors of the glucocorticoid receptor</td>
<td>(Maeda et al., 2002)</td>
</tr>
<tr>
<td>Stat-1</td>
<td>Cell proliferation and apoptosis</td>
<td>(Levy and Darnell, 2002)</td>
</tr>
<tr>
<td>YY-1</td>
<td>Embryogenesis, growth, differentiations, proliferation and response to genotoxic stimuli</td>
<td>(Gordon et al., 2006)</td>
</tr>
<tr>
<td>ATF-2</td>
<td>Inflammation, metabolic regulation, oncogenesis</td>
<td>(Maekawa et al., 2010)</td>
</tr>
<tr>
<td>CREB</td>
<td>Inflammation, cell cycle regulation, cancer</td>
<td>(Ha, 2004)</td>
</tr>
<tr>
<td>p53</td>
<td>DNA repair, cell cycle regulation (G1/S), apoptosis</td>
<td>(Sionov and Haupt, 1999)</td>
</tr>
</tbody>
</table>

Table 2. Transcription factors regulated by PARP-1
3.2.1.1. Transcriptional events in inflammation mediated by PARP-1

Studies performed with PARP-1 knockout mice revealed that genetic deletion of PARP-1 inhibits the propagation of inflammation which was later verified in a large number of diseases (reviewed in (Virag and Szabo, 2002)). The disregulation of different transcription factors represents the basis for the protection against inflammatory diseases. Some transcription factors involved in the regulation of inflammatory process are redox-sensitive and usually require the enzymatic activation of PARP-1. The dominant transcription factors activated during inflammation are NF-κB and AP-1.

NF-κB is a protein complex that controls the transcription from specific promoters. NF-κB exists in virtually all cell types and is involved in cellular responses to stimuli such as stress, cytokines or free radicals. NF-κB plays key role in regulating inflammatory processes. NF-κB family consists of multiple members such as c-Rel, RelA, RelB, NF-kB1 and NF-kB2. NF-κB consists of two protein subunits, p50 and p65, which are present in the cytosol of non-stimulated cells as homo- or heterodimers of the above proteins. The active transcription factor contains a nuclear localization signal, targeting it to the nucleus for DNA binding. In non-stimulated cells this signal is masked by the action of a monomeric inhibitor protein IκB, resulting in the cytoplasmic accumulation of the transcription factor. Cytokine stimulation induces a signal transduction cascade in which the key step is the phosphorylation of IκB by IκB kinase (IKK), which results in the release of NF-κB from IκB, followed by nuclear translocation and transcription of NF-κB-dependent genes. Several studies have been revealed the interaction between NF-κB and PARP-1 (Aguilar-Quesada et al., 2007; Chiarugi and Moskowitz, 2003; Oliver et al., 1999). PARP-1 may influence NFκB transactivation by several means. Primarily, upon the loss of PARP-1 NFκB binding to DNA is hampered (Oliver, 1999). It is under debate whether the enzymatic activation of PARP-1 is required or dispensable for NF-κB (Hassa and Hottiger, 2002; Oliver et al., 1999). Finally, the PI3/Akt and MAP kinase signalling pathways may also modify NF-κB transactivation (Veres et al., 2004).

The heterodimeric complex AP-1 is a basic leucine zipper protein formed by the dimerization of its components c-Fos, c-Jun or ATF-2. ATF-2 is a cAMP-responsive element-binding transcription factor (Livingstone et al., 1995) that forms homomer
complex with c-Jun and binds to activator protein-1 sites (Reimold et al., 2001). The genes regulating the cellular concentration of the transcription factor are induced by extracellular stress, linking AP-1 activity to the cell stress response. AP-1 can therefore be viewed as a critical intermediary in many signal transduction pathways. Under oxidative stress, activated p38 MAPK rapidly translocates from the cytoplasm to the nucleus and phosphorylates its substrates such as transcription factors, e.g. cAMP-response element-binding protein (CREB), ATF-2 and c-Jun or other kinases, such as mitogen- and stress-activated protein kinase (Ha, 2004). The AP-1 transcription factor can bind to DNA and can start the transcription of inflammatory genes. ATF-2 has been shown to be regulated by PARP-1 (Ha, 2004). Genetic deletion of ATF-2 leads to impaired AP-1 activation and consequently to reduced expression of cytokines (TNF-α, IFN-γ, IL-1, IL-6, or MCP-1α) and adhesion factors (E-selectin and P-selectin) (Reimold et al., 2001). Deficient AP-1 activation upon PARP-1 ablation has already been described in a murine model of colitis to provide a protective phenotype (Zingarelli et al., 2004). PARP-1 can influence AP-1 transcription activity via MAP kinase phosphatase 1 (MKP-1) under oxidative stress conditions (Racz et al., 2010) that is an attractive model to explain our findings on altered AP-1 transactivation in PARP-1 knockout mice. Namely, PARP-1 regulate transcription of MKP-1 which phosphorylate the p38 MAPK and JNK/SAPK impacting on the phosphorylation of AP-1 subunits such as ATF-2 and c-Jun in the nucleus (Racz et al., 2010).

Inflammatory cytokines such as TNFα, IL-1β are central regulators of the inflammatory process orchestrated by Th1 cells. These cytokines induce the expression of other cytokines and chemokines (chemoattractants), adhesion molecules, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 and matrix metalloproteinases. If PARP-1 is inhibited or knocked out, the expression of inflammatory cytokines is suppressed (MIP-1α, MIP-2, IL1β, TNFα, and MCP-1). These chemokines and cytokines were described to be NF-κB dependent (Espinoza et al., 2007; Haddad et al., 2006; Oliver et al., 1999; Virag and Szabo, 2002). Therefore, the suppression of NF-κB activity provides a likely explanation for the reduced expression of these genes. Cytokines and chemokines possess central role in the propagation of inflammation. Changes in chemokine and cytokine expression represent a mechanism for reduced migration of inflammatory cells. Adherence, extravasation and migration of
inflammatory cells is facilitated by adhesion proteins, such as I-CAM, V-CAM, L-CAM, selectins expressed on the surface of endothelial. Inhibition of PARP-1 blocks the expression of these adhesion molecules and reduce cellular infiltration (Zingarelli et al., 1999). Importantly, these genes are also regulated by NF-κB and AP-1 (Espinoza et al., 2007; Haddad et al., 2006).

Another important inflammatory gene regulated by PARP-1 is iNOS that is responsible for NO synthesis under inflammatory conditions (Lowenstein et al., 1994; Moncada et al., 1991). NO combines with superoxide to form peroxynitrite causing lipid peroxidation, protein oxidation and DNA strand breakage (Ischiropoulos et al., 1992). iNOS is regulated via NF-κB activation, hence PARP inhibition markedly reduces iNOS expression and nitrosative stress (Haddad et al., 2006).

MMPs are indispensable for tissular movement of cells. MMPs may be activated by free radical-induced structural changes (Le et al., 2007). Several studies have shown that MMP activation during inflammation (e.g. MMP-9 activation in skin inflammation) can be prevented by knocking out PARP-1 or by pharmacological PARP-1 inhibition (Bai et al., 2009; Kauppinen and Swanson, 2005; Koh et al., 2005; Oumouna-Benachour et al., 2007). Moreover, tissue inhibitors of MMPs (TIMPs) that reduce MMP activity are inversely regulated than MMPs upon inflammation (Bai et al., 2009; Oumouna-Benachour et al., 2007). PARP inhibition is capable of restoring the balance between MMPs and TIMPs (Bai et al., 2009). It has not been determined as of yet whether these effects are related to direct gene expression changes or represent cellular invasion of tissues.

3.2.1.2. PARP-1 in the transcriptional control of oxidative energy metabolism

Sirtuins (SIRTs) – homologs of the yeast sir2 (silent mating-type information regulation 2) - are NAD$^+$-dependent class III histone deacetylases (HDAC III). Sirtuin protein family has seven members. Among these proteins, the nuclear protein SIRT1 is the closest homolog of yeast sir2. SIRT1 is induced by caloric restriction and have been implicated in influencing aging and regulating transcription, stress resistance, as well as energy efficiency (Guarente and Picard, 2005; Oberdoerffer et al., 2008).

SIRT1 has a multiple role in the regulation of metabolism and can influence metabolism depending the level of nutrient availability. SIRT1 is capable of regulating
glucose and lipid metabolism in different metabolic tissues such as skeletal muscle, liver, brown adipose tissue (BAT), white adipose tissue and brain. In the pancreas SIRT1 activity regulates insulin secretion (Rodgers et al., 2008). In these tissues, SIRT1 interacts PGC-1α and FOXO1 and upon activation it deacetylates and activates them (Feige and Auwerx, 2008; Lagouge et al., 2006; Rodgers et al., 2008). In this way, SIRT1 mediates the gene expression of mitochondrial biogenesis, fatty acid oxidations/syntheses, oxidative phosphorilation, adaptive thermogenesis, gluconeogenesis and glycolysis genes. Hence, SIRT1 can fine tune the energy homeostasis of the whole body (Fig. 10.).

Figure 10. Summary of SIRT1 mediated metabolic pathway via PGC-1α and FOXO1.
By responding to metabolic alterations SIRT1 mediates key transcription factors of oxidative metabolism through which it translates energy status into gene expression programs in (A) liver, (B) BAT and (C) skeletal muscle. Reproduced from (Finck and Kelly, 2006).
SIRT1 can be activated by fasting (Rodgers et al., 2005), exercise (Canto et al., 2009) or low glucose availability (Fulco et al., 2008). Under these conditions the level of glucose in blood decreases, while the intracellular NAD\(^+\) levels is increased in the metabolic tissues: liver, skeletal muscle and BAT. SIRT1 is activated by increased NAD\(^+\) level and deacetylates transcription factors PGC-1\(\alpha\) and FOXO (Feige and Auwerx, 2008; Lagouge et al., 2006; Rodgers et al., 2005). Deacetylation activates these transcription factors, whereby they induce mitochondrial biogenesis mitochondrial biogenesis, fatty acid oxidation, oxidative phosphorylation in skeletal muscle (Gerhart-Hines et al., 2007), adaptive thermogenesis in BAT (Uldry et al., 2006) and moreover gluconeogenesis in liver (Finck and Kelly, 2006). At the same time the genes of fatty acid synthase are down-regulated in liver and skeletal muscle. Together these processes lead to fatty acid oxidation (ketone-body production) and glucose production in these tissues. Moreover, in skeletal muscle SIRT1 activation leads to muscle fiber type switch. Muscle fiber type switch elevates the quantity of type I (oxidative) fibers characterized by a higher mitochondrial content.

The dependence of SIRT1 on NAD links its enzymatic activity directly to the energy status of the cell via sensing cellular NAD\(^+\)/NADH ratio. PARP-1 can influence NAD\(^+\) levels since its activation consumes cellular NAD\(^+\) (Schraufstatter et al., 1986). Moreover, PARP-1 is a faster and more efficient NAD\(^+\) consumer than SIRT1 (Knight and Chambers, 2001; Malik et al., 2009; Smith et al., 2009). These data suggest that PARP-1 activity may impact on gene transcription through modulating SIRT1 activity. PARP-1 and -2 have a baseline PARP activity that is initiated by constant oxidative stress stemming from mitochondrial oxidative metabolism (Barja, 1992). Therefore, these PARP enzymes constantly turn over NAD\(^+\) even under non-stressed conditions. Recent studies revealed that through modulating NAD\(^+\) PARP-1 impacts on other NAD\(^+\)-dependent enzymes, such as SIRT1 (Cai et al., 2006; Caito et al., 2010; El Ramy et al., 2009; Kolthur-Seetharam et al., 2006; Rajamohan et al., 2009). Moreover, it is important to note that NAD\(^+\) is compartmentalized in cell, namely, nuclear, cytosolic and mitochondrial NAD\(^+\) pools. NAD\(^+\) between these pools cannot exchange (Houtkooper et al., 2010). Since PARP-1 and SIRT1 are nuclear proteins their activity probably use only nuclear NAD\(^+\) hence influencing gene expression in nucleus only.
4. Literary overview and aims

PARP-1 has been shown to influence transcription and oxidative stress at multiple levels. We set out to investigate the effects of PARP-1 depletion on transcription in different models. On one hand, we utilized a skin inflammation model, oxazolone-induced contact hypersensitivity. In this model we have already shown the prominent role of oxidative stress (Szabo et al., 2001) and PARP activation (Bai et al., 2009). However, the exact molecular mechanism through which PARP-1 or -2 influence inflammation is unknown. On the other hand, we set out to investigate how PARP-1 may act on SIRT1 and oxidative metabolism. As pointed out in the introduction, the interdependence of PARP-1 and SIRT1 have already been revealed (Cai et al., 2006; Caito et al., 2010; El Ramy et al., 2009; Kolthur-Seetharam et al., 2006; Rajamohan et al., 2009), however the metabolic impact of their interplay and its molecular mechanism have not been studied yet.

Our aims were the following:
I. Investigation of the role of PARP-1 in oxazolone-induced contact hypersensitivity (CHS):
   - Defining the respective role of PARP-1 in oxazolone-induced CHS.
   - Characterization of oxidative stress in oxazolone-induced CHS.
   - Characterization of transcriptional changes during oxazolone-induced CHS (proinflammatory cytokines, chemokines, adhesion molecules and transcription factors).
II. Characterization of gene expression changes after PARP-1 depletion or inhibition in
   - skeletal muscle, BAT, liver
   - murine embryonic fibroblasts
   - pharmacology inhibition of PARP-1 with PJ34.
5. Materials and Methods

5.1. Methods

5.1.1. In vivo studies

All animal experiments were approved by the local ethical committee (9/2008/DE MÁB) and were performed according to EU and national guidelines.

In oxazolone-induced contact hypersensitivity reaction, homozygous PARP-1 (Menissier-de Murcia et al., 1997) and PARP-2 knockout (KO) mice and their respective wild-type (WT) littermates on C57/Bl6 background were used. Female mice were randomized into four groups (WT non-sensitized, WT-sensitized, KO non-sensitized, KO sensitized) and were sensitized and challenged.

PMA-induced irritative dermatitis model was performed as described in (Bai et al., 2009). Briefly, PMA (10 µl, 0.05% w/v) was smeared onto both sides of the ears of female mice (6 animals per group), ear swelling was determined together with all other biochemical measurements 24 hours later.

In metabolic studies, male PARP-1+/+ and PARP-1−/− mice on a C57/Bl6 background were used. Mice were housed separately, had ad libitum access to water and standard rodent chow (10 kcal% of fat, Safe, Augy, France) and were kept under a 12 h dark-light cycle.

In case of PJ34 treatment, mice received each 12 h (at 7:00 and 19:00) 10 mg/kg PJ34 by intraperitoneal injection for 5 continuous days. In all studies animals were killed either CO₂ inhalation or cervical dislocation after 6 h of fasting (starting at 8:00), and tissues were collected each time (between 14:00 and 15:00) and processed as specified below.

5.1.2. Preparation of MEF cells

MEFs (primary mouse embryonic fibroblasts) were isolated by standard procedures (Isolation and handling of MEFs accompanying protocol to "Mouse embryonic stem (ES) cell culture - basic procedures" by Boris Gerber). After crossing heterozygous PARP-1+/− mice, we sacrificed the pregnant female at day 13 post coitum by cervical dislocation. Dissected out the uterine horns, briefly rinsed them in 70% (v/v) ethanol and placed into a petri dish containing PBS. We separated each embryo from its
placenta and surrounding membranes, cut away brain and dark red organs, washed with fresh PBS, to remove as much blood as possible. After it, we used a minimal amount of PBS and razor blades, finely minced the embryos until they became "pipettable", then suspended cells/tissue in several ml of trypsin-EDTA (Lonza) and incubated with gentle shaking at 37°C for 15 min. After incubation we transferred suspension to a 50 ml falcon tube and added about 2 volumes of fresh medium and we removed remaining pieces of tissue by low speed centrifugation (5 min, 1000 g) - resuspended the resulting cell pellet in warm MEF medium (Gibco) and plated out at 1 embryo equivalent per 10 cm dish. We changed medium on the following day. The fibroblasts were the only cells that attached to the dishes. Cells grew confluent within one or a few days.

5.1.3. Histology and microscopy

Haematoxylin-eosine (HE) histochemistry was performed as described earlier (Szabo et al., 2001). Immunohistochemistry was performed on paraffin sections (7 µm) were treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides for 1 h in 2% goat serum (in PBS). To detect nitrotyrosine, rabbit polyclonal anti-nitrotyrosine antibody (Sigma) was applied for 2 h in a dilution of 1:1000 at room temperature. (Control sections were incubated with normal rabbit.) Following extensive washing (five times 5 min) with PBS, immunoreactivity was detected with a biotinylated goat anti-rabbit secondary antibody and the avidin-biotin-peroxidase complex (ABC), both supplied in the Vector Elite kit (Vector Laboratories, Burlingame, CA). Color was developed using nickel-DAB substrate. Sections were counterstained with nuclear fast red for 2 min, dehydrated, and mounted in Permount medium. In the case of neutrophil elastase (Santa Cruz, Santa Cruz, CA, USA) and anti-PAR murine monoclonal antibody (BD Biosciences, San Jose, CA, USA) were also used the above mentioned protocols.

5.1.4. Myeloperoxidase activity assay

The activity of myeloperoxidase (MPO), an indicator of neutrophil accumulation, was determined in tissue homogenates prepared in 0.5% hexa-decyl-trimethylammonium bromide in 10 mM MOPS buffer. The supernatants of the
homogenates were mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C, using a Labsystems Multiskan MS plate reader (Analytical Instruments, Minneapolis, USA). MPO activity was expressed as the percentage of the MPO activity determined in the samples of WT non-sensitized animals.

5.1.5. MMP activity assay

Ears were homogenized in DQ buffer (50 mM Tris (pH 7.6), 150 mM NaCl, and 5 mM CaCl₂) by Ultra Turrax, and then homogenate was cleared by centrifugation. To 100 µl supernatant and 80 µl DQ buffer 20 µl of fluorescein-conjugated gelatine (DQ gelatine, Invitrogen, Carlsbad, CA, USA) was added, samples were incubated at room temperature 30 minutes and fluorescein fluorescence was determined using a Victor V3 fluorimeter (Beckton-Dickinson, Franklin Lakes, NJ, USA). Fluorescence values were normalized for protein content.

5.1.6. 8-oxo-2’-deoxyguanosine (8-OHdG) determination

8-OHdG determination was performed using a commercial kit from Cell Biolabs (San Diego, CA, USA) according to the manufacturer’s instructions. After ear homogenisation by Ultra Turrax, we extracted DNA by standard phenol-chloroform methods from samples. Extracted DNA were dissolved in nuclease free water at 1-5 mg/mL. DNA samples were converted to single-stranded DNA by incubating the sample at 95°C for 5 minutes and rapidly chilling on ice. DNA samples were digested by incubating the denatured DNA with 5-20 units of nuclease P1 for 2 hrs at 37 °C in 20 mM sodium acetate, pH 5.2, and following with treatment of 10 units of alkaline phosphatase for 1 hr at 37 °C in 100 mM Tris, pH 7.5. The reaction mixture was centrifuged for 5 minutes at 6000 g and the supernatants were used for the 8-OHdG ELISA assay. Each 8-OHdG sample were measured in duplicate. 50 µL of unknown sample or 8-OHdG standard were added to the wells of the 8-OHdG conjugate coated plate. Plate was incubated at room temperature for 10 minutes on an orbital shaker. 50 µL of the diluted anti-8-OHdG antibody were added to each well, incubate at room temperature for 1 hour on an orbital shaker. After it we washed microwell strips 3 times
with PBS. After washing 100 µL of the diluted secondary antibody-enzyme conjugate were added to all wells. Incubate at room temperature for 1 hour on an orbital shaker and after washed again the plate warm 100 µL of substrate solution were added to each well and incubated at room temperature on an orbital shaker about 10 minutes. After stopping the enzyme reaction by adding 100 µL of stop solution into each well, results were read immediately on a spectrophotometer using 450 nm as the primary wavelength. We read off the data on standard curve and data were normalized to total DNA content represented in ng/mg DNA concentration.

5.1.7. Protein carbonyl assay

Protein carboxylation was assessed using a commercial kit from Cayman Chemicals (Ann Harbour, MI, USA) according to the manufacturer’s instructions.

We dissected 200-300 mg of tissue and rinsed with a PBS to remove any red blood cells or clots. The ears were homogenized in 2 ml of cold buffer (50 mM PBS, ph 6.7, 1 mM EDTA). Samples centrifuged at 10,000 x g for 15 minutes at 4 °C. The supernatant diluted to 10 µg/mL in 1X PBS. Each protein sample and BSA standard assayed in duplicate or triplicate. We pretreated lysate with nuclease to remove nucleic acids contaminations followed by ammonium sulfate precipitation of high percentage saturation and removed precipitates by centrifuging at 6000 g for 10 minutes at 4°C. 100 µL of 10 µg/mL protein samples, including reduced/oxidized BSA standards, were added to the 96-well protein binding plate. Plates were incubated at 37°C for 2 hours and washed wells 3 times with 250 µL 1X PBS per well. After the last wash, we removed excess wash solution, added 100 µL of the DNPH working solution, and incubated for 45 minutes at room temperature in the dark. In the next step wells were washed with 250 µL of 1X PBS/Ethanol (1:1, v/v) and incubated on an orbital shaker for 5 minutes. After incubation we repeated washing a total of 5 times with 250 µL of 1X PBS. We added 200 µL of blocking solution per well and incubate for 2 hours at room temperature on an orbital shaker and wash again 3 times with 250 µL of 1X PBS. 100 µL of the diluted anti-DNP antibody were added to all wells and incubate for 1 hour at room temperature on an orbital shaker. The strip wells were washed 3 times with 1X PBS and added 100 µL of the diluted HRP conjugated secondary antibody to all wells and incubate for 1 hour at room temperature on an orbital shaker. In the next step after
repeated washing, warm substrate solution was added to each well and incubated at room temperature on an orbital shaker. Actual incubation time was 10 minutes. After stopping the enzyme reaction by adding 100 µL of stop solution to each well, results were read immediately. We used 450 nm as the primary wave length and calculated the average absorbance of each sample and control. We subtracted the average absorbance of the controls from the average absorbance of the samples (corrected absorbance: CA) and determined the concentration of the carbonyls by inserting the CA in the following equation:

$$\text{Protein carbonyl (nmol/ml)} = \frac{(\text{CA})}{(0.011 \text{ uM-1})} \times \frac{500 \text{ ul}}{200 \text{ ul}}$$

The calculated data then were normalized to protein content and represented in nmol/mg total protein concentration.

### 5.1.8. Lipid peroxidation assay

Lipid peroxidation was measured using a commercial kit to detect HNE-protein adducts from Cell Biolabs (San Diego, CA, USA) according to the manufacturer's instructions. After ear homogenised in cold 1X PBS by Ultra Turrax, we diluted unknown protein sample to 10 µg/mL in 1X PBS. Each protein sample and HNE-BSA standard were assayed in duplicate or triplicate. We added 100 µL of the 10 µg/mL protein samples or reduced/HNE-BSA standards to the 96-well protein binding plate and incubated at 37 ºC for at least 2 hours. After incubation wells were washed 2 times with 250 µL 1X PBS per well. After the last wash 200 µL of assay diluent was added to each well and incubated for 2 hours at room temperature on an orbital shaker. In next step we washed 3 times with 250 µL of 1X PBS with plate and added 100 µL of the diluted anti-HNE-His antibody to all wells and incubated for 1 hour at room temperature on an orbital shaker. After it plate was washed 3 times with 1X PBS and added 100 µL of the diluted secondary antibody-HRP conjugate to all wells and incubate for 1 hour at room temperature on an orbital shaker. After washing, warm 100 µL of substrate solution were added to each well and incubated it at room temperature on an orbital shaker. Actual incubation time was 10 minutes and stopped the enzyme reaction by adding 100 µL of stop solution to each well. Absorbance of each well was read immediately on a microplate reader using 450 nm as the primary wave length. Reduced BSA standard were absorbance blank. The exact value of each sample was
read off standard curve, normalized to protein, and represented them in mg/mg protein concentration.

5.1.9. Total RNA preparation, reverse transcription and qPCR

Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNase, and 2 µg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (Qiagen, Valencia, CA, USA). 10X diluted cDNA were used for quantitative PCR (qPCR) reactions. The qPCR reactions were performed using an ABI 7500 thermal cycler (Applied Biosciences, Foster City, CA, USA) and qPCR Supermix (Applied Bioscience) with the primers summarized in Table 3.

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GGG AGC CTG AGA AAC GGC GGG TCG GGA GTG GGT AAT TTT</td>
</tr>
<tr>
<td>36B4</td>
<td>AGA TTC GGG ATA TGC TGT TGG AAA GCC TGG AAG AAG GAG GTC</td>
</tr>
<tr>
<td>ACC1</td>
<td>GAC AGA CTG ATC GCA GAG AAA G TGG AGA GCC CCA CAC ACA</td>
</tr>
<tr>
<td>ACC2</td>
<td>CCC AGC CGA GTT TGT CAC T GGC GAT GAG CAC CTT CTC TA</td>
</tr>
<tr>
<td>ACO</td>
<td>CCC AAC TGT GAC TTC CAT T GGC ATG TAA CCC GTA GCA CT</td>
</tr>
<tr>
<td>ATP5g1</td>
<td>GCT GCT TGA GAG ATG GGT TC AGT TGG TGT GGC TGG ATC A</td>
</tr>
<tr>
<td>COX17</td>
<td>CGT GAT GCG TGC ATC ATT GA CAT TCA CAA AGT AAG GCA CCA CC</td>
</tr>
<tr>
<td>Cyclophyllin</td>
<td>TGG AGA GCA CCA AGA CAG ACA TGC CGG AGT CCA CAA TGAT</td>
</tr>
<tr>
<td>Cyt C</td>
<td>TCC ATC AGG GTA TCC TCT CC GGA GGC AAG CAT AAG ACT GG</td>
</tr>
<tr>
<td>Dio2</td>
<td>GCA CGT CTC CAA TCC TGA AT TGA ACC AAA GGT GAC CAC CA</td>
</tr>
<tr>
<td>eNOS</td>
<td>GCA ATC TTC GTT CAG CCA TCA C AGA GCT CAG TGA TCT CCA CGT TG</td>
</tr>
<tr>
<td>ERRα</td>
<td>ACT GCC ACT GCA GGA TGA G CAC AGC CTC AGC ATC TTC AA</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>GGC AGA GTG AGA TTT GAA GGA TG GGA CTT CAG CGT CAC TTT GGT AG</td>
</tr>
<tr>
<td>G6Pase</td>
<td>CCG GAT CTA CCA CCT TGC TCA CT T TAC CTA GAA TCC AAG CGC GAA AC</td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GK</td>
<td>ACA TTG TGC GCC GTG CCT GTG AA</td>
</tr>
<tr>
<td>I-CAM</td>
<td>CTT TCG ATC TTC CAG CTA CCA TC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CAA CCA ACA AGT GAT ATT CTC CAT G</td>
</tr>
<tr>
<td>L-CAM</td>
<td>GGC AGA GTG AGA TTT GAA GGA TG</td>
</tr>
<tr>
<td>iNOS</td>
<td>GAA GTG CAA AGT CTC AGA CAT GG</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>GCC GGC TCT ATC CTC CTT TG</td>
</tr>
<tr>
<td>MCAD</td>
<td>GAT CGC AAT GGG TGC TTT TGA TAG AA</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CTC AGC CAG ATG CAG TTA ACG</td>
</tr>
<tr>
<td>mCPT1</td>
<td>TTG CCC TAC AGC TGG CTC ATT TCC</td>
</tr>
<tr>
<td>MHCI</td>
<td>GAG TAG CTC TTG TGC TAC CCA GC</td>
</tr>
<tr>
<td>MHCIIA</td>
<td>GCA AGA AGC AGA TTC AGA AAC</td>
</tr>
<tr>
<td>MHCIIIX</td>
<td>GCA ACA GGA GAT TTC TGA CCT CAC CCA GAG ATG CTC CTT C</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CTC TGC AAC CAA GTC TTC TCA GC</td>
</tr>
<tr>
<td>MIP-2</td>
<td>CTC CAG CCA CAC TTC AGC CTA G</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CAT TCG CGT GGA TAA GGA GT</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>CTG ACG AAG GCC ACT TTG CAC CTC TG GCA CAA GAT CCC GGT CAA GTA CCA GCC</td>
</tr>
<tr>
<td>Ndufa2</td>
<td>GCA CAC ATT TCC CCA CAC TG</td>
</tr>
<tr>
<td>Ndufa3</td>
<td>TAC CAC AAA CGC AGC AAA CC</td>
</tr>
<tr>
<td>Ndufb5</td>
<td>CTT CGA ACT TCC TGC TCC TT GGC CCT GAA AAG AAC TAC G</td>
</tr>
<tr>
<td>PARP-1</td>
<td>GGA GCT GCT CAT CTT CCA CC</td>
</tr>
<tr>
<td>PEPCK</td>
<td>CCA CAG CTG CTG CAG AAC A</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>AAG TGT GGA ACT CTC TGG AAC TG</td>
</tr>
<tr>
<td>PPARα</td>
<td>CCT GAA CAT CGA GTG TCG AAT AT</td>
</tr>
</tbody>
</table>
Table 3. List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SREBP1</td>
<td>GGC CGA GAT GTG CGA ACT TTG TTG ATG AGC TGG AGC ATG T</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>CGT TTC TTT GGG GTT TCT GA TTT ATC ACT AAC AAT ATA GAC AGC CAC TCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>CAT CTT CTC AAA ATT CGA GTG ACA A TGG GAG TAG ACA AGG TAC AAC CC</td>
</tr>
<tr>
<td>Troponin I</td>
<td>CCA GCA CCT TCA GCT TCA GGT CCT TGA T TGC CGG AAG TTG AGA GGA AAT CCA AGA T</td>
</tr>
<tr>
<td>UCP1</td>
<td>GGC CCT TGT AAA CAA CAA AAT AC GGC AAC AAG AGC TGA CAG TAA AT</td>
</tr>
<tr>
<td>UCP2</td>
<td>TGG CAG GTA GCA CCA CAG G CAT CTG GTC TTG CAG CAA CTC T</td>
</tr>
<tr>
<td>UCP3</td>
<td>ACT CCA GCG TCG CCA TCA GGA TTC T TAA ACA GGT GAG ACT CCA GCA ACT T</td>
</tr>
<tr>
<td>V-Cam</td>
<td>TAC CAG CTC CCA AAA TCC TG TCT GCT AAT TCC AGC CTC GT</td>
</tr>
</tbody>
</table>

5.1.10. Transcription factor transactivation studies

Transfactor Extraction kit (Clontech, Mountain View, CA, USA) was used for nuclear protein extraction according to the manufacturer’s instructions as described below. Ears were frozen in liquid nitrogen and were ground into fine powder in a mortar cooled in liquid nitrogen. All further procedures were performed on ice with ice-cold reagents and equipment. The powdered sample was re-suspended in a small volume of ice-cold PBS and was consequently centrifuged at 450 g for 5 min at 4 °C. The pellet was resuspended in lysis buffer (10 mM HEPES, pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT and protease inhibitor cocktail, Roche) and was disrupted repeatedly pressing through a syringe fitted with a No. 26 gauge needle (obtained from 10 times each). The disrupted tissue lysate was then centrifuged at 11,000 g for 20 min, the supernatant removed and the nuclear pellet resuspended in extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT and protease inhibitor cocktail). The nuclei were then disrupted with a syringe fitted with a No. 26 gauge needle (10 times each) and centrifuged for 5 min at 21,000 g. Protein concentration of the supernatant (nuclear extract) was measured with Bradford protein assay. For the quantification of transcription factor transactivation, the TransFactor Inflammatory Profiling-1 kit was utilized (Clontech). The kit enables the colorimetric determination of the DNA binding of the active transcription factors using
an ELISA-based technique. 10 µg ear nuclear extract was loaded into each well and the measurement was performed according to the Manufacturer’s instructions. The results are expressed as fold increase of the WT non-sensitized control samples.

5.1.11. mtDNA analysis

Select mouse tissues were homogenized and digested with Proteinase K overnight in a lysis buffer for DNA extraction by conventional phenol-chloroform method for direct analysis of liberated nucleic acids. Quantitative PCR was performed in duplicate, using 10 µM each primer (mtDNA specific PCR (16S rRNA), forward 5’- CCG CAA GGG AAA GAT GAA AGA C -3’, reverse 5’- TCG TTT GGT TTC GGG GTT TC -3’; and nuclear specific PCR (hexokinase 2 gene, intron 9), forward 5’- GCC AGC CTC TCC TGA TTT TAG TGT -3’, reverse 5’- GGG AAC ACA AAA GAC CTC TTC TGG -3’)) and LightCycler 480 SYBR Green I Master PCR Kit (Roche) in a LightCycler 480 Real-Time PCR system (Roche) with a program of 10 minutes at 95 °C, followed by 50 cycles of 15 seconds at 95 °C, 20 seconds at 60 °C. Single-product amplification was verified by an integrated post-run melting curve analysis. Exponential amplification efficiency was verified during each PCR run using a standard dilution series made from pooled samples. Results were calculated from the difference in threshold cycle (ΔCT) values for mtDNA and nuclear specific amplification (Lagouge et al., 2006).

5.1.12. Statistical analysis

Results were expressed as means ± SEM. Statistical significance between groups was determined by Student’s t-test, p<0.05 was considered as significant. Error bars represent ± SEM.
6. Results

6.1. Role of PARP-1 in oxazolone-induced contact hypersensitivity

6.1.1. Characterization of the oxazolone-induced contact hypersensitivity reaction in PARP-1−/− mice

Ear swelling was determined 24 hours after OXA challenge. OXA challenge in PARP-1+/+ mice caused twelve fold ear swelling compared to vehicle-sensitized animals (0.875 ± 0.07 μm vs. 10.5 ± 0.91 μm), while OXA-induced ear swelling was significantly reduced in the PARP-1−/− mice (1.4 ± 0.52 μm vs. 7.33 ± 1.23 μm) (Fig. 11A).

To assess immune cell infiltration, myeloperoxidase (MPO) activity was evaluated. MPO activity, indicative of the neutrophil infiltration, showed similar changes to ear swelling. PARP-1−/− mice were protected against the OXA-evoked increase in MPO activity (Fig. 11B). The degree of protection provided by the PARP-1−/− phenotype was similar to the one previously observed in mice treated with the PARPinhibitor, PJ34 (Bai et al., 2009).

Figure 11. PARP-1−/− mice are protected against the oxazolone-induced CHS reaction.
(A) Oxazolon (10 l, 0.5% w/v in acetone:olive oil mixture, 1:4 ratio) was spread on both sides of the ears of female mice PARP-1 (n=6 CTL PARP-1+/+; n=6 OXA PARP-1+/+; n=7 CTL PARP-1−/−; n=9 OXA PARP-1−/−, 12 weeks) one week after sensitization. Ear thickness was measured before and 24 hours after challenge by a calliper and was expressed as the difference of the values. (B) At 24 hours after OXA challenge, ears were removed and MPO activity was determined in the supernatants of ear homogenates spectrophotometrically at 650 nm 37 °C. Absorbance were normalized to protein and expressed as % of PARP-1+/+ non-sensitized mice. Asterisks indicate significant difference between cohorts, where * p<0.05; *** p<0.001.
Oxazolone has strong irritative properties, therefore we set out to investigate whether PARP-1 might influence irritative dermatitis for which we applied a 12-O-tetradecanoyl-phorbol 13-acetate (PMA)-induced irritative dermatitis model. Similarly, to our previous observations with the PARP inhibitor compound PJ34, the PARP-1\(^{-/-}\) phenotype also conferred partial protection against the PMA-induced irritative dermatitis both at the level of ear swelling (34.17 ± 0.73 µm vs. 30.63 ± 0.51 µm) and MPO activity (Fig. 12A, B). We detected marked cellular infiltration upon PMA-induction (Fig. 12D) in line with strong induction of matrix metalloproteinase (MMP) activity that was reduced in PARP-1\(^{-/-}\) mice (Fig. 12C). Apparently, both the irritative and the immunological components are affected by the loss of PARP-1.

![Graphs showing ear swelling and MPO activity](image1)

**Figure 12.** PARP-1\(^{-/-}\) mice are protected against the PMA-induced irritative dermatitis.

PMA (10 µl, 0.05% w/v) was smeared onto both sides of the ears of female mice PARP-1 (n=6 CTL PARP-1\(^{+/+}\); n=6 OXA PARP-1\(^{+/+}\); n=7 CTL PARP-1\(^{-/-}\); n=9 OXA PARP-1\(^{-/-}\); 12 weeks) After 24 hours, (A) ear swelling and (B) MPO activity was determined. Absorbance were normalized to protein and expressed as % of PARP-1\(^{+/+}\) non-sensitized mice. MMP activity (C) was measured in ear homogenates normalized for protein content. (D) Formalin-fixed, 7 µm paraffin-embedded tissue sections were stained with haematoxylin and eosin (HE).
Changes in MPO activity suggested similar changes in inflammatory cell infiltration therefore we investigated HE colored histology sections. Histology examination revealed inflammatory cell infiltration in the ear upon OXA challenge which was markedly reduced in the PARP-1<sup>−/−</sup> mice (Fig. 13A). This is in good correlation with ear swelling and MPO activity as presented on Fig 10. Using cell type-specific markers, we identified predominant neutrophil infiltration. In line with the suppressed inflammatory response, neutrophil infiltration was markedly reduced in the PARP-1<sup>−/−</sup> mice (Fig. 13B).

Figure 13. Genetic ablation of PARP-1 suppresses inflammatory cell immigration. (A) Formalin-fixed, 7 μm paraffin-embedded tissue sections were stained with HE and (B) for neutrophil elastase, with 4,6-diamidino-2-phenylindole (DAPI) nuclear counterstained as described in Materials and Methods. The arrows point at the neutrophil elastase-positive cells.
6.1.2. Lack of oxidative/nitrosative stress in PARP-1−/− mice

Leukocytic infiltration is usually accompanied by the production of reactive oxygen and nitrogen species, such as hydrogen peroxide and peroxynitrite. Moreover, these oxidants were shown to influence redox-sensitive transcription factors that prompted us to investigate oxidative stress and its related biochemical events in OXA-induced CHS.

As the endothelial and the inducible nitric oxide synthase enzymes (eNOS and iNOS, respectively) can be considered as the most important sources of NO in the ear under inflammatory conditions, their expression was assessed. Although eNOS expression was lower in the PARP-1−/− mice as compared to PARP-1+/+, eNOS expression did not change upon OXA-treatment (Fig. 14A). Therefore eNOS is probably not the major source of NO under inflammatory conditions. Similarly to eNOS, iNOS expression was lower in the PARP-1−/− animals than in PARP-1+/+ mice. Importantly, iNOS expression increased markedly upon OXA-challenge, while only mild induction was observed in the OXA-treated PARP-1−/− mice (Fig. 14A). Apparently, iNOS appears to be the major source of NO in our model system. This is in line with other findings obtained in different models of inflammation.

Nitrosative stress is indicated by the formation of protein tyrosine nitration, which could be observed in the ears of wild type but not that of PARP-1−/− mice (Fig. 14B). Nitrotyrosine staining could be detected in the infiltrating cells and in the keratinocytes with strongest immunopositivity seen in the microabscessi, where widespread keratinocyte death has previously been described.
Figure 14. Reduced nitrosative stress in PARP-1-/- mice.

(A) Expression of eNOS and iNOS were determined by qRT-PCR (n=6 CTL PARP-1+/+; n=6 OXA PARP-1+/+; n=7 CTL PARP-1-/-; n=9 OXA PARP-1-/-; 12 weeks). Expression values were normalized to the geometric mean of 36B4, cyclophyllin and 18S expression then to the values of non-sensitized PARP-1+/+ mice.

(B) Nitrotyrosine immunohistochemistry was performed on formalin-fixed, 7 μm paraffin-embedded tissue sections as described in Materials and Methods. Asterisks indicate significant difference between cohorts, where * p<0.05; *** p<0.001.
Nitrosative stress is accompanied by the enhanced production of reactive oxygen species. In CHS, increased level of ROS was indicated by increased lipid, protein and DNA base oxidation (as shown by protein carbonylation (oxa PARP-1+/− 14,23 ± 3,06; oxa PARP-1−/− oxa 8,32 ± 2,90), protein-4-hydroxy-2-nonenal (HNE) adduct (oxa PARP-1+/− 12,42 ± 4,95; oxa PARP-1−/− 7,59 ± 0,97)and 8-OHdG (oxa PARP-1+/− 3,69 ± 0,21; oxa PARP-1−/− 2,31 ± 0,25) formation) that was all reduced in the PARP-1−/− mice (Fig. 15A-C).

Figure 15. Reduced oxidative stress and DNA damage in PARP-1−/− mice
(A) HNE-adduct, (B) protein carbonyl and (C) 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined in the ear extracts of PARP-1+/− and PARP-1−/− mice (n=6 CTL PARP-1+/−; n=6 OXA PARP-1+/−; n=7 CTL PARP-1−/−; n=9 OXA PARP-1−/−; 12 weeks) as described in Materials and Methods. Asterisks indicate significant difference between cohorts, where *p< 0,05; ***p<0,001.

Oxidative and nitrosative stress can induce DNA breakage and PARP activation. Therefore, we set out to investigate DNA strand breakage by TUNEL assay. DNA strand breaks appeared in keratinocytes, endothelial cells and leukocytes (Fig. 16A) in PARP-1+/− mice. There were high number of TUNEL-positive cells in the microabscessi suggesting intense oxidative stress (Fig. 16A). The number of TUNEL-positive cells was reduced in the PARP-1−/− subjects. DNA strand breaks lead to PARP-1 activation resulting in the formation of PAR. A nuclear PAR signal could be detected in the PARP-1+/− mice which was absent in the PARP-1−/− animals (Fig. 16B). PAR was present in most cell types in the ear.
Figure 16. Poly(ADP-ribose) polymerase (PARP) activity is decreased in PARP-1 -/- mice. (A) TUNEL assay and (B) PAR immunohistochemistry were performed on formalin-fixed, paraffin-embedded tissue sections as described in Materials and Methods. n.s.: non-sensitized. The arrows point at PAR-positive nuclei.
6.1.3. PARP-1 mediated gene expression in oxazolone-induced contact hypersensitivity

The infiltration and migration of polymorphonuclear leukocytes is facilitated by appearance of proinflammatory cytokines and chemokines. Therefore, we investigated their expression. On the course of the CHS reaction, we observed the induction of MIP-1α, MIP-2, IL-1β, MCP-1 and TNFα. A reduced expression of all of these cytokines/chemokines has been observed in the PARP-1\( ^{-/-} \) mice (Fig. 17A.).

Cellular extravasation and infiltration under inflammatory conditions require the concerted expression of different cell adhesion molecules such as I-CAM, L-CAM, V-CAM and E-Selectin. OXA challenge induced I-CAM, L-CAM, V-Cam and E-Selectin expression in the WT mice that was absent in the PARP-1\( ^{-/-} \) mice (Fig. 17B).

In addition to adhesion molecules, leukocytes also express MMPs in order to facilitate their movements in tissues. MMPs are secreted as zymogens that are subsequently activated by either proteolytic cleavage or free-radical-induced structural changes. We have observed the induction of MMP-9 upon OXA challenge in the PARP-1\(+/-\) mice. However, MMP-9 induction was impaired in the PARP-1\(-/-\) mice (Fig. 17C).

MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMP1-4). Interestingly, TIMP2 was down-regulated in PARP-1\(+/-\) mice, which may support higher MMP-9 activity (Fig. 17C). On the contrary, TIMP2 expression was not reduced in PARP-1\(-/-\) mice.

PARP-1 interacts with a plethora of transcription factors and modulates their activity, hence gene expression. We hypothesized that the absence of PARP-1 protein and the consequent loss of PARP-1 activity may be the underlying mechanism for the altered expression of inflammatory mediators. We assessed the activation of a number of transcription factor in the ears. We have observed the strong activation of two redox-sensitive transcription factors, p65, a member of the NF-κB family and activating transcription factor-2 (ATF-2) upon OXA-sensitization in the PARP-1\(+/-\) mice (Fig. 17D). Interestingly, the activation of other members of the REL family such as p50 and c-Rel was not detectable. The activation of p65 was completely absent in the PARP-1\(-/-\) mice. ATF-2 showed a similar pattern to p65, though its activity was only partially reduced in the knockout mice (Fig. 17D).
Figure 17. Expression of proinflammatory genes is suppressed in PARP-1/-/- mice.
Expression of (A) a set of proinflammatory cytokines, (B) adhesion molecules, (C) MMP-9 and TIMP-2 were determined by qRT-PCR. (D) Transcription factor activation was determined using TransFactor kit (n=6 CTL PARP-1+/-; n=6 OXA PARP-1+/-; n=7 CTL PARP-1-/-; n=9 OXA PARP-1-/-; 12 weeks). Expression values were normalized to the geometric mean of 36B4, cyclophyllin and 18S expression then to the values of non-sensitized PARP-1+/- mice. Asterisks indicate significant difference between cohorts, where * p<0.05; *** p<0.001. Abbreviations are in the text. Primer sequences are listed in primer Table 3.
6.2 Transcriptional changes affected by the PARP-1 SIRT1 interdependence

6.2.1. Brown adipose tissue and muscle, but not liver from PARP-1−/− mice have higher mitochondrial content

Large amount of literature data suggest (Cai et al., 2006; Caito et al., 2010; El Ramy et al., 2009; Pillai et al., 2005; Rajamohan et al., 2009) that PARP-1 and SIRT1 interact and hence PARP-1 may interfere with transcriptional activities of SIRT1 which was further supported by the metabolic changes observed in skeletal muscle and BAT upon the genetic deletion of PARP-1. To gain insight into these changes we assessed the expression of genes responding to SIRT1 that was shown by assessing the acetylation level of PGC-1α (Fig. 2D and F in (Bai et al., 2011)) in different in vivo and cellular models. First we investigated BAT with transmission electron microscopy that (Fig 1M in (Bai et al., 2011)) revealed higher mitochondrial content in PARP-1−/− BAT, which was further corroborated by the increased mitochondrial DNA content (Fig. 18A). This higher mitochondrial DNA content in PARP-1−/− mice was in line with increased expression of genes involved in mitochondrial uncoupling (UCP1 and UCP3), fatty acid oxidation (MCAD) and respiration (Ndufa2, Ndufa3, Ndufb5, Cyt C, COX17) (Fig.18B). Additionally, the expression of Deiodinase-2 (Dio2) was higher in PARP-1−/− mice, suggesting increased thyroid hormone activation in the BAT (Fig. 18B). PARP-1−/− skeletal muscle we observed a switch towards a more oxidative profile. Succinate dehydrogenase (SDH) staining (Fig. 1P in (Bai et al., 2011)) and the expression of muscle fiber isotype Troponin I (Trop I), Myosin heavy chain I (MHC1) (Fig. 18C) indicated an increase in the number of oxidative fibers, characterized by a higher mitochondrial content than glycolytic fibers.
Figure 18. Increased mitochondrial mRNA expression in brown adipose tissue (BAT) and gastrocnemius muscle of PARP-1−/− mice. (A) BAT mitochondrial DNA (mtDNA) and (B) mRNA levels of the indicated genes were determined by RT-qPCR in PARP-1+/+ and −/− male mice (n=8/9; 46 weeks). (C) In gastrocnemius muscles, mRNA levels of the indicated genes were measured by RT-qPCR. Expression values were normalized to cyclophyllin expression. * indicates statistical difference vs. PARP-1+/+ mice at p<0.05. Abbreviations can be found in the text. Primer sequences are listed in Table 3.

Another crucial tissue for whole body metabolism is the liver. We investigated the expression of a large set of genes, containing genes of the SIRT1 transcriptome, encoding for transcription factors: Estrogen receptor related-α (ERRα), Peroxisome proliferator activated receptor-γ (PPARy), Peroxisome proliferator- activated receptor-gamma co-activator 1α (PGC-1α), Sterol regulatory element-binding protein (SREBP1) (Fig. 19A) and proteins involved in mitochondrial respiration (Ndufa2, Ndufa3, cyt c, ATP5g1), fatty acid oxidation (MCAD, ACO), fatty acid synthesis (ACC1, ACC2 and Malic enzyme) and glucose metabolism (PEPCK, GK, G6Pase) (Fig. 19B). The lack of significant changes in the expression of this metabolic gene set suggested that the absence of PARP-1 has only a minor metabolic impact in the liver, potentially explained by the very low expression of PARP-1 in the liver relative to skeletal muscle or BAT (Fig. 19C).
Figure 19. Gene expression pattern of different metabolic genes and Parp-1 in metabolic tissues.

(A); (B) mRNA expression levels of selected genes were quantified by RT-qPCR reactions in the liver of PARP-1+/+ and −/− male mice (n=9/9; 46 weeks). (C) mRNA expression of PARP-1 were quantified in different metabolic tissues of C57/B16 male mice (n=5). Expression values were normalized to cyclophyllin expression. Asterisks indicate significant difference between the respective tissue and liver, where *** p<0.001. Abbreviations can be found in the text. Primer sequences are listed in Table 3.
6.2.2. Reduced PARP-1 activity in murine embryonic fibroblasts promotes oxidative metabolism.

Given the relation between PARP-1 and SIRT1 upon the somatic ablation of PARP-1 we next evaluated whether reducing PARP-1 activity in cells could equally bring about the improvement of energy metabolism. For this purpose, we used of MEF cells from PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mice. The reduction of PARP activity in this cell model perfectly recapitulated all expression changes observed in vivo findings (Suppl. Fig. 3. in (Bai et al., 2011)), such as higher expression of genes involved in mitochondrial function (e.g. PGC-1α, Ndufb5, Cyt C, COX17, UCP-2, mCPT-1, ACO) (Fig. 20B).

Figure 20. Assessment of mitochondrial expression in PARP-1<sup>+/+</sup> and <sup>-/-</sup> MEFs.
In PARP-1<sup>+/+</sup> and <sup>-/-</sup> primary MEFs (n=3/3) mitochondrial DNA content (A) and mRNA expression (B) was determined. Expression values were normalized to cyclophyllin expression. Abbreviations are listed in the text. Asterisks indicate significant difference between cohorts, where * p<0.05. Abbreviations can be found in the text. Primer sequences are listed in Table 3.
6.2.3. **Pharmacological inhibition of PARP-1 activity enhances oxidative metabolism via SIRT1.**

Since genetic ablation of PARP-1 increased the expression of SIRT1-related genes, we turned to investigate whether *in vivo* pharmacological inhibition of PARP-1 could have similar effects.

We injected mice with PJ34 (10 mg/kg) twice a day for 5 days. In muscle, the increase in mitochondrial gene expression (Ndufa2, Ndufb5, UCP2 and UCP3) induced by PJ34 was accompanied by an increase in myoglobin mRNA levels, which facilitate oxygen delivery into muscle fibers (Fig. 21). All of these data indicate that PJ34 treatment phenocopies also *in vivo* part of the oxidative features (Suppl. Fig. 4 in Bai et al., 2011) induced by PARP-1 gene deletion.

![Figure 21. Pharmacological PARP-1 inhibition in vivo phenocopies SIRT1 activation.](image)

C57/Bl6 male mice were injected with PJ34 (2 x 10 mg/kg/day i.p.) or saline (n=10/10; 12 weeks) and were sacrificed after 5 days of injections. Then mRNA expression levels of selected genes in gastrocnemius muscle were quantified by RT-qPCR reactions. Expression values were normalized to cyclophyllin expression. Abbreviations are listed in the text. * indicates statistical vs. vehicle-treated group at p<0.05. Abbreviations can be found in the text. Primer sequences are listed in Table 3.
7. Discussion

Several studies have proven the major role of PARP-1 in the regulation of transcription (Kraus and Lis, 2003). Our aim was to elucidate the transcriptional role of PARP-1 in relation to skin hypersensitivity inflammatory reaction and to SIRT1 mediated transcription.

7.1. Role of PARP-1 in oxazolone-induced contact hypersensitivity

Several research groups have suggested the importance of PARP-1 in numerous inflammatory processes including some skin inflammations (Virag et al., 2002). The role of PARP activity in CHS has previously been demonstrated by our group (Bai et al., 2009).

PARP inhibition decrease inflammation in mouse model of CHS, where PJ34, a PARP inhibitor, conferred protection (Bai et al., 2009). PJ34 is a panPARP inhibitor and since both PARP-1 and PARP-2 are active members of the PARP family (Sims et al., 1981), both are likely to regulate CHS. In the present work we identified PARP-1 as the main PARP enzyme mediating CHS, since the genetic ablation of PARP-1 proved to be protective against CHS. The level of protection provided by PARP-1 ablation suggests that PARP-1 is the sole PARP involved in CHS. However, there are other PARPs and PARG that exert immune functions (e.g. macroPARPs (Hakme et al., 2008a) or PARG (Frizzell et al., 2009)) that may have additional minor roles.

Oxazolone has strong irritative effect. The involvement of the irritative component was assessed in PMA-induced dermatitis, where PARP-1 provided partial protection. Apparently, the irritative component affects most PARP-1-mediated effects of CHS (MMP activation, infiltration).

It is an interesting notion that PARP-1 ablation did not fully block oedema formation, we observed reduction only by roughly 30%. Apparently the reduction in oedema formation correlated with lower immune function in PARP-1−/− mice, however we have no knowledge about the origin of the remaining 70% that seems PARP-1 independent. It is tempting to speculate whether neurogenic inflammation may account for that portion of ear swelling. Neurogenic inflammation may have important role in airway and skin inflammatory processes (Meggs, 1993). During certain inflammatory processes chemical sensitizers, such as oxazolone, has been described to induce
neurogenic inflammation (Richardson and Vasko, 2002). Therefore it is tempting to speculate that neurogenic inflammation may occur also in our model system.

Upon the induction of CHS we have observed neutrophil infiltration that was accompanied by increased oxidative stress as indicated by increased MPO activity, protein carbonyl production, HNE and 8-OHdG levels. Nitrosative stress was also elevated as illustrated by increased iNOS expression and nitrotyrosine staining similarly to previous findings (Szabo et al., 2001). The high level of reactive species may lead to DNA breakage and PARP activation in keratinocytes, endothelial cells and leukocytes.

In order to characterize inflammatory processes we measured inflammatory molecules such as MIP-1α and-2, IL-1β, TNF-α, and MCP-1. They were induced in CHS and were reduced in PARP-1/− mice. These chemokines/cytokines are known mediators of CHS (Olmos et al., 2007; Saint-Mezard et al., 2004) and are of different origin (dermal cells, mast cells, or Langerhans cells) possessing pleiotropic effects (Biedermann et al., 2000) leading to the (1) upregulation of cellular adhesion molecules on the surface of the endothelium (McHale et al., 1999); and (2) the recruitment, activation, and proliferation of inflammatory cells (Homey et al., 1998; Lim et al., 2008), culminating in enhanced inflammation.

Moreover, we found different adhesion molecules (I-CAM, L-CAM, V-CAM, and E-selectin) regulated by PARP-1 (Fuchs et al., 2001; Kelly et al., 2007). These molecules support the adherence of leukocytes onto the endothelium. Consequently, decrease in the expression of adhesion molecules impairs infiltration of inflammatory cells, as we demonstrated in PARP-1/− mice. Our observations correspond to the result of Zingerelli and co-workers showing that reduced PARP-1 levels diminish adhesion molecule expression, leading to decreased inflammatory infiltration in murine colitis (Zingarelli et al., 1999). To facilitate diapedesis, leukocytes secrete MMPs in order to help their movements in tissues. MMPs are secreted as zymogens that are subsequently activated by either proteolytic cleavage or free radical-induced structural changes (Le et al., 2007). It has been shown that upon PARP-1 inhibition, or depletion, the activity and protein levels of MMP-9 increases in oxazolone induced CHS (Bai et al., 2009; Kauppinen and Swanson, 2005; Koh et al., 2005). TIMP-1 - 4 regulate the activity of MMP. We have observed the downregulation of TIMP-2 upon OXA challenge, that further enhance MMP-9 activity. However, TIMP-2 downregulation did not take place
in PARP-1−/− mice. This pattern is in alignment with the effects of pharmacological PARP inhibition that also blocked TIMP-2 downregulation (Bai et al., 2009; Oumouna-Benachour et al., 2007).

The concerted changes of iNOS, chemokines, adhesion factors, MMP-9 and TIMP-2 suggest common roots at the level of gene transcription. PARP-1 interacts with numerous transcription factors and modulates their activity. Our hypothesis was that the absence of PARP-1 protein and loss of its activity may be responsible for the transcriptional alterations of inflammatory mediators. In CHS we have identified the activation of two redox-sensitive transcription factors, p65, a member of the NF-κB family and activating transcription factor-2 (ATF-2) from AP-1 family that were suppressed in PARP-1−/− mice.

The defective p65 activation is in line with the reduced expression of numerous NF-κB target genes such as iNOS, adhesion factors and cytokines (Oliver et al., 1999). The direct molecular interaction between PARP-1 and NF-κB has already been described (Hassa and Hottiger, 1999; Oliver et al., 1999). However, the exact molecular mechanism through which PARP-1 affects NF-κB, is not clear yet. It seems that PARP-1 affects DNA binding of NF-κB, but it is of question whether PARP-1 activation is crucial for NF-κB transcriptional activity.

Concerning the AP-1 pathway, PARP-1 can affect phosphorylation of ATF-2 and c-Jun through MKP-1 (Racz et al., 2010). MKP-1 through phosphorylating p38MAPK and JNK/SAPK can influence the activity of AP-1. Therefore the aforementioned changes in phosphorylation levels represent a likely model to explain how hampered ATF-2 activation lead to the repression of inflammation.

DNA breakage may also contribute to the transcriptional regulation through PARP-1. Estrogen receptor activation has been reported to lead to DNA nicking. These DNA nicks need to be repaired for the effective ER–related transcription. PARP-1 activation is crucial for the resolution of these nicks (Ju et al., 2006). Therefore it is tempting to speculate that a similar mechanism may also participate in the activation of NF-κB or ATF-2.

PARP-1 has a central role in the control of oxidative stress in the elicitation phase of CHS (Fig. 22), and its inhibition or genetic deletion disrupts the self-
intensifying propagation of inflammation. Altogether, these results provide further support for the possible therapeutic applicability of PARP inhibitors in CHS.

**Figure 22.** The possible signalling mechanism leading to NFkB or AP-1 – activation and rearrangement of inflammatory gene expression on the course of CHS reaction. NF-κB and AP-1 have crucial roles in the regulation of cytokine induced gene expression. Upon the depletion of PARP-1 NF-κB transactivation decrease hence inflammatory gene expression is quenched. The lack of PARP-1 may block the phosphorilation of ATF-2 or c-Jun thereby AP-1 cannot be formed in the nucleus, hampering gene expression.
7.2. Transcriptional effects of the PARP-1 SIRT1 interplay

Multiple groups have identified links between SIRT1 and PARP-1, where activation of SIRT1 suppresses PARP-1 activity (El Ramy et al., 2009; Kolthur-Seetharam et al., 2006; Pillai et al., 2005; Rajamohan et al., 2009). Physiological observations revealed that PARP-1−/− mice have less body fat than PARP-1+/+ littermates. PARP-1−/− mice consumed more O2 than PARP-1+/+ mice. PARP-1−/− mice also maintained higher body temperature upon cold exposure, were more glucose tolerant and had a trend towards lower fasting blood glucose levels despite similar insulin levels. These observations were affirmed upon high fat feeding experiments where PARP-1−/− retained glucose tolerance and insulin sensitivity. In line with the amelioration of metabolic parameters, improved mitochondrial content and activity was observed.

As PARP-1 is a major NAD+ consumer (Sims et al., 1981), we speculated that the lack of PARP-1 activity might increase NAD+ content, in turn activating SIRT1. Our group demonstrated that NAD+ levels increased in BAT and muscle of PARP-1 knockout mice (Fig. 2B, 2C in (Bai et al., 2011)). At the same time, increased PGC-1α and FOXO1 deacetylation and the induction of SIRT1-specific genes were revealed in BAT and muscle (Fig. 2D, 2E in (Bai et al., 2011)), which is an indicator of enhanced SIRT1 activation. We observed similar increase of SIRT1 activity in PARP-1 depleted HEK293T and MEF cells where we encountered with similar gene expression changes as in PARP-1−/− mice. In both cases, mitochondrial content and oxidative metabolism was also enhanced. Furthermore, in mice treated with PJ34, PARP activity was suppressed and SIRT1 activity was induced by decreased PGC-1α acetylation and induction of genes of oxidative metabolism (Fig. 4H, 4I in (Bai et al., 2011)). Importantly, most of the metabolic effects elicited by PARP-1 depletion were lost when SIRT1 was simultaneously knocked-down (Fig. 4J in (Bai et al., 2011)).

It is possible that endogenous oxidants leaking out of mitochondria (in the BAT and muscle due to high oxidative metabolism (Barja, 1992) maintain a high basal PARP activity that leads to a constant NAD+ turnover. Therefore, when NAD+ degradation by PARP-1 is inhibited, it causes significant increases in cellular NAD+. Our group demonstrated that attenuation of PARP-1 activity impacts on metabolic homeostasis by increasing intracellular NAD+ content (Bai et al., 2011) and SIRT1 activity (Bai et al.,
SIRT1 activation then prompts the deacetylation and activation of key metabolic transcriptional regulators such as PGC-1α and FOXO1 (Bai et al., 2011), and subsequently increases mitochondrial content and oxidative metabolism.

Technically, reciprocal regulation exists between PARP-1 and SIRT1, because they both may limit NAD⁺ availability for each other. Moreover, due to differences in enzyme kinetics, PARP-1 limits NAD⁺ availability for SIRT1. The $K_M$ for NAD⁺ of the purified enzymes is significantly lower for PARP-1 than for SIRT1 (Knight and Chambers, 2001; Malik et al., 2009; Smith et al., 2009). Similarly, PARP-1 has a much higher (~20-fold) $k_{cat}/K_M$ for NAD⁺ than SIRT1 (Smith et al., 2009). It indicates that PARP-1 is a faster and more efficient NAD⁺ consumer than SIRT1. Under basal conditions PARP-1 activity accounts for the most part of cellular NAD⁺ consumption, which would maintain NAD⁺ at limiting levels for SIRT1 activity. Supporting this notion, overactivation of PARP-1 is enough to deplete intracellular NAD⁺ dramatically and hence decrease SIRT1 activity. The theoretical prediction that PARP-1 deletion would allow NAD⁺ levels to increase and potentiate SIRT1 activation is supported by our experimental observations. Moreover, our results indicate that the interplay between both proteins could be exploited pharmacologically in metabolic diseases. Since several SIRT1 substrates, such as PGC-1α, FOXOs or p53, are crucial metabolic regulators, it is not surprising that the activation of SIRT1 by increased NAD⁺ availability enhances mitochondrial biogenesis and oxidative metabolism (Canto and Auwerx, 2009; Gross et al., 2008; Vousden and Ryan, 2009). In this manner PARP-1 plays an indirect role in the regulation of gene expression for cell energy homeostasis by controlling SIRT1 activation. Fig. 23 depicts the complexity of this regulatory circuitry.
PGC-1α and FOXO1 have crucial role in the regulation of oxidative metabolism through modulating gene expression. The lack of PARP-1 increase the NAD⁺ content in cells, stimulating the activity of SIRT1. This leads to deacetylating of PGC-1α and FOXO1. These transcription factors increase the expression of oxidative metabolic genes leading to elevated metabolic rate and energy deliberation.

Despite the clear impact of PARP inhibition on SIRT1 activity and the involvement of SIRT1 in the phenotypes elicited by reductions in PARP activity, we cannot rule out the possibility that PARP inhibition also affects energy metabolism through SIRT1-independent mechanisms. Future research will have to clarify the nature of these SIRT1-independent effects of PARP inhibition on metabolism. Given the fact that PARP-1 is a nuclear protein, it will be particularly interesting to explore whether PARylation can directly modulate the activity of nuclear proteins and transcriptional regulators related to energy metabolism. It is of interest that the expression of the other PARP enzymes is unaltered in our PARP-1⁻/⁻ mice indicating the lack of compensation upon the loss of PARP-1.
The utilization of PARP inhibitors raises the possibility of occurrence of adverse effects. The lack of PARP-1 has been associated with genomic instability upon serious DNA damage evoked by oxidative stress, γ-irradiation or alkylating agents (Bai et al., 2007; Menissier-de Murcia et al., 1997; Virag and Szabo, 2002). Therefore, prolonged PARP-1 inhibition may lead to the accumulation of DNA damage in the genome. However previous reports have shown (Allinson et al., 2003), that despite the role of PARP-1 in DNA repair, no apparent DNA damage was observed in our PARP-1−/− mice under basal conditions. Moreover, PARP inhibitors were well tolerated in clinical trials (Fong et al., 2009; Jagtap and Szabo, 2005). Together, this identifies PARP-1 as an attractive novel target to manage metabolic disease. Our work on one hand implicates the PARP-1 inhibition by riveting link between PARP-1 and SIRT1. The interplay between PARP-1 and SIRT1 might have implications in a number of additional areas, ranging from circadian rhythm, cellular proliferation and longevity, which warrant future investigation.
8. Summary

PARP-1 plays important roles in the regulation of transcription. It may act on chromatin condensation, transcription factors, or function as insulator. In these cases, PARP-1 directly controls gene expression via binding to other DNA-binding proteins (e.g., transcription factors) or PARyling them (e.g., histone proteins). However, several lines of evidence suggest that PARP-1 functions as a promoter-specific cofactor for transcription factor-dependent gene expression (e.g., NF-κB), or may indirectly control gene expression through modulating other NAD⁺-dependent enzymes (e.g., SIRT1).

In the present work, we investigated the role of PARP-1 in the regulation of contact hypersensitivity reaction and in SIRT1-mediated mitochondrial alterations. Oxazolon-induced CHS was quenched in PARP-1−/− mice, as indicated by reduced neutrophil infiltration, oxidative and nitrosative stress. Under inflammation, cytokines, chemokines, adhesion molecules and matrix metalloproteinase-9 were induced, which was strongly suppressed in PARP-1−/− mice. Furthermore, we identified two inflammatory transcription factors: p65 and ATF-2, which were induced in CHS but were suppressed in PARP-1−/− mice. p65 is the member of the NF-κB while ATF-2 is the member of the AP-1 family of transcription factors. Both transcription factors were described to be PARP-1-dependent, therefore the impaired activation of NF-κB and ATF-2 may explain the reduced inflammation in the PARP-1−/− mice.

In the other study, we investigated PARP-1 and SIRT1 interaction on energy metabolism and its impact on gene expression. We observed increased mitochondrial content and higher expression of oxidative metabolism genes (mitochondrial respiration, fatty acid oxidation, glucose metabolism) in BAT and skeletal muscle of PARP-1−/− mice. These observations were confirmed in PARP-1−/− MEF cells, where similar expression changes were observed. Moreover, pharmacological inhibition of PARP-1 has phenocopied the above gene expression alterations. Apparently, alterations in NAD⁺ content and the consequent SIRT1 activation lay behind these alterations.
9. Összefoglalás

PARP-1 fontos faktora a transzkripció szabályozásának. Szerepet játszhat a kromatin kondenzációban, transzkripciós faktor aktivációjában, vagy mint insulator m ködhet. Ezekben az esetekben a PARP-1 közvetlenül szabályozza a génexpressziót más DNS-köt fehérjékhez (pl.: transzkripciós faktorokhoz) való kapcsolódáson keresztül vagy PARiláció útján (pl.: hiszton fehérjéknél). Azonban több bizonyíték mutat arra, hogy a PARP-1 els sorban promoter-specifikus kofaktorként m ködik közre a génexpresszió szabályozásában vagy közvetetten szabályozhatja a génexpressziót például egyéb NAD⁺-függ enzimeken keresztül (SIRT1).


A PARP-1 SIRT-1 kölcsönhatás vizsgálata során a génexpresszió és a metabolikus egyensúly összefüggését vizsgáltuk. Modelljeinkben magasabb fokú mitokondriális biogenezist és az oxidatív metabolizmus génjeinek (mitokondriális légzési lánc, zsírsav oxidáció, glükóz metabolizmus) megnövekedett expresszióját figyeltük meg barna zsír szövetben és vázizomban. Ezeket a megfigyeléseinket megerősítettük PARP-1⁻/⁻ MEF sejtékben, ahol szintén hasonló expressziós változásokat kaptunk. Továbbá a PARP-1 farmakológiai gátlása a fentiekkel azonos expressziós változásokat indukált. Úgy t nik, hogy a NAD⁺ koncentráció és a SIRT1 aktiváció változása felel s ezekért a jelenségekért.
10. References


List of publications related to the dissertation


List of other publications


DOI: http://dx.doi.org/10.1016/j.bmc.2007.03.064
IF: 2.862

European J. Org. Chem. 2006 (18), 4242-4256, 2006,
DOI: http://dx.doi.org/10.1002/ejoc.200600073
IF: 2.769

DOI: http://dx.doi.org/10.1016/j.bmc.2004.07.013
IF: 2.018

The Candidate’s publication data submitted to the Publication Database of the University of Debrecen have been validated by Kényez Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

04 April, 2011
11. Keywords

Inflammation, oxidative stress, redox-sensitive transcription factors, PARP-1 SIRT1, NAD+, PGC-1α, FOXO1, NF-κB, ATF-2
12. Acknowledgement

I wish to thank my supervisor Dr. Péter Bai for helping and guiding my work during my Ph.D study.

I would like to thank Prof. Pál Gergely, head of Department of Medical Chemistry and Prof. László Virág, head of laboratory for giving all the support for my work.

Many thanks to all the people in the Department of Medical Chemistry, especially our group: Magdolna Szántó, Dr. Katalin Erdélyi, Dr. Csaba Hegedűs, Petra Lakatos and Katalin Kovács for their scientific and personal support. I greatly appreciate the excellent technical assistance of Ms. Erzsébet Herbály, Ms. Julianna Hunyadi and Ms. Anita Jeney Szabó.

I am much obliged to Prof. Johan Auwerx and Dr. Carles Cantó for their help and support.

I would like to thank Dr. Valérié Schreiber for donating the PARP-1 and PARP-2 knockout strains.

I am very grateful for Ms Erzsébet Herbály who processed tissue blocks and performed histochemistry and immunohistochemistry, Dr. Csaba Hegedűs who helped me by CHS and irritative dermatitis models. I would like thank to my family for encouragement and support.
13. Appendix

This thesis is built on the following publications:

IF: 5,543

IF: 17,35
Genetic Ablation of PARP-1 Protects Against Oxazolone-Induced Contact Hypersensitivity by Modulating Oxidative Stress

Attila Brunyánszki¹, Csaba Hegedűs¹,², Magdolna Szántó¹, Katalin Erdélyi¹,², Katalin Kovács¹, Valérie Schreiber³, Szabolcs Gergely⁴, Borbála Kiss⁵, Éva Szabó⁵, László Virág¹,² and Péter Bai¹,²

Contact hypersensitivity (CHS) reaction is a form of delayed-type of hypersensitivity caused by contact allergens such as oxazolone (OXA). In previous studies it has been shown that poly(ADP-ribose) polymerase (PARP) inhibition reduces the extent of inflammation in CHS. We aimed to shed light on the molecular events causing the protective effect of PARP inhibitors. PARP-1 and -2 knockout mice were sensitized by abdominal delivery of OXA, and a week later CHS was induced by applying OXA on the ears of the mice. PARP-1⁻/⁻ mice were protected against OXA-induced CHS in contrast to PARP-2⁻/⁻ mice. In PARP-1⁻/⁻ mice, neutrophil infiltration was reduced in line with the suppressed expression of proinflammatory cytokines, cell adhesion factors, and matrix metalloproteinase-9, which is likely because of impaired activation of NF-κB p65 and activating transcription factor-2, the two redox-sensitive transcription factors. Moreover, reduced nitrosative and oxidative stress was observed under inflammatory conditions in the PARP-1⁻/⁻ mice when compared with PARP-1⁺/⁺. In conclusion, PARP-1 activation is necessary for proinflammatory gene expression through which PARP-1 enhances neutrophil infiltration and hence oxidative/nitrosative stress, forming a vicious circle, and further aggravating the inflammatory process. Our data identify PARP-1 as a possible target in CHS.

INTRODUCTION

The poly(ADP-ribose) polymerase (PARP) superfamily consists of 17 members, with some of them implicated in the regulation of the immune response. PARP-1 and PARP-2 belong to the subgroup that can be activated in vivo by DNA single-strand breaks or in vitro by DNase I-treated DNA or aberrant DNA forms (de Murcia and Menissier-de Murcia, 1994; Ame et al., 1999). Because of its abundance and high catalytic activity, PARP-1 is responsible for most of the cellular PARP activity after DNA damage (Shieh et al., 1998). PARP-1 activation results in the cleavage of NAD⁺ substrate and the synthesis and attachment of poly(ADP-ribose) (PAR) polymers to different acceptor proteins (Schraufstatter et al., 1986; Schreiber et al., 2006).

Both PARP-1 and PARP-2 have been described to influence inflammatory processes through modulating numerous transcription factors (Hassa and Hottiger, 2008; Yelamos et al., 2008). PARP-1 interacts with a large number of proinflammatory transcription factors, and the beneficial effects of PARP-1 ablation on inflammatory damage have been shown in multiple disease models such as colitis, arthritis, uveitis, and pancreatitis (Virag and Szabo, 2002). Recent data suggest that PARP-2 may also affect immune functions. PARP-2⁻/⁻ mice suffer compromised thymopoiesis that leads to impaired survival of CD4⁺ CD8⁺ double-positive thymocytes and consequently to weaker systemic T-cell functions (Yelamos et al., 2006). In addition, PARP-2 associates with transcription factors (such as thyroid transcription factor-1 and peroxisome proliferator activated receptor-γ) and with protein factors influencing the histone code, pointing toward a plethora of possible alterations in gene expression (Bai et al., 2007; Quenet et al., 2008; Yelamos et al., 2008).

Contact hypersensitivity (CHS) is a form of T cell-mediated delayed type of hypersensitivity reaction caused by small-molecular-weight molecules (haptens) that bind to host proteins to form a complete allergen (Grabbe and Schwarz, 1998). The CHS reaction can be divided into the sensitization and elicitation phase. In the elicitation phase, proinflammatory cytokines and chemokines recruit different inflammatory...
cells (e.g., neutrophil granulocytes) (Olmos et al., 2007). Infiltration is accompanied by oxidative stress due to the formation of superoxide, hydrogen peroxide, nitric oxide, peroxynitrite, and further reactive species (Morita et al., 1996; Rowe et al., 1997; Olmos et al., 2007; Korkina and Pastore, 2009), leading to DNA breakage and PARP activation (Szabo et al., 2001). Recently, we showed that the inhibition of PARP activation has beneficial effects during the CHS reaction (Bai et al., 2009). In this study we set out to identify the PARP isoform mediating CHS and to characterize the molecular events during the elicitation phase of the CHS reaction.

RESULTS AND DISCUSSION
PARP-1−/−, but not PARP-2−/−, mice are protected against OXA challenge
We evaluated the ear swelling, as a measure of inflammatory edema, 24 hours after oxazolone (OXA) challenge. OXA challenge in wild-type (WT) mice caused a 4- to 5-fold ear swelling compared with vehicle-sensitized animals, whereas ear swelling was significantly reduced in the PARP-1−/− mice. In contrast, there was no significant difference between the OXA-sensitized PARP-2+/+ and the OXA-sensitized PARP-2−/− mice (Figure 1a).

The myeloperoxidase (MPO) activity, indicative of the neutrophil infiltration, showed similar changes to ear swelling, PARP-1−/−, but not PARP-2−/−, mice were protected against the OXA-evoked increase in MPO activity (Figure 1b). The degree of protection provided by the PARP-1−/− phenotype was similar to the one previously observed in mice treated with the PARP inhibitor, PJ34 (Bai et al., 2009).

PARP-1−/−, but not PARP-2−/−, mice are protected against the irritative component of CHS
In addition to the antigen-specific reaction, an irritative component is usually also present in contact allergies (Grabbe et al., 1996). Therefore, we applied a 12-O-tetradecanoyl-phorbol 13-acetate-induced irritative dermatitis model (Bai et al., 2009) to investigate whether the antigen-specific and/or the nonspecific irritant response is affected by PARP-1 and -2. Similar to our previous observations with the PARP inhibitor compound PJ34 (Bai et al., 2009), the PARP-1−/− phenotype also conferred partial protection against the 12-O-tetradecanoyl-phorbol 13-acetate-induced irritative dermatitis, both at the level of ear swelling and MPO activity (Figure 2a and b). In contrast, genetic ablation of PARP-2 did not affect irritative dermatitis. We detected marked cellular infiltration upon 12-O-tetradecanoyl-phorbol 13-acetate induction (Figure 2d), in line with strong induction of matrix metalloproteinase (MMP) activity that was reduced in PARP-1−/− mice (Figure 2c). As our data indicate that PARP-2 has no functional role in CHS or in irritative dermatitis, we focused our mechanistic investigation on the role of PARP-1 and omitted the PARP-2 knockout strain from all further studies.

Genetic ablation of PARP-1 suppresses inflammatory cell immigration in CHS reaction
Histology examination revealed a marked infiltration of the ear with inflammatory cells upon OXA challenge that was reduced in the PARP-1−/− mice (Figure 3a). This is in good correlation with ear swelling and MPO activity as presented in Figure 1.

CHS is characterized by a mixed infiltration of polymorphonuclear leukocytes, monocytes (Olmos et al., 2007), and T cells. Staining for cell type-specific markers revealed predominant neutrophil infiltration in the connective tissue. In line with the suppressed inflammatory response, neutrophil infiltration was markedly reduced in the PARP-1−/− mice (Figure 3b).

We went on to investigate the expression of proinflammatory cytokines and chemokines. On the course of the allergic inflammation, we observed the induction of macrophage inflammatory protein-1α and -2, IL-1β, tumor necrosis factor-α, and monocyte chemotactic protein-1. A reduced expression of all of these cytokines/chemokines has been observed in the PARP-1−/− mice (Figure 4a). These chemokines/ cytokines are known mediators of CHS (Saint-Mezard et al., 2004; Olmos et al., 2007) and are of different origin (dermal cells, mast cells, or Langerhans cells) possessing pleiotropic

---

**Figure 1.** PARP-1−/−, but not PARP-2−/−, mice are protected against the oxazolone-induced CHS reaction. (a) Ear thickness was measured before and 24 hours after challenge by a caliper, and ear swelling was expressed as the difference of the two values. (b) At 24 hours after OXA challenge, ears were removed and relative MPO activity was determined. *P<0.05; ***P<0.001; significant difference between cohorts. CHS, contact hypersensitivity; MPO, myeloperoxidase; OXA, oxazolone; PARP, poly(ADP-ribose) polymerase.

---
effects (Biedermann et al., 2000) leading to the (1) upregulation of cellular adhesion molecules on the surface of the endothelium (McHale et al., 1999a, b); and (2) the recruitment, activation, and proliferation of inflammatory cells (Homey et al., 1998; Lim et al., 2008), culminating in inflammation.

As cellular extravasation and infiltration under inflammatory conditions require the concerted expression of different cell adhesion molecules such as I-CAM, L-CAM, V-CAM, and E-selectin (Fuchs et al., 2001; Kelly et al., 2007), we examined their expression. As expected, OXA challenge induced I-CAM, L-CAM, V-Cam, and E-selectin expression in the WT mice that was absent in the PARP-1/C0/C0 mice (Figure 4b). Similar observations have previously been made in several oxidative stress-related pathologies, in which inhibition or genetic ablation of PARP-1 reduced the expression of these adhesion molecules, leading to decreased inflammatory infiltration (Zingarelli et al., 1999). Importantly, there is a strong correlation between the expression of the above-mentioned chemokines/cytokines (e.g., monocyte chemotactic protein-1 and tumor necrosis factor-α) and cellular adhesion molecules as all rely on NF-κB activation (Oliver et al., 1999; Virag and Szabo, 2002; Haddad et al., 2006; Espinoza et al., 2007).

In addition to adhesion molecules, leukocytes also secrete MMPs to facilitate their movements in tissues. MMPs are expressed as zymogens that are subsequently activated either by proteolytic cleavage or free radical-induced structural changes (Le et al., 2007). We have observed the induction of MMP-9 upon OXA challenge in the PARP-1+/+ mice. However, MMP-9 induction was impaired in the PARP-1−/− mice (Figure 4c). Our observation is in line with other models of inflammation, in which MMP-9 expression was found to be reduced on pharmacological PARP inhibition or by treatment with small interfering RNA against PARP-1 (Kauppinen and Swanson, 2005; Koh et al., 2005). MMP activity is controlled by tissue inhibitors of metalloproteinases 1–4. Interestingly, tissue inhibitor of metalloproteinase-2 was downregulated in PARP-1+/+ mice, which may explain
MMP-9 activation (Figure 4c). On the contrary, its expression was not reduced in PARP-1/C0/C0 mice. It is noteworthy that pharmacological PARP inhibition has previously also been found to prevent downregulation of this tissue inhibitor (Oumouna-Benachour et al., 2007; Bai et al., 2009).

PARP-1 interacts with a plethora of transcription factors and modulates their activity, and hence gene expression. Therefore, we hypothesized that the absence of PARP-1 protein and the consequent loss of PARP-1 activity may be the underlying mechanism for the altered expression of inflammatory mediators. We assessed the activation of a number of transcription factor in the ears. We have observed the strong activation of two redox-sensitive transcription factors, p65, a member of the NF-κB family and activating transcription factor-2 (ATF-2), on OXA sensitization in the PARP-1+/+ mice (Figure 4d). Interestingly, the activation of other members of the REL family, p50 and c-Rel, was not detectable. The activation of p65 was completely absent in

**Figure 4.** Expression of proinflammatory genes is suppressed in the PARP-1−/− mice. Expression of (a) a set of proinflammatory cytokines, (b) adhesion molecules, (c) MMP-9 and TIMP-2 were determined with qRT-PCR. (d) Transcription factor activation was determined using the TransFactor kit. *P<0.05; ***P<0.001, significant difference between cohorts. ATF-2, activating transcription factor-2; MCP-1, monocyte chemotactic protein-1; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PARP, poly(ADP-ribose) polymerase; qRT-PCR, quantitative real-time reverse transcriptase-PCR; TIMP-2, tissue inhibitor of metalloproteinase-2; TNF-α; tumor necrosis factor-α.
the PARP-1\(^{-/-}\) mice. ATF-2 showed a similar pattern to p65, although its activity was only partially reduced in the knockout mice (Figure 4d). The defective p65 activation is in line with the reduced expression of numerous NF-κB target genes such as inducible nitric oxide synthase (iNOS), adhesion factors, and cytokines (Haddad et al., 2006; Espinoza et al., 2007). The direct molecular interaction between PARP-1 and NF-κB has already been described (Hassa and Hottiger, 1999; Oliver et al., 1999). ATF-2 is a cAMP-responsive element-binding transcription factor that forms complex with c-Jun or itself and binds to activator protein-1 sites (Reimold et al., 2001). ATF-2 has been shown to be regulated by PARP-1 (Ha, 2004). Genetic deletion of ATF-2 leads to impaired activator protein-1 activation and consequently to reduced expression of cytokines (tumor necrosis factor-α, IFN-γ, IL-1, IL-6, or monocyte chemotactic protein-1) and adhesion factors (E-selectin and P-selectin) (Reimold et al., 2001). Deficient activator protein-1 activation upon PARP-1 ablation has already been described in a murine model of colitis providing aprotective phenotype (Zingarelli et al., 2004). Apparently, the combined effects of PARP knockout on ATF-2 and NF-κB may culminate in the changes described in this study.

The lack of oxidative/nitrosative stress in the PARP-1\(^{-/-}\) mice

Leukocytic infiltration is usually accompanied by the production of reactive oxygen and nitrogen species, such as hydrogen peroxide and peroxynitritre, a reactive nitrogen species, formed in the reaction of nitric oxide and superoxide (Beckman and Koppenol, 1996).

In the ear, the endothelial NOS (eNOS) and iNOS enzymes can be considered as the most important sources of nitric oxide under inflammatory conditions; therefore, we assessed their expression. Although eNOS expression was lower in the PARP-1\(^{-/-}\) mice when compared with PARP-1\(^{+/+}\), it did not change upon OXA challenge (Figure 5a). Therefore, eNOS cannot be considered as the major source of nitric oxide under inflammatory conditions. Similar to eNOS, iNOS expression was lower in the PARP-1\(^{-/-}\) animals than in PARP-1\(^{+/+}\) mice under normal conditions. Importantly, iNOS expression increased markedly upon OXA challenge, whereas only mild induction was observed in the OXAtreated PARP-1\(^{-/-}\) mice (Figure 5a). Apparently, iNOS seems to be the major source of nitric oxide in our model system. This is in line with other findings obtained in different models of inflammation (Virag and Szabo, 2002).

Nitrosative stress is indicated by the formation of protein tyrosine nitration that could be observed in the ears of WT but not PARP-1\(^{-/-}\) mice (Figure 5b). Nitrotyrosine staining could be detected in the infiltrating cells and in the keratinocytes, with strongest immunopositivity observed in the microabscesses, in which widespread keratinocyte death has previously been described (Ormerod et al., 1997; Szabo et al., 2001; Cals-Grierson and Ormerod, 2004; Olmos et al., 2007).

We assessed the level of oxidative stress that generally accompanies nitrosative stress. We detected increased lipid, protein, and DNA base oxidation (as shown by protein carbonylation, protein-4-hydroxy-2-nonenal adduct, and 8-OHdG formation) that was all reduced in the PARP-1\(^{-/-}\) mice (Figure 6a-c). Oxidative and nitrosative stress can induce DNA breakage and PARP activation (Virag and Szabo, 2002; Hassa and Hottiger, 2008). Therefore, we set out to investigate DNA strand breakage by TUNEL assay. DNA strand breaks appeared in keratinocytes, endothelial cells, and leukocytes (Figure 6d) in PARP-1\(^{+/+}\) mice. There was a high number of TUNEL-positive cells in the microabscesses, suggesting intense oxidative stress (Figure 6d). The number of TUNEL-positive cells was reduced in the PARP-1\(^{-/-}\) subjects. DNA strand breaks led to PARP-1 activation, resulting in the formation of PAR (Virag and Szabo, 2002; Hassa and Hottiger, 2008). A nuclear PAR signal could be detected in the PARP-1\(^{+/+}\) mice that was absent in the PARP-1\(^{-/-}\) animals (Figure 6e). PAR was present in all cell types in the ear.

Our data show that PARP-1 is a regulator of inflammation in CHS; however, other members of the PARP family (except for PARP-2) might also be implicated. The group of macroPARPs (PARP-9/Bal1, PARP-14/Bal2, and PARP-15/Bal3) also possess immunological roles (reviewed in Hakme et al., 2008), suggesting that their involvement cannot be excluded in cutaneous inflammatory processes. However, PAR glycohydrolase, which is responsible for PAR degradation, is more likely candidate for regulating CHS. Despite the opposing biochemical role, PAR glycohydrolase often shares similar transcriptional function with PARP-1 (Frizzell et al., 2009) and it affects inflammatory gene expression (Rapizzi et al., 2004). Tannins are inhibitors of PAR glycohydrolase (Ying and Swanson, 2000), and it is tempting to hypothesize that the effectiveness of the commonly used tannin-containing external products in dermatology may be related to their PAR glycohydrolase inhibitory effects. These data obtained in PARP-1\(^{-/-}\) mice are very similar to previous results obtained with the administration of a PARP inhibitor compound in the elicitation phase of CHS (Bai et al., 2009). It is important to note the important difference between pharmacological PARP inhibition and PARP-1 knockout. On one hand, the pharmacological inhibitor causes general PARP inhibition affecting all PARP isoforms, and on the other, application of pharmacological inhibitor after the sensitization phase allows us to determine the enzymes’ role on the elicitation phase. In the knockout study, however, the absence of PARP-1 may affect both the sensitization and the elicitation phase. For example, uptake and presentation of antigens by dendritic cells is a cornerstone of efficient sensitization. As both ATF-2 and NF-κB have important roles in dendritic cell function (Stepnik and Arkusz, 2003; Sasaki and Alba, 2007), it cannot be excluded that the sensitization phase of CHS is also impaired in the PARP-1\(^{-/-}\) mice. This possibility, however, would require further investigation.

In the effector phase, PARP-1 modulates several key steps of inflammation such as: (1) cellular infiltration, (2) expression of chemokines, (3) adhesion molecules, (4) MMPs, and (5) the
redox-sensitive p65 and ATF-2 transcription factors. In a possible scenario, the increasing level of oxidative/nitrosative stress enhances PARP-1 activation that in turn boosts all the previously described processes, further aggravating oxidative/nitrosative stress and creating a vicious circle. In fact, PARP-1 has a central role in the control of oxidative stress in the elicitation phase of CHS, and its inhibition or genetic deletion disrupts the self-intensifying propagation of inflammation. Altogether, these results provide further support for the possible therapeutic applicability of PARP inhibitors in CHS.

**MATERIALS AND METHODS**

**Materials**

OXA and all chemicals were from Sigma-Aldrich (St Louis, MO) unless stated otherwise.

**Animal studies**

All animal experiments were approved by the local ethical committee (9/2008/DE MAB) and were performed according to the European Union and national guidelines. Homozygous female PARP-1 and PARP-2 knockout mice and their respective WT littermates on C57/Bl6 background were used (Menissier-de Murcia et al., 2008).

**Figure 5. Reduced nitrosative stress in poly(ADP-ribose) polymerase (PARP)-1-/- mice.** (a) Inducible and endothelial nitric oxide synthase (iNOS and eNOS) expression was determined by quantitative real-time reverse transcriptase PCR (qRT-PCR). (b) Nitrotyrosine immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. NS, nonsensitized. *P<0.05; ***P<0.001, significant difference between cohorts (linear contrast adjustment was applied on image b). Scale bar = 20 μm.
et al., 1997; Menissier-de Murcia et al., 2003). CHS model was performed as described in Szabo et al. (2001), and irritative dermatitis model was performed as described in Bai et al. (2009).

Histology and microscopy
Hematoxylin–eosin staining and immunohistochemistry was performed on paraffin-fixed 7 μm tissue sections as described in Szabo et al. (2001).

MPO activity measurement
The measurement of MPO activity has been described in Virag et al. (2004).

MMP activity measurement
Ear homogenates were incubated with fluorescein-labeled gelatin (Invitrogen, Carlsbad, CA) and then fluorescein fluorescence was determined and normalized for protein content.

Characterization of oxidative stress
Commercial kits from Cell Biolabs (Sand Diego, CA) were used for determining 8-OHdG and 4-hydroxy-2-nonalen. Protein carbonylation was determined using a commercial kit from Cayman Chemicals (Ann Harbor, MI). Samples were processed according to the manufacturer’s instructions.

mRNA preparation and reverse transcription and quantitative PCR
Total RNA preparation, and reverse transcription and quantitative PCR were performed as described in Bai et al. (2007). Normalized expression values were expressed as fold increase compared with samples from WT nonsensitized mice. Primers are summarized in Supplementary Table S1 online.

Transcription factor transactivation studies
For nuclear protein extraction, the TransFactor Extraction kit (Clontech, Mountain View, CA), and for transactivation studies, TransFactor Inflammatory Profiling-1 kit (Clontech) were used according to the manufacturer’s instructions. The results are expressed as fold increase compared to WT non-sensitized samples.

Statistical analysis
Results were expressed as means ± SEM. Statistical significance between groups was determined using Student’s t-test, and P<0.05 was considered as significant. Error bars represent ± SEM.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work was supported by a Bolyai and SNSF fellowship to PB, a FEBS fellowship to AB, OTKA NN178498, K73003, K60780, K62009, TAMOP-4.2.2-08/1-2008-0019, NKTH (Baross program), Mecenatura (DE OEC Mec-1/2008), Ambassade de France en Hongrie and Ministère des Affaires Étrangères, Hungarian Science and Technology Foundation (FR-26/2009), Association pour la Recherche sur le Cancer, Centre National de la Recherche Scientifique, Université de Strasbourg, and Agence Nationale de la Recherche. We acknowledge the help of Mrs Erzsébet...
Herbály in processing histology samples and Mrs Júlia Hunyadi for technical assistance.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

**REFERENCES**


Lim YM, Moon SJ, An SS et al. (2008) Suitability of macrophage inflammatory protein-1beta production by THP-1 cells in differentiating skin sensitizers from irritant chemicals. *Contact Dermatitis* 58:193-8


Rapizzi E, Fossati S, Moroni F et al. (2004) Inhibition of poly(ADP-ribose) glycohydrolase by gallotannin selectively up-regulates expression of PARPs, a small but powerful family of poly-ADP-ribose polymerases. *Front Biosci* 13:3046-82


Schrafuatter IU, Hinshaw DB, Hyslop PA et al. (1986) Oxidant injury of cells. DNA strand-breaks activate polyadenosine diprophosphate-ribosyl polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J Clin Invest* 77:1312-20


Supplementary material to Brunyánszki et al. “Genetic ablation of PARP-1 protects against oxazolone-induced contact hypersensitivity by modulating oxidative stress”

Materials

Oxazolone and all chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Animal studies

All animal experiments were approved by the local ethical committee (9/2008/DE MÁB) and were performed according to EU and national guidelines. Homozygous PARP-1 and PARP-2 knockout (KO) mice and their respective wild-type (WT) littersmates on C57/B16 background were used in our experiments (Menissier-de Murcia et al., 1997; Menissier-de Murcia et al., 2003). Female mice were randomized into four groups (WT non-sensitized (n=6), WT sensitized (n=6), KO non-sensitized (n=7), KO sensitized (n=9)) and were sensitized and challenged as described previously (Szabo et al., 2001). PMA–induced irritative dermatitis model was performed as described in (Bai et al., 2009). Briefly, PMA (10 ml, 0.05% w/v) was smeared onto both sides of the ears of female mice (6 animals per group), ear swelling was determined together with all other biochemical measurements 24 hours later.

Histology and microscopy

Haematoxylin-eosine (HE) histochemistry was performed as described in (Szabo et al., 2001). Immunohistochemistry was performed using anti-nitrotyrosine (Sigma), CD-3 (Millipore-Upstate, Billerica, MA, USA), CD-68 (Serotec, Raleigh, NC, USA), neutrophil elastase (Santa Cruz, Santa Cruz, CA, USA) rabbit polyclonal antibodies and anti-PAR murine monoclonal antibody (BD Biosciences, San Jose, CA, USA) on paraffin-fixed 7 µm tissue sections as described in (Szabo et al., 2001).

Myeloperoxidase activity measurement

The measurement of myeloperoxidase (MPO) activity has been described in (Virag et al., 2004). MPO activity was expressed as the percentage of the MPO activity determined in the samples of WT non-sensitized animals.
**MMP activity measurement**

Ears were homogenized in DQ buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5 mM CaCl₂) buffer by Ultra Turrax, then homogenate was cleared by centrifugation. To 100 µl supernatant and 80 µl DQ buffer 20 µl of fluorescein-conjugated gelatin (DQ gelatine, Invitrogen, Carlsbad, CA, USA) was added, samples were incubated at room temperature 30 minutes and fluorescein fluorescence was determined using a Victor V3 fluorimeter (Beckton-Dickinson, Franklin Lakes, NJ, USA). Fluorescence values were normalized for protein content.

**8-OHdG determination**

8-OHdG determination was performed using a commercial kit from Cell Biolabs (San Diego, CA, USA) according to the manufacturer’s instructions.

**Protein carbanoylation assay**

Protein carbanoylation was assessed using a commercial kit (Cayman Chemicals, Ann Harbor, MI, USA) according to the manufacturer’s instructions.

**Lipid peroxidation assay**

Lipid peroxidation was measured using a commercial kit to detect HNE-protein adducts from Cell Biolabs (San Diego, CA, USA) according to the manufacturer’s instructions.

**mRNA preparation, reverse transcription and qPCR**

Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNase, and 2 µg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (Qiagen, Valencia, CA, USA). 10X diluted cDNA was used for quantitative PCR (qPCR) reactions. The qPCR reactions were performed using an ABI 7500 thermal cycler (Applied Biosciences, Foster City, CA, USA) and qPCR Supermix (Applied Bioscience) with the primers summarized in Supplementary Table 1. Expression values were normalized to the geometric mean of the expression of three control genes (18S, 36B4 and cyclophyllin) (Bai et al., 2007). mRNA expression levels were expressed as fold increase compared to samples from WT non-sensitized mice.
Transcription factor transactivation studies

For nuclear protein extraction the Transfactor Extraction kit (Clontech, Mountain View, CA, USA) was used according to the manufacturer’s instructions as described bellow.

Ears were frozen in liquid nitrogen and were ground into fine powder in a mortar cooled in liquid nitrogen. All further procedures were performed on ice with ice-cold reagents and equipment. The powdered sample was re-suspended in a small volume of ice-cold PBS and was consequently centrifuged at 450 g for 5 min at 4°C. The pellet was resuspended in lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT and protease inhibitor cocktail) and was disrupted repeatedly pressing through a syringe fitted with a No. 27 gauge needle (10 times each). The disrupted tissue lysate was then centrifuged at 11,000 g for 20 min, the supernatant removed and the nuclear pellet resuspended in extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT and protease inhibitor cocktail). The nuclei were then disrupted with a syringe fitted with a No. 27 gauge needle (10 times each) and centrifuged for 5 min at 21,000 g. Protein concentration of the supernatant (nuclear extract) was measured with Bradford protein assay.

For the quantification of transcription factor transactivation, the TransFactor Inflammatory Profiling-1 kit was utilized (Clontech). The kit enables the colorimetric determination of the DNA binding of the active transcription factors using an ELISA-based technique. 10 µg ear nuclear extract was loaded into each well and the measurement was performed according to the Manufacturer’s instructions. The results are expressed as fold increase of the WT non-sensitized control samples.

Statistical analysis

Results were expressed as means ±SEM. Statistical significance between groups was determined by Student’s t-test, p<0.05 was considered as significant. Error bars represent +/- SEM.
### Supplementary Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GGG AGC CTG AGA AAC GGC</td>
</tr>
<tr>
<td></td>
<td>GGG TCG GGA GTG GGT AAT TTT</td>
</tr>
<tr>
<td>36B4</td>
<td>AGA TTC GGG ATA TGC TGT TGG</td>
</tr>
<tr>
<td></td>
<td>AAA GCC TGG AAG AAG GAG GTC</td>
</tr>
<tr>
<td>Cyclophyllin</td>
<td>TGG AGA GCA CCA AGA CAG ACA</td>
</tr>
<tr>
<td></td>
<td>TGC CGG AGT CGA CAA TGAT</td>
</tr>
<tr>
<td>eNOS</td>
<td>GCA ATC TTC GTT CAG CCA TCA C</td>
</tr>
<tr>
<td></td>
<td>AGA GCT CAG TGA TCT CCA CGT TG</td>
</tr>
<tr>
<td>iNOS</td>
<td>GAAGTGCAAAGGTCTCAGACATGG</td>
</tr>
<tr>
<td></td>
<td>GATTCTGGAAACATTCTGTGCCTGTC</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>GGCAGAGTGAGATTGAAGGATAG</td>
</tr>
<tr>
<td></td>
<td>GGACTTTCAGCGTCACTTTGGTAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CAA CCA ACA AGT GAT ATT CTC CAT G</td>
</tr>
<tr>
<td></td>
<td>GAT CCA CAC TCT CCA GCT GCA</td>
</tr>
<tr>
<td>I-CAM</td>
<td>CTT TCG ATC TTC CAG CTA CCA TC</td>
</tr>
<tr>
<td></td>
<td>CTG CTG TTT GTG CTC TCC TG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CTC AGC CAG ATG CAG TTA AC</td>
</tr>
<tr>
<td></td>
<td>CTC TCT CTT GAG CTT GGT GAC A</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CTC TGC AAC CAA GTC TTC TCA GC</td>
</tr>
<tr>
<td></td>
<td>AAG GCT GCT GGT TTC AAA ATA GTC</td>
</tr>
<tr>
<td>MIP-2</td>
<td>CTC CAG CCA CAC TTC AGC CTA G</td>
</tr>
<tr>
<td></td>
<td>CGT CAC ACT CAA GCT CTG GAT G</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CAT TCG CGT GGA TAA GGA GT</td>
</tr>
<tr>
<td></td>
<td>ACC TGG TTC ACC TCA TGG TC</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>CGT TTC TTT GGG GTT TCT GA</td>
</tr>
<tr>
<td></td>
<td>TTT ATC ACT AAC AAT ATA GAC AGC CAC TCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>CAT CTT CTC AAA ATT CGA GTG ACA A</td>
</tr>
<tr>
<td></td>
<td>TGG GAG TAG ACA AGG TAC AAT CC</td>
</tr>
<tr>
<td>V-Cam</td>
<td>TAC CAG CTC CCA AAA TCC TG</td>
</tr>
<tr>
<td></td>
<td>TCT GCT AAT TCC AGC CTC GT</td>
</tr>
</tbody>
</table>
References


PARP-1 Inhibition Increases Mitochondrial Metabolism through SIRT1 Activation

Péter Bai,1,2 Carles Cantó,3 Hugues Oudart,4 Attila Brunyánszki,2 Yana Cen,5 Charles Thomas,3 Hiroyasu Yamamoto,3 Aline Huber,1 Borbála Kiss,1 Riekelt H. Houtkooper,3 Kristina Schoonjans,3 Valérie Schreiber,1 Anthony A. Sauve,6 Josiane Menissier-de Murcia,1 and Johan Auwerx3,*

1Biotechnologie et Signalisation Cellulaire, UMR7242 CNRS, Université de Strasbourg, ESBS, Illkirch, France
2Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary
3Laboratory of Integrative and Systems Physiology, École Polytechnique Fédérale de Lausanne, Switzerland
4CEPE, CNRS UPR9010, Strasbourg, France
5Department of Pharmacology, Weill Cornell Medical College, New York, NY 10021, USA
*Correspondence: admin.auwerx@epfl.ch
DOI 10.1016/j.cmet.2011.03.004

SUMMARY

SIRT1 regulates energy homeostasis by controlling the acetylation status and activity of a number of enzymes and transcriptional regulators. The fact that NAD+ levels control SIRT1 activity confers a hypothetical basis for the design of new strategies to activate SIRT1 by increasing NAD+ availability. Here we show that the deletion of the poly(ADP-ribose) polymerase-1 (PARP-1) gene, encoding a major NAD+-consuming enzyme, increases NAD+ content and SIRT1 activity in brown adipose tissue and muscle. PARP-1−/− mice phenocopied many aspects of SIRT1 activation, such as a higher mitochondrial content, increased energy expenditure, and protection against metabolic disease. Also, the pharmacologic inhibition of PARP in vitro and in vivo increased NAD+ content and SIRT1 activity and enhanced oxidative metabolism. These data show how PARP-1 inhibition has strong metabolic implications through the modulation of SIRT1 activity, a property that could be useful in the management not only of metabolic diseases, but also of cancer.

INTRODUCTION

Intracellular NAD+ levels control the activity of the type III deacetylase SIRT1, allowing it to act both as a metabolic sensor and effector (Yu and Auwerx, 2009). Overexpression studies indicated how activation of SIRT1 or of its orthologs extends lifespan in lower eukaryotes and protects against high-fat-diet (HFD)-induced metabolic disease in mice (Yu and Auwerx, 2009). These attractive properties spurred a quest to identify small-molecule SIRT1 agonists that could be used in situations of metabolic stress and damage. This strategy identified compounds like resveratrol or SRT1720 (Howitz et al., 2003; Mline et al., 2007), whose ability to directly interact with and activate SIRT1 is, however, debated (Pacholec et al., 2010). Therefore, a strong interest exists to develop alternative strategies to activate SIRT1. Given the NAD+ dependency of SIRT1, another potential way to activate it is by increasing NAD+ availability. This could be achieved by specifically inhibiting other NAD+-consuming activities.

Poly(ADP-ribose) polymerase (PARP)-1 is a major cellular NAD+ consumer (Sims et al., 1981). PARP-1 is activated upon binding to damaged or abnormal DNA (Durkacz et al., 1980) and catalyzes the formation of poly(ADP-ribose) polymers (PAR) onto different acceptor proteins, including PARP-1 itself (autoPARylation), using NAD+ as substrate (Adamietz, 1987). PARP-1 activation depletes cellular NAD+ levels, using it to form PAR (Sims et al., 1981). This led us to test the influence of PARP-1 on SIRT1 activity and metabolic homeostasis. Our results show how a reduction/ablation of PARP-1 activity boosts NAD+ levels and SIRT1 activity, which in turn enhances mitochondrial content and function, culminating in a solid protection against metabolic disease.

RESULTS

PARP-1−/− Mice Are Leaner and Have Increased Energy Expenditure

Chow-fed PARP-1−/− mice (de Murcia et al., 1997) weighed less (Figure 1A) and accumulated less fat than PARP-1+/+ littermates upon aging (Figure 1B), despite eating significantly more (Figure 1C). During indirect calorimetry, PARP-1−/− mice consumed more O2 (Figure 1D), suggesting higher energy expenditure (EE). Resting EE was not different (Figure S1A), indicating that the increase was due to changes at night, when mice are active. Accordingly, PARP-1−/− mice were more active at night (Figure S1B). In addition, the respiratory quotient was also higher in PARP-1−/− mice during the dark phase, indicating enhanced glucose oxidation during the feeding period (Figure 1E). PARP-1−/− mice also maintained a higher body temperature upon cold exposure (Figure 1F), were more glucose tolerant (Figure 1G), and had a trend toward lower fasting blood glucose levels (4.30 ± 0.17 mM in PARP-1−/− mice versus 3.98 ± 0.18 mM in PARP-1+/+ mice; p = 0.058), despite similar insulin levels (data not shown). During euglycemic-hyperinsulinemic clamp, glucose infusion rates or hepatic glucose production were similar to PARP-1+/+ mice,
but supporting the idea of their better glucose tolerance, glucose uptake in PARP-1−/− muscle trended up (Figures S1C–S1E). In line with the lower fat mass and improved glucose tolerance, serum triglycerides (1.04 ± 0.07 mM in PARP-1+/+ versus 0.84 ± 0.05 mM in PARP-1−/− mice; p = 0.048) and free fatty acids (FFA) (0.93 ± 0.09 mEq/l in PARP-1+/+ versus 0.72 ± 0.03 mEq/l in PARP-1−/− mice; p = 0.040) were reduced in PARP-1−/− mice.

PARP-1 Is Induced by Nutrient Availability and Contributes to HFD-Induced Diabesity

The metabolic impact of PARP-1 deletion made us evaluate whether nutrient scarcity (fasting) or overload (HFD) affects PARP-1 activity. Despite similar PARP-1 protein levels, a 24 hr fast sharply reduced PARP-1 autoPARylation levels, which reflect global PARP activity (Adamietz, 1987), suggesting a lower enzymatic activity (Figure 1H). In contrast, HFD robustly

Figure 1. Phenotyping the PARP-1−/− Mice

(A) Body weight (BW) evolution in PARP-1+/+ and −/− mice (n = 8/9).
(B) Epididymal white adipose tissue mass.
(C) Average food consumption.
(D and E) \( \text{O}_2 \) consumption (D) and respiratory quotient (RQ) (E) of PARP-1+/+ and −/− mice (n = 9/9) determined by indirect calorimetry.
(F) Body temperature after exposure to 4°C (n = 6/5).
(G) Oral glucose tolerance test (OGTT) (n = 5/5) and the area under curve (AUC).
(H) PARP-1 autoPARylation (arrow), analyzed in 100 μg of protein extract from gastrocnemius muscles of 16-week-old mice fed ad libitum or fasted (24 hr).
(I) Expression of the genes indicated (N), and the abundance of mitochondrial complexes (O) in 25 μg of total protein extracts.
(J) BW evolution in PARP-1+/+ and −/− mice (n = 10/10) fed a CD (circles) or HFD (squares) from 8 weeks of age.
(K and L) OGTT (K) and an insulin tolerance test (L) in HFD-fed PARP-1+/+ and −/− mice at 16 weeks of age (n = 10/10). The AUC of the OGTT is shown on the top right.
(M–O) BAT from PARP-1+/+ and −/− mice on CD was extracted and mitochondrial biogenesis was analyzed by transmission electron microscopy (M), mRNA expression of the genes indicated (N), and the abundance of mitochondrial complexes (O) in 25 μg of total protein extracts.
(P) SDH staining of sections from gastrocnemius and soleus of PARP-1+/+ and −/− mice on CD.
(Q and R) Gastrocnemius was also used to analyze mRNA levels of the indicated genes (Q) and the abundance of mitochondrial complexes in 25 μg of total protein extracts (R). White bars represent PARP-1+/+ mice; black bars represent PARP-1−/− mice. Values are expressed as mean ± SEM unless otherwise stated. * indicates statistical difference versus PARP-1+/+ mice at p < 0.05. For abbreviations, see Table S1.
increased PARP-1 protein levels and activity (Figure 1I), indicating a positive correlation between PARP-1 activity and nutrient availability.

As nutrient availability induces PARP-1 activity and PARP-1 deletion prompts a leaner phenotype, we next explored how PARP-1−/− mice responded to HFD-induced metabolic disease. PARP-1−/− mice gained less weight after 2 months of HFD (Figure 1J), due to a lower fat accumulation (Figure S1F). Moreover, PARP-1−/− mice on HFD were more glucose tolerant (Figure 1K) insulin sensitized (Figure 1L) and had lower serum FFAs (0.66 ± 0.05 mEq/l versus 0.53 ± 0.03 mEq/l; p = 0.026).

Mitochondrial Activation in Brown Adipose Tissue and Muscle from PARP-1−/− Mice

The above results suggested improved mitochondrial activity in key metabolic tissues of PARP-1−/− mice, such as skeletal muscle and brown adipose tissue (BAT). PARP-1−/− mice had a relatively higher amount of BAT, with a more intense red appearance (Figure S1G). Transmission electron microscopy revealed higher mitochondrial content in PARP-1−/− BAT (Figure 1M), which was further corroborated by the increased mitochondrial DNA content (Figure S1H) and mRNA expression of genes involved in mitochondrial respiration (Ndufa2, Ndufb2, Ndufb5, Cyt C, COX17), uncoupling (UCP1, UCP3), fatty acid oxidation (MCAD), and thyroid hormone activation (Dio2). Mitochondrial biogenesis was also evidenced by the higher protein content of subunits from different respiratory complexes in the BAT from PARP-1−/− mice (Figure 1O).

Also, PARP-1−/− skeletal muscle had a marked oxidative profile. Succinate dehydrogenase (SDH) staining (Figure 1P) and the expression of muscle fiber isoforms genes (Trop I, MHCI) (Figure 1Q) exposed an increase in oxidative fibers with a high mitochondrial content. As in BAT, the increased mitochondrial content was linked to an induction of the mRNA (Figure 1Q) and protein levels (Figure 1R) of mitochondrial components. In contrast to BAT and muscle, the expression of key metabolic genes was not altered in PARP-1−/− livers (Figure S1I), reflecting a minor role of PARP-1 in the liver, probably due to its very low expression (Figure S1J).

Higher NAD+ Content and SIRT1 Activity in BAT and Muscle from PARP-1−/− Mice

The PARP-1−/− mice phenocopy many features seen after SIRT1 activation (Yu and Auwerx, 2009). As PARP-1 is a major NAD+ consumer (Sims et al., 1981), we speculated that the lack of PARP-1 activity might increase NAD+ content, in turn activating SIRT1. Illustrating how PARP-1 drives most PARP activity, the ablation of PARP-1 reduced PARylation in both BAT and muscle (Figure 2A). The expression of the other PARP enzymes was not increased in PARP-1−/− BAT and muscle (Figures S2A and S2B), explaining the lack of compensation on PARylation. Confirming previous studies (Allinson et al., 2003; Fong et al., 2009), terminal dUTP nick-end labeling indicated that DNA damage was not increased in PARP-1−/− tissues (data not shown). In line with the attenuated NAD+"-consuming PARP activity, NAD+ content was robustly increased in PARP-1−/− BAT and muscle (Figure 2B), while the levels of nicotinamide (NAM), a NAD+-derived metabolite that inhibits sirtuin activity (Bitterman et al., 2002), remained unaffected (Figure 2C).

We next tested if the increase in NAD+ correlated with SIRT1 activation. Indicative of SIRT1 activation, and supporting the higher mitochondrial content, PGC-1α acetylation levels in BAT and muscle of PARP-1−/− mice were reduced by ~40% and ~90%, respectively (Figures 2D and 2E). The acetylation of another SIRT1 target, forkhead box O1 (FOXO1), was also reduced by ~60% in BAT and ~40% in muscle (Figures 2D and 2E), supporting the idea that PARP-1 deficiency leads to SIRT1 activation. Remarkably, SIRT1 protein was also robustly induced in PARP-1−/− BAT and muscle (Figures 2D and 2E), further amplifying SIRT1 activity.

As altered NAD+ levels could also potentially impact other sirtuins, we also tested the activity of SIRT2 and SIRT3, which act as cytoplasmic and mitochondrial sirtuins, respectively. The acetylation level of tubulin, a SIRT2 target (North et al., 2003), was not altered in muscle from PARP-1−/− mice (Figure 2F). Likewise, the acetylation of complex I, a target for SIRT3 (Ahn et al., 2008), even showed a slight tendency to increase in PARP-1−/− muscles (Figure 2G). These results indicate that not all sirtuins are activated in PARP-1−/− tissues.

Reduced PARP-1 Activity in Cellular Models Enhances Oxidative Metabolism

Next, we knocked down PARP-1 in HEK293T cells to evaluate whether an acute reduction in PARP-1 activity enhances oxidative metabolism. In these conditions, PARP-1 protein levels were reduced by ~80%, and the low PARP-1 autoPARylation demonstrated that PARP activity was largely blunted (Figure 3A). The reduced PARP activity was associated with enhanced NAD+ content and SIRT1 function, as illustrated by decreased PGC-1α acetylation (Figures 3B and 3C). Importantly, this happened despite unchanged SIRT1 protein levels (Figure 3C) or changes in the activity of SIRT2 or SIRT3, as manifested in tubulin, Ndufa9, or total mitochondrial acetylation levels (Figures S3A–S3C). The induction of SIRT1 and PGC-1α activity culminated in a robust increase in mitochondrial DNA content (Figure 3D), mitochondrial-related gene expression (Figure 3E), and O2 consumption (Figure 3F). Importantly, most of the metabolic effects elicited by PARP-1 depletion were lost when SIRT1 was simultaneously knocked down (Figures 3D–3F).

In line with the results in HEK293T cells, the expression of genes involved in mitochondrial function, mitochondrial DNA content, and O2 consumption were also induced in PARP-1−/− compared to PARP-1−/− MEFs (Figures S3D–S3F). Consistent with our observations in tissues from PARP-1−/− mice, SIRT1 protein was also induced in PARP-1−/− MEFs (Figure S3G).

Pharmacological PARP Inhibition Enhances Oxidative Metabolism via SIRT1

To test the relation between SIRT1 and PARP-1 activities, we exposed C2C12 myotubes to H2O2 (500 μM, 6 hr), a well-known inducer of PARP-1 activity (Schraufstatter et al., 1986). H2O2 treatment vigorously increased PARP-1 (Figure 4A) and global protein PARylation levels (Figure 4B), as manifested by the slow migrating bands, in the absence of changes in PARP-1 levels (Figures 4A and 4B). Importantly, SIRT1 was not PARylated in response to H2O2 (Figure 4A), indicating that it is not a PARylation substrate. As reported (Schraufstatter et al., 1986), the H2O2-induced increase in PARP-1 activity sharply
depleted NAD⁺ content (Figure 4C), but did not affect SIRT1 protein levels (Figures 4A and 4B). This lower NAD⁺ availability limited SIRT1 activity, as reflected in PGC-1α hyperacetylation (Figure 4D). Interestingly, the inhibition of PARP activity with PJ34 (Garcia Soriano et al., 2001) rescued the drop in NAD⁺ and recovered SIRT1 function during H₂O₂ exposure (Figures 4B–4D). These results indicate that PARP-1 activation restrains SIRT1 activity and that PARP inhibitors relieve this limitation.

PARP-1 activity is not necessarily linked to DNA damage, and it has been shown to fluctuate in a circadian fashion (Asher et al., 2010). Therefore, we wondered whether prolonged PARP inhibition, even in the absence of DNA damage, would favor NAD⁺ accumulation and, potentially, SIRT1 activity. Supporting this premise, PARP inhibition by PJ34 (Figure 4E) or a structurally unrelated compound, TIQ-A (data not shown), gradually raised NAD⁺ levels, becoming significant after 24 hr. At that time, PARP activity, but not PARP-1 protein levels, was robustly decreased (Figure 4F). PJ34 increased NAD⁺ levels dose dependently, in correlation with SIRT1 activity, as illustrated by PGC-1α deacetylation (Figure 4G). Similar effects also happened in vivo, as treatment of mice with PJ34 (10 mg/kg, b.i.d. for 5 days) blunted basal PARP activity in muscle (Figure 4H), while increasing NAD⁺ and SIRT1 activity (Figure 4I). Despite the short duration of the treatment, serum triglyceride (1.21 ± 0.08 mM vehicle versus 1.11 ± 0.04 mM PJ34; p = 0.08) and FFA levels (1.59 ± 0.06 mEq/l vehicle versus 1.44 ± 0.03 mEq/l PJ34; p = 0.03) were reduced in PJ34-treated mice. Of note, while compounds like resveratrol impact SIRT1 through AMP-activated protein kinase (AMPK) (Canto et al., 2010), PJ34 did not alter AMPK activity, as reflected by the unchanged acetyl-CoA carboxylase (ACC) phosphorylation in either C2C12 myotubes (Figure 4J) or gastrocnemius muscle (Figure 4H). PJ34 did not affect SIRT1 protein levels either (Figures 4H and 4J), but robustly induced the expression of mitochondrial and lipid oxidation genes, both in C2C12 myotubes and muscle (Figures 4K and 4L). Consistent with PGC-1α deacetylation and activation,
PJ34 promoted the recruitment of PGC-1α to target gene promoters (e.g., PDK4) (Figure S4A). Finally, the activation of SIRT1/PGC-1α by PJ34 culminated in higher O₂ consumption rates (Figure 4M), testifying for enhanced oxidative metabolism.

The effect of PJ34 on PGC-1α acetylation in C2C12 myotubes was blunted upon SIRT1 knockdown (Figure 4J). The role of SIRT1 in mediating PJ34-induced PGC-1α deacetylation was further confirmed in SIRT1−/− MEFs, where PJ34 was unable to decrease PGC-1α acetylation (Figure S4B). In line with impaired PGC-1α activation, mitochondrial gene expression and O₂ consumption were largely unresponsive to PJ34 upon SIRT1 depletion in C2C12 cells (Figures 4K and 4M) and in SIRT1−/− MEFs (Figures S4C and S4D), indicating that SIRT1 is a key mediator of PJ34 action. However, PJ34 also had SIRT1-independent effects, as reflected by the persistent increase in UCP3 mRNA even after the SIRT1 knockdown (Figure 4K). This could be explained by the fact that PJ34 does not regulate UCP3 expression by recruitment of PGC-1α to its promoter (Figure S4A). The pharmacological inhibition of PARP recapitulates the phenotypic characteristics of the PARP−1−/− mice and reveals that these effects are largely mediated by SIRT1.

**DISCUSSION**

The difficulty of identifying compounds that specifically and directly bind and activate SIRT1 led us to test whether the modulation of NAD⁺ availability could be an alternative path to activation of SIRT1. Our present work supports this concept by showing how the attenuation of PARP-1, another NAD⁺-consuming enzyme, increases intracellular NAD⁺ levels and enhances SIRT1 activity. This prompts the deacetylation and activation of key metabolic transcriptional regulators, such as PGC-1α and FOXO1, leading to increased mitochondrial content and metabolism.

Our data suggest that PARP-1 limits NAD⁺ availability for SIRT1 activation. This concept originates from the differences...
in the $K_M$ and $k_{cat}/K_M$ of both enzymes for NAD$^+$, which indicate that PARP-1 is a faster and more efficient NAD$^+$ consumer than SIRT1 (Knight and Chambers, 2001; Smith et al., 2009). Therefore, it is likely that PARP-1 activity maintains NAD$^+$ at limiting levels for SIRT1 function. The prediction that PARP-1 deletion would increase NAD$^+$ levels and activate SIRT1 is perfectly matched by our data. While previous work already speculated on a link between PARP-1 and sirtuin activities (Kothur-Seetharam et al., 2006; Pillai et al., 2005), our study expands the consequences of this link to energy homeostasis. In apparent discrepancy, one report (Devalaraja-Narashimha and Padanilam, 2010) suggested that PARP-1$^{-/-}$ mice could be more susceptible to HFD-induced obesity. However, that study used mice on an SV129 background, which are less suited for metabolic studies than C57BL/6J mice. The convergent results of our genetic, physiological, pharmacological, and in vitro studies clearly support our conclusions.

Results from our lab indicate that the activation of SIRT1 after a bout of exercise or cold exposure is not linked to decreased PARP-1 activity (data not shown). Rather, only robust and/or protracted changes, such as pharmacological (PJ34) or genetic (knockdown or deletion) PARP-1 inhibition, influence SIRT1 activity. While PARP-1 might not always participate in the physiological modulation of SIRT1 activity, our data suggest that the
interplay between both proteins could be exploited pharmacologically. Our work illustrates the way in which SIRT1 is a key mediator of the PARP-1-deficient phenotype, but the link between PARP-1 and SIRT1 activities is still unclear. Several models used in this work show that reducing PARP-1 activity controls SIRT1 function, independent of changes in SIRT1 protein levels. In all these cases, the levels of NAD+, the rate-limiting coenzyme for SIRT1, correlated with SIRT1 activity, suggesting that NAD+ availability might influence SIRT1 activity. If this were true, boosting NAD+ content through alternative strategies should elicit similar metabolic phenotypes to those of the PARP-1−/− mice. Supporting this notion, deletion of another NAD+ consumer, CD38, also activates SIRT1 (Aksoy et al., 2006), resulting in protection against HFD-induced obesity (Barbosa et al., 2007). Nutrient scarcity and AMPK activation also lead to increased NAD+ levels and SIRT1 activation coupled to the induction of oxidative metabolism (Cantó et al., 2009, 2010). This correlative evidence indicates that the increased NAD+ availability might be a key mechanism by which PARP deficiency activates SIRT1. However, we cannot exclude the possibility that PARP inhibition also impacts SIRT1 via other means, even though our results rule out direct PARYlation of SIRT1 as the mechanism (Figure 4A). In addition, SIRT1 content was increased in PARP-1−/− tissues and MEFs, further amplifying SIRT1 activity. The reason for this increase in SIRT1 levels remains elusive, but is independent of changes in SIRT1 mRNA (P.B., C.C., and J.A., unpublished data).

Of note, PARP-1 depletion affects the activity of SIRT1, but not of SIRT2 and SIRT3, which occupy nonnuclear compartments. If increased SIRT1 activity was mainly driven by changes in NAD+, the unchanged SIRT2 and SIRT3 activities in PARP-1−/− tissues suggest that the increase in NAD+ is either not enough to enhance SIRT2 and SIRT3 activities or that it only happens in specific cellular compartments, supporting an independent regulation of different subcellular NAD+ pools (Yang et al., 2007). Alternatively, PARP-1 and SIRT1 activities might not be linked by changes in NAD+, and some yet-unfound mechanism drives the specificity toward this sirtuin.

Some results indicate that the effects of PARP deficiency cannot be completely explained by SIRT1 (Figure 4K). Future research will have to clarify the nature of these SIRT1-independent effects of PARP inhibition on metabolism. It will be interesting to explore whether PARYlation can directly modulate the activity of key metabolic transcriptional regulators, as PARP-1 may contribute to nuclear processes other than DNA repair (Krishnakumar and Kraus, 2010).

PARP inhibitors are currently in clinical development as antitumor drugs (Fong et al., 2009). While our data encourages a possible utilization of PARP inhibitors as therapeutic agents to activate SIRT1 and promote oxidative metabolism, this should be taken cautiously. PARP-1 has key roles in genomic maintenance, and while neither this nor previous studies (Allinson et al., 2003) detected enhanced DNA damage in PARP-1−/− mice under basal conditions, it cannot be ignored that PARP-1−/− mice are sensitive to ionizing radiation (de Murcia et al., 1997). Hence, it will be important to analyze the impact of aging and metabolic disease on DNA damage to establish the therapeutic potential and limitations of PARP inhibition.

**EXPERIMENTAL PROCEDURES**

Detailed materials and procedures can be found in the Supplemental Information.

**Animal Experiments**

Pure C57BL/6J male mice were used for the study. PARP-1−/+ and PARP-1−/− were described (de Murcia et al., 1997). Mice were housed separately, had ad libitum access to water and chow (10 kcal% of fat) (SAFE, Augy, France) or HFD (60 kcal% of fat) (Research Diets, Inc., New Brunswick, NJ), and were kept in a 12 hr dark/light cycle. Animal experiments were carried out according to local, national, and EU ethical guidelines. Animals were sacrificed after 6 hr of fast, and tissues were collected and processed as specified.

**NAD+ and NAM Determination**

NAD+ levels in cultured cells were determined using an enzymatic method (EnzyChrom, BioAssays Systems, Hayward, CA), whereas for tissues (Figure 4A), NAD+ and NAM levels were determined as described (Sauve et al., 2005).

**Statistics**

All data were verified for normal distribution. Statistical significance was assessed by Student’s t test for independent samples. Values are expressed as mean ± SEM unless otherwise stated.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, four figures, and three tables and can be found with this article online at doi:10.1016/j.cmet.2011.03.004.

**ACKNOWLEDGMENTS**

This work was supported by fellowships awarded by Bolyai (P.B.), SNSF (P.B.), EMBO (C.C.), FBS (A.B.), and NWO (R.H.H.), as well as grants from the NKTH, OTKA (NFF7/498, IN80481), Mecenatura (DE OEC Mec-1/2008), the NIH (DK59820 and DK73466), the ERC (2008-AdG-23118), the CNRS, ANR EQIDE (22S732Y), and the Ellison Medical Foundation New Scholar in Aging 2007.

Received: April 12, 2010

Revised: May 5, 2010

Accepted: February 24, 2011

Published: April 5, 2011

**REFERENCES**


PARP-1 Inhibition Increases Mitochondrial Metabolism through SIRT1 Activation

Péter Bai, Carles Cantó, Hugues Oudart, Attila Brunyánszki, Yana Cen, Charles Thomas, Hiroyasu Yamamoto, Aline Huber, Borbála Kiss, Riekelt H. Houtkooper, Kristina Schoonjans, Valérie Schreiber, Anthony A. Sauve, Josiane Menissier-de Murcia, and Johan Auwerx

Figure S1, Related to Figure 1. Increased Spontaneous Locomotor Activity and Energy Expenditure in the PARP-1−/− Mice during Night

(A) Oxygen consumption was determined in PARP-1+/+ and −/− male mice (n=6/6) as described in the Materials and Methods. TEE – total energy expenditure, REE – resting energy expenditure.
(B) Spontaneous activity was determined during indirect calorimetry in CLAMS using \textit{PARP-1}^{+/+} and \textit{PARP-1}^{-/-} male mice (n=9/9).

(C-E) Peripheral and hepatic insulin responsiveness of \textit{PARP-1}^{+/+} and \textit{PARP-1}^{-/-} male mice was assessed by euglycemic-hyperinsulinemic clamp. (C) Glucose infusion rates (GIR), (D) hepatic glucose production (HGP) and (E) glucose uptake in different tissues are all shown as mean +/- SEM.

(F) The total WAT mass, individual WAT depots, and organ weights were determined upon autopsy (E – epididymal, SC – subcutaneous, P – perirenal).

(G) BAT was photographed and weighed after autopsy of \textit{PARP-1}^{+/+} and \textit{PARP-1}^{-/-} male mice (11.5 months of age, n=8/9 males). BAT content (relative to total body weight), is shown at the bottom of the image.

(H) BAT mitochondrial DNA (mtDNA) was quantified by qPCR in the BAT.

(I) mRNA expression levels of selected genes were quantified by RT-qPCR reactions in the liver of \textit{PARP-1}^{+/+} and \textit{PARP-1}^{-/-} male mice (n=9/9). Abbreviations are listed in the text and in Table S1.

(J) mRNA expression of \textit{PARP-1} were quantified in different metabolic tissues of C57Bl/6J male mice (n=5) (black bar – liver; dashed bar – BAT; white bar – skeletal muscle; grey bar – testis). Asterisks indicate significant difference at * p<0.05; *** p<0.001.
Figure S2, Related to Figure 2. Gene Expression Pattern of the Different Members of the PARP Family in the BAT and Gastrocnemius Muscle

(A-B) RT-qPCR reactions were performed on cDNA populations from the BAT (A) and the gastrocnemius muscle (B) of PARP-1+/− and −/− male mice (n=7/5). Asterisks indicate significant difference between cohorts, where * p<0.05; *** p<0.001.
Figure S3, Related to Figure 3. Assessment of Protein Acetylation in HEK293T Cells and Measurement of Mitochondrial Function and Protein Levels in PARP-1<sup>+/+</sup> and <sup>-/-</sup> MEFs (A-C). HEK293T were transfected with either control or PARP-1 shRNAs. 48 hrs later, total and mitochondrial extracts were obtained.

(A) 50 mg of total extracts were used to test tubulin acetylation levels.

(B) 400 mg of total extracts were used to immunoprecipitate Ndufa9, using 3 mg of antibody, and evaluate lysine acetylation on the immunoprecipitates.

(C) 50 ug of mitochondrial extracts were used to test global lysine acetylation and porin levels (as input).

(D-F) In PARP-1<sup>+/+</sup> and <sup>-/-</sup> primary MEFs (n=3/3) mRNA expression (D), mitochondrial DNA content (E), oxygen consumption (F) pACC, SIRT1 and PARP-1 protein levels (G) was determined. Abbreviations are listed in the text. Asterisks indicate significant difference between cohorts, where * p<0.05, ** p<0.01.
Supplementary Figure 4

Figure S4, Related to Figure 4. Assessment of Mitochondrial Function upon Pharmacological PARP Inhibition in C2C12 Cells and SIRT1−/− MEFs

(A) Promoter occupancy of PGC-1α was quantified after PJ34 treatment on the PDK4 and UCP-3 promoters (1 μM, 48h) (n=3/3).
(B-D) PGC-1α acetylation, ACC phosphorylation, SIRT1 protein levels (B), expression of mitochondrial protein mRNAs (C) and O₂ consumption (D) were determined in SIRT1+/+ and −/− MEF cells, treated with PBS (Veh) or PJ34 for 48 hrs.
Table S1. qRT-PCR Primers for Quantification of Gene Expression

All primers are designed for murine sequences unless otherwise specified.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S (human)</td>
<td>5'-CGG CTA CCA CAT CCA AGG AA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCT GTA TTA TTA TTA GTC ACT ACC T-3'</td>
</tr>
<tr>
<td>ACC1 acetyl-CoA carboxylase -1</td>
<td>5'-GAC AGA CTG ATC GCA GAG AAA G-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGG AGA GCA CAC ACA -3'</td>
</tr>
<tr>
<td>ACC2 acetyl-CoA carboxylase-2</td>
<td>5'-CCC AGC CGA GTT TGT CAC T-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGC GAT GAG CAC CTT TCC TA-3'</td>
</tr>
<tr>
<td>ACO Acyl-CoA oxidase</td>
<td>5'-CCC AAC TGT GAC TTC CAT T-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGC GAT GGC TGG ATC A-3'</td>
</tr>
<tr>
<td>ATP5g1</td>
<td>5'-GCT GTG TGA GAG ATG GGT TC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGT TGG TGT GGC TGG ATC A-3'</td>
</tr>
<tr>
<td>COX17</td>
<td>5'-CGT GAT GCG TGC ATC ATT GA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAT TCA CAA AGT AGG CCA CC-3'</td>
</tr>
<tr>
<td>Cyclophyllin B</td>
<td>5'-TGG AGA GCA CCA AGA CAG ACA -3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGG CCG AGT CGA CAA TGA T-3'</td>
</tr>
<tr>
<td>cyt c Cytochrome C</td>
<td>5'-TCC ATC AGG GTA TCC TCT CC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGA GGC AAG CAT AAG ACT GG-3'</td>
</tr>
<tr>
<td>Dio2 Deiodinase-2</td>
<td>5'-GCA CGT CTC CAA TCC TGA AT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGA ACC AAA GGT GAC CAC CA-3'</td>
</tr>
<tr>
<td>ERRα estrogen receptor related receptor α</td>
<td>5'-ACTGCCACTGCAAGGATAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CACAGCCTCAGCATCTTCAA-3'</td>
</tr>
<tr>
<td>GK Glucokinase</td>
<td>5'-ACA TTG TGC GCC GTG CCT GTG AA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGC CTG CGC ACA CTG GGC TGA AA-3'</td>
</tr>
<tr>
<td>G6Pase - glucose-6 phosphatase</td>
<td>5'-CCG GAT CTA CCT TGC TGC TCA CTT T-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TAG CAG GTA GAA TCC AAG CGC GAA AC-3'</td>
</tr>
<tr>
<td>L-CPT-1 (human) Liver Carnitine Palmitoyl Transferase</td>
<td>5'-GAG GCG AGA ACA CCA GCT TC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCG GAT GTG GTC AAA G-3'</td>
</tr>
<tr>
<td>MCD malonyl-CoA decarboxylase</td>
<td>5'-TGG ATG GCT GAC AGC AGC CTC AA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTG AGG ATC TGC TCG GAA ACT TTG-3'</td>
</tr>
<tr>
<td>MCAD (human) Medium chain acyl-coenzyme A dehydrogenase</td>
<td>5'-AGA ATT GGC TTA TGG ATG TAC AGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TTT GTT GAT CAT TTC CAG CAA TAA T-3'</td>
</tr>
<tr>
<td>MCAD Medium chain acyl-coenzyme A dehydrogenase</td>
<td>5'-GAT CGC AAT GGG TGC TTT TGA TAG AA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGC TGA TTG GCA ATG TCT CCA GCA AA-3'</td>
</tr>
<tr>
<td>mCPT1 Carnitine Palmitoyl Transferase-1</td>
<td>5'-TTT CCC TAC AGC TGG CTC ATT TCC -3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCA CCC AGA TGA TGG GGA TAC TGT-3'</td>
</tr>
<tr>
<td>MHC1 Myosin heavy chain 1</td>
<td>5'-GAG TAG CTC TTG TGC TAC CCA GC -3'</td>
</tr>
<tr>
<td></td>
<td>5'-AAT TGC TTT ATT CGT CCA CC -3'</td>
</tr>
<tr>
<td>MHCIIA Myosin heavy chain 2A</td>
<td>5'-GCA AGA AGC AGA TCC AGA AAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGT CTT CTT CTG TGT AAG TAA GC-3'</td>
</tr>
<tr>
<td>MHCIIIX Myosin heavy chain 2X</td>
<td>5'-GCA ACA GGA GAT TCC TGA CCT CAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCA GAG ATG CCT CTG CTT C-3'</td>
</tr>
<tr>
<td>Ndufa2</td>
<td>5'-GCA CAC ATT TCC CCA CAC TG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCC AAC CTG CCC ATT CTG AT-3'</td>
</tr>
<tr>
<td>Ndufb3</td>
<td>5'-TAC CAC AAA CGC AGC AAA CC-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>5'-Sequence 3'</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td><strong>Ndubf5</strong></td>
<td>5'-CTT CGA ACT TCC TGC TCC TT-3'</td>
</tr>
<tr>
<td><strong>PARP-1 (human)</strong></td>
<td>5'-GCT CCT GAA CAA TGC AGA CA-3'</td>
</tr>
<tr>
<td><strong>PARP-1</strong></td>
<td>5'-GGA GCT GCT CAT CTT CAA CC-3'</td>
</tr>
<tr>
<td><strong>PARP-2</strong></td>
<td>5'-GGA AGG CGA GTG CTA AAT GAA-3'</td>
</tr>
<tr>
<td><strong>PARP-3</strong></td>
<td>5'-CCT GCT GAT AAT CGG GTC AT-3'</td>
</tr>
<tr>
<td><strong>PARP-4</strong></td>
<td>5'-GTT AAA TTT TGC ACT GGA G-3'</td>
</tr>
<tr>
<td><strong>PARP-5a</strong></td>
<td>5'-TAG AGG CAT CGA AAG CTG GT-3'</td>
</tr>
<tr>
<td><strong>PARP-5b</strong></td>
<td>5'-GTC CCA TGGCAA CGA GAC GTT CA-3'</td>
</tr>
<tr>
<td><strong>PARP-6</strong></td>
<td>5'-TTT CCA GCC ATC GAA TAA GG-3'</td>
</tr>
<tr>
<td><strong>PARP-7</strong></td>
<td>5'-AAA ACC CCT GGA AAT CAA CC-3'</td>
</tr>
<tr>
<td><strong>PARP-8</strong></td>
<td>5'-CCA CTT GGC TTT CAC AA-3'</td>
</tr>
<tr>
<td><strong>PARP-9</strong></td>
<td>5'-CCC AAT TGG CAG AGT GGC AGG TAA-3'</td>
</tr>
<tr>
<td><strong>PARP-10</strong></td>
<td>5'-ACA GCT CAG GGT AGA GAT GC-3'</td>
</tr>
<tr>
<td><strong>PARP-11</strong></td>
<td>5'-CAA GAT CCT GCA GAT GCA AA-3'</td>
</tr>
<tr>
<td><strong>PARP-12</strong></td>
<td>5'-CGG ATC CAG AAC ATG GGC-3'</td>
</tr>
<tr>
<td><strong>PARP-14</strong></td>
<td>5'-GGC AAA CGC AAT GGA ACT AT-3'</td>
</tr>
<tr>
<td><strong>PARP-16</strong></td>
<td>5'-CGG ATC CAG AAC ATG GGC-3'</td>
</tr>
<tr>
<td><strong>PDK4</strong></td>
<td>5'-AAA GGA CAG GAT GGA AGG AAT CA-3'</td>
</tr>
<tr>
<td><strong>PEPCK</strong></td>
<td>5'-CCA CAG CTG CTG CAG AAG CA-3'</td>
</tr>
<tr>
<td><strong>PGC-1α</strong></td>
<td>5'-AAG TGT GGA ACT CTC TGG AAC TG-3'</td>
</tr>
<tr>
<td><strong>PPARα (human)</strong></td>
<td>5'-TCA TCA AGA AGA CGA GTC G-3'</td>
</tr>
<tr>
<td><strong>PPARα</strong></td>
<td>5'-CCT GAA CAT CGA GTG TCG AAT AT-3'</td>
</tr>
<tr>
<td><strong>SIRT1 (human)</strong></td>
<td>5'-TAG GCG GCT TGA TGG TAA TC-3'</td>
</tr>
</tbody>
</table>
### Table S2. Primers for mtDNA Determination

<table>
<thead>
<tr>
<th></th>
<th>5'-TCT GGC ATG TCC CAC TAT CA-3'</th>
</tr>
</thead>
</table>
| **SREBP1** *sterol regulatory element binding transcription factor 1* | 5'-GGC CGA GAT GTG CGA ACT-3'  
5'-TTG TTG ATG AGC TGG AGC ATG T-3' |
| **Trop I** *Troponin I*                                           | 5'-CCA GCA CCT TCA GCT TCA GGT CCT TGA T-3'  
5'-TGC CGG AAG TTG AGA GGA AAT CCA AGA T-3' |
| **UCP1** *Uncoupling protein-1*                                   | 5'-GGC CCT TGT AAA CAA CAA AAT AC-3'  
5'-GGG AAC AGC TGA CAG TAA AT-3' |
| **UCP2** *Uncoupling protein-2*                                    | 5'-TGG CAG GTA GCC CCA CAG G-3'  
5'-CAT CTG GTC TTT CAG CAA CTC T-3' |
| **UCP3 (human)** *Uncoupling protein-3*                           | 5'-GTG ACC TAC GAC ATC CTC AAG G-3'  
5'-GCT CCA AAG GCA GAG ACA AAG-3' |
| **UCP3** *Uncoupling protein-3*                                    | 5'-ACT CCA GGG TCG CCA TCA GGA TTC T-3'  
5'-TAA ACA GGT GAG ACT CCA GCA ACT T-3' |

### Table S3. ChIP Primers

|               | 5'-AAC CCT CCT CCC TCT CAC CCT-3'  
5'-ACA CCA ATC AGC TCA GAG AA-3' |
|---------------|-----------------------------------|
| **PDK4**                                               | 5'-GAA TGT CAG GCC TCT AAG AA-3'  
5'-CAG GAG GTG TGT GAC AGC AT-3' |

<table>
<thead>
<tr>
<th></th>
<th>5'-CAT TAA ACA GTC TAC AAA ACA TAT-3'</th>
</tr>
</thead>
</table>
| **UCP-3**                                             | 5'-GTG GTG CTA TAG ATG ATA TTT TAA ATT G-3'  
5'-CAT TAA ACA GTC TAC AAA ACA TAT-3' |
Supplemental Experimental Procedures

Animal Experiments

To monitor body weight, mice were weighed and the food consumption was measured each week on the same day. In case of PJ34 treatment, mice received each 12h (at 7:00 and 19:00) 10 mg/kg PJ34 by intraperitoneal injection for 5 continuous days. Oral glucose tolerance test, intraperitoneal insulin tolerance test, free fatty acid (FFA) and triglycerides were determined as described (Lagouge et al., 2006). Plasma insulin and was determined in heparinized plasma samples using specific ELISA kits (Mercodia). Thermoadaptation was performed as described (Lagouge et al., 2006). We measured O₂ consumption, CO₂ production, and spontaneous locomotor activity in an open-circuit indirect calorimetry system (Sabre systems, Las Vegas, NV, USA) over 24-48h as described (Dali-Youcef et al., 2007; Lagouge et al., 2006; Watanabe et al., 2006). Energy expenditure was obtained by using an energy equivalent of 20.1 J / ml O₂. The respiratory quotient was the ratio of CO₂ production over O₂ consumption. During actimetry, beamline crossings were summarized each 15 minutes. The sum of beamline crosses for each 15 min period were plotted against time and AUC was calculated for each mouse that was averaged in each experimental cohort.

Euglycemic-hyperinsulinemic clamps were performed in PARP-1−/− and +/+ male mice (n = 4 mice per genotype; age = 4 months) exactly as previously described (Feige et al., 2008). In all studies animals were killed (at 14:00) either after CO₂ inhalation or cervical dislocation after 6h of fasting (starting at 8:00), and tissues were collected and their weight was expressed relative to the body weight.

Histology and Microscopy

Haematoxylin-eosine (HE) and succinate dehydrogenase (SDH) staining was performed on frozen-section- and paraffin-fixed 7 μm sections, as described (Lagouge et al., 2006). Transmission electron microscopy (TEM) investigation was performed on glutaraldehyde-
fixed, osmium tetroxyde stained ultrafine sections (Watanabe et al., 2006). Aspecific binding of the secondary antibody was controlled on sections where the primary antibody was omitted.

**Cell Culture, Transfection, Adenoviral Infection and Mitochondrial Characterization**

HEK293T, MEF and C2C12 cells were cultured in DMEM (4,5 g/l glucose, 10% FCS). *PARP-1<sup>+/+</sup>* and *PARP-1<sup>-/-</sup>* MEFs were prepared as described in (Menissier-de Murcia et al., 1997). *SIRT1<sup>+/+</sup>* and *SIRT1<sup>-/-</sup>* MEFs were kindly provided by Fred Alt (Chua et al., 2005). HEK293T cells were transfected using JetPei reagent (*Polyplus Transfections*, Illkirch, France) according to the manufacturer’s instructions. C2C12 cells were differentiated in DMEM (4,5 g/L glucose, 2% horse serum) after reaching confluency for 2 days, followed by 2 days of PJ34 treatment (10 μM). PARP-1 shRNA constructs were described in (Shah et al., 2005). Human PARP-1 and SIRT1 siRNAs were obtained from Dharmacon (Thermo Scientific). The adenovirus encoding for FLAG-HA-PGC-1α, control and SIRT1 shRNAs were a kind gift from Pere Puigserver and were used (MOI = 100) in C2C12 myotubes as described (Canto et al., 2009). DNA strand breaks were quantified by TUNEL assays according to the manufacturer’s instructions (*Millipore*).

**mRNA and mtDNA Analysis**

Total RNA was prepared using TRIzol (*Invitrogen*) according to the manufacturer’s instructions. RNA was treated with DNase, and 2 μg of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (*Qiagen*, Valencia, CA, USA). 50X diluted cDNA was used for RT-quantitative PCR (RT-qPCR) reactions (Bai et al., 2007). The RT-qPCR reactions were preformed using the Light-Cycler system (*Roche Applied Science*) and a qPCR Supermix (*Qiagen*) with the primers summarized in Table S1. mtDNA quantification was performed as described (Lagouge et al., 2006) with the primers indicated in Table S2.
**Immunoprecipitation, SDS-PAGE, Western Blotting**

Cells were lysed in lysis buffer (50 mM Tris, 100 mM KCl, EDTA 1 mM, NP40 1%, nicotinamide 5 mM, Na-butyrate 1 mM, protease inhibitors pH 7.4). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The origin of the primary and secondary antibodies used can be found bellow. Reactions were developed by enhanced chemiluminescence (Amersham, Little Chalfont, UK). PGC-1α, FOXO1 and Ndufa9 acetylation levels were analyzed by immunoprecipitation from cellular or nuclear lysates of tissues with anti-PGC-1α (Millipore), anti-FOXO1 (Cell Signalling, Danvers, MA, USA) and anti-Ndufa9 (Abcam) antibody followed by Western blot using an acetyl-lysine antibody (Cell Signalling) that was normalized to total PGC-1α/FOXO1/Ndufa9 levels. In HEK293T cells HA-tagged PGC-1α was overexpressed and was immunoprecipitated using an anti-HA. In C2C12 myotubes, FLAG-HA tagged PGC-1α was introduced through adenoviral delivery 2 days before treatments, then IP was performed using anti-FLAG antibody and samples were processed as described. All blots were quantified by densitometry using ImageJ software.

**Antibodies Used for Western Blot Applications**

PARP-1 (Erdelyi et al., 2009), PAR (Alexis, Lausanne, Switzerland), SIRT1 (Millipore), FOXO1 (Cell Signalling), haemagglutinin (Sigma), p-ACC (Upstate), Complex I (Ndufa9) (Abcam), Complex IV (COXI) and V (α subunit) (Molecular probes), FLAG (Sigma), and actin (Sigma) were detected using a polyclonal rabbit antibodies. The secondary antibody was IgG-peroxidase conjugate (Sigma, 1:10000).

**Poly(ADP-Ribose) Detection**

PAR was detected either by using a monoclonal anti-PAR antibody (Alexis) and Mouse-on-mouse kit (Vector Laboratories) on 7 μm formalin-fixed tissues using as described in (Garcia et al., 2001) or by Western blotting of total protein lysates (Erdelyi et al., 2009).
Chromatin Immunoprecipitation (ChIP)

ChIP was performed according to (Bai et al., 2007). FLAG-HA-PGC-1α (Rodgers et al., 2005) was introduced by adenoviral transfer into C2C12 myotubes after 48h of differentiation and cells were cultured for an additional 2 days. Cell were then exposed to 1 µM PJ34 in saline for 24h. Thereafter ChIP was performed using anti-FLAG (Sigma) and anti-TNF-R1 (Santa Cruz) as described (Bai et al., 2007). Pelleted DNA was quantified by qPCR using the primers against PDK4 and UCP-3 promoters flanking the nuclear receptor site (Table S3). The results were normalized for the signal of the respective inputs (vehicle/PJ34–treated) and were expressed as a percentage. The signal of anti-TNF-R1 (non-specific antibody) was subtracted from the anti-FLAG signal (specific) and the specific signal was plotted.

NAD⁺ and NAM Determination

NAD⁺ levels in cultured cells were determined using a commercial kit (Enzynchrom, BioAssays Systems, CA). For tissue samples NAD⁺ and NAM levels were determined as described in (Sauve et al., 2005). In brief, to a weighed aliquot of frozen pulverized tissue we added as standards, O¹⁸-NAD⁺ (typically 2,00 nmol) and O¹⁸-NAM (typically 2,00 nmol). 70 µL of ice-cold 7% perchloric acid was then added and the sample was vortexed and sonicated three times, then centrifuged. Clear supernatant was removed and neutralized by additions of 3 M NaOH and 1 M phosphate buffer (pH=9), then centrifuged. Clear supernatant was injected onto HPLC C-18 column with 20 mM ammonium acetate eluent to separate NAD⁺ and NAM from other cellular components, NAD⁺ and NAM peaks (260 nm absorbance) were collected. Collections were lyophilized to dryness and subjected to MALDI-TOF analysis. For NAD⁺ measurement, ratio of intensities for m/z = 664 and 666 peaks, corresponding to¹⁶O- and ¹⁸O-NAD⁺ isotopomers, was multiplied by 2,00 nmol and then divided by tissue weight to determine NAD⁺ concentration in the sample. For NAM the ratio of intensities for m/z = 123 and 125 peaks, corresponding to ¹⁶O- and ¹⁸O-NAM isotopomers, was multiplied by 2,00
nmol and then divided by tissue weight to determine NAM concentration in the sample. Corrections were applied for isotopic abundance.

**Oxygen Consumption in Cultured Cells**

Cellular O$_2$ consumption was measured using a Seahorse bioscience XF24 analyzer with thirty biological replicates per condition, in 24 well plates at 37°C, exactly as described (Canto et al., 2009). C2C12 were infected with an adenovirus encoding for FLAG-HA-PGC-1$\alpha$ and either scramble or SIRT1 shRNAs 48h previous to O$_2$ consumption measurements. Then myotubes were treated with 1 $\mu$M PJ34 for 48h.

**Supplemental References**


