Poly (ADP-ribose) polymerase activation in circulating leukocytes

Ph.D. Thesis

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Abbreviations

3-AB	3-aminobenzamide
5-AIQ	5-amino- isoquinolinone
8-OHdG	8-hydroxy-2'deoxyguanosine
ACE	angiotensin converting enzyme
AHR	aryl hydrocarbon receptor
AIF	apoptosis inducing factor
ALT	alanine aminotransferase
AP-2	activator protein-2
ART	ADP-ribosyl transferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BER	base-excision repair
C	caspase
CaMk	calmodulin-dependent protein kinase
CDK	cycline-dependent kinase
CK	creatine kinase
CLP	cecal ligation and puncture
CPK	creatine phosphokinase
CRA	right coronary artery
DNA	deoxyribonucleic acid
EBNA-1	Epstein–Barr nuclear antigen-1
EDTA	ethylenediaminetetraacetic acid
ECG	electrocardiogram
ERK2	extracellular signal-regulated kinase2
Erα	estrogen receptor α
GOT	glucotamic-oxaloacetic transaminase
HUVEC	human umbilical vein endothelial cell
i.p.	intraperitoneal
IAB	intra-aortic balloon pump
ICAM	Intracellular adhesion molecule
IHD	ischemic heart disease

IL	interleukin
iNOS	inducable nitric oxide synthase
IPC	ischemic preconditioning
IRAP	insulin-responsive aminopeptidase
ISO	1,5-dihydroxy-isoquinoline
LAD	left anterior descending artery
LCx	left circumflex artery
LDH	lactate dehydrogenase
LM	left main coronary artery
LPS	lipopolysaccharide (*Escherichia coli* endotoxin)
LV	left ventricular
MAPK	mitogen-activated protein kinase
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
MVP	major vault protein
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NFR	nuclear fast red
NF-κB	uclear factor- κB
NLS	nuclear homing signal
NO	nitric oxide
NT	nitrotyrosine
NuMA	nuclear and mitotic apparatus
OM	obtuse marginal
PAR	poly (ADP-ribose)
PARG	poly (ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PCI	percutaneous coronary intervention
R1	region1
ROS	reactive oxygen species
RRV	right retroventricularis
SD	standard deviation
SEM	standard error of the mean

SSBR	single-strand break repair
STAT6	signal transducer and activator of transcription-6
STEMI	ST-elevation myocardial infarction
TCDD	2,3,7,8-tetrachlorodi benzo-p-dioxin
TEF-1	transcriptional enhancer factor-1
TEP	telomerase-associated protein
TGF	transforming growth factor
TIMI	thrombolysis in myocardial infarction
TNF-α	tumor necrosis factor-α
TRF	telomeric-repeat binding factor
VEGF	vascular endothelial growth factor
VRNA	untraslated vault RNA

1. Introduction

1.1. Poly (ADP-ribose) polymerase (PARP) superfamily

The transfer of ADP-ribose to proteins was originally discovered in association with bacterial toxins, such as Diphtheria or Perussis toxin. Mono (ADP-ribosyl)ation is the enzymatic transfer of ADP-ribose from βNAD^+ (β nicotinamide adenine dinucleotide) to specific amino acid of the acceptor protein, which process is catalyzed by cellular ADP-ribosyl transferases (ARTs) and certain bacterial toxins. There are two subclasses of cellular enzymes: the ectoenzymes that modify cell surface molecules; and the intracellular enzymes that act on proteins involved in cell signaling and metabolism (1-3).

Poly (ADP-ribosyl)ation of proteins is a post-translational modification mediated by poly (ADP-ribose) polymerases (PARPs), that uses NAD^+ as substrate to form the negatively charged polymer of poly (ADP-ribose) (PAR). The binding of PAR to proteins is exceptionally strong. Panzeter et al. found that PAR-histone complexes are resistant to phenol partitioning, strong acids, chaotropes, detergents, and high salt concentrations (4). A specific PAR-binding consensus sequence was identified in several important DNA (deoxyribonucleic acid) damage checkpoint proteins. The 20-amino acid motif contains two conserved regions: a basic amino acid rich cluster and a pattern of hydrophobic amino acids interspersed with basic residues. These PAR-binding sequences overlap functionally important domains that are responsible for protein-protein interactions, DNA binding, nuclear localization signaling, nuclear export signaling, and protein degradation (5).

PARPs constitute a large and growing family of 18 proteins, encoded by different genes and displaying a conserved catalytic domain (Figure 1.) (1, 6).

1.1.1. DNA-damage-dependent PARPs (PARP-1, PARP-2)

PARP-1 is an abundant nuclear enzyme present in eukaryotic cells with the exemption of yeast and neutrophil granulocytes. It is a molecular sensor of DNA damage; its catalytic activity is stimulated more than 500-fold on binding to DNA breaks. Its protein acceptors are associated with chromatin structure and DNA metabolism, and the enzyme also PARylates itself in an automodification reaction.

PARP-1 is important in the survival and genomic integrity of cells; however it has detrimental effects in various inflammatory processes, cardiac and cerebral ischemia (1, 3, 7, 8).

PARP-2 is also activated by DNA-damage. Although PARP-1 and 2 hetrodimerize and share severe common nuclear binding partners, their characteristics and functions are similar but not fully overlap. While PARP-1 targets DNA nicks, PARP-2 prefers DNA gaps. PARP-1 preferentially modifies histone H1, PARP-2 prefers histone H2B as an acceptor. However both PARP-1 and PARP-2 knockout mice are viable PARP-1/PARP-2 double-gene disruption leads to embryonic lethality (1, 3, 9).

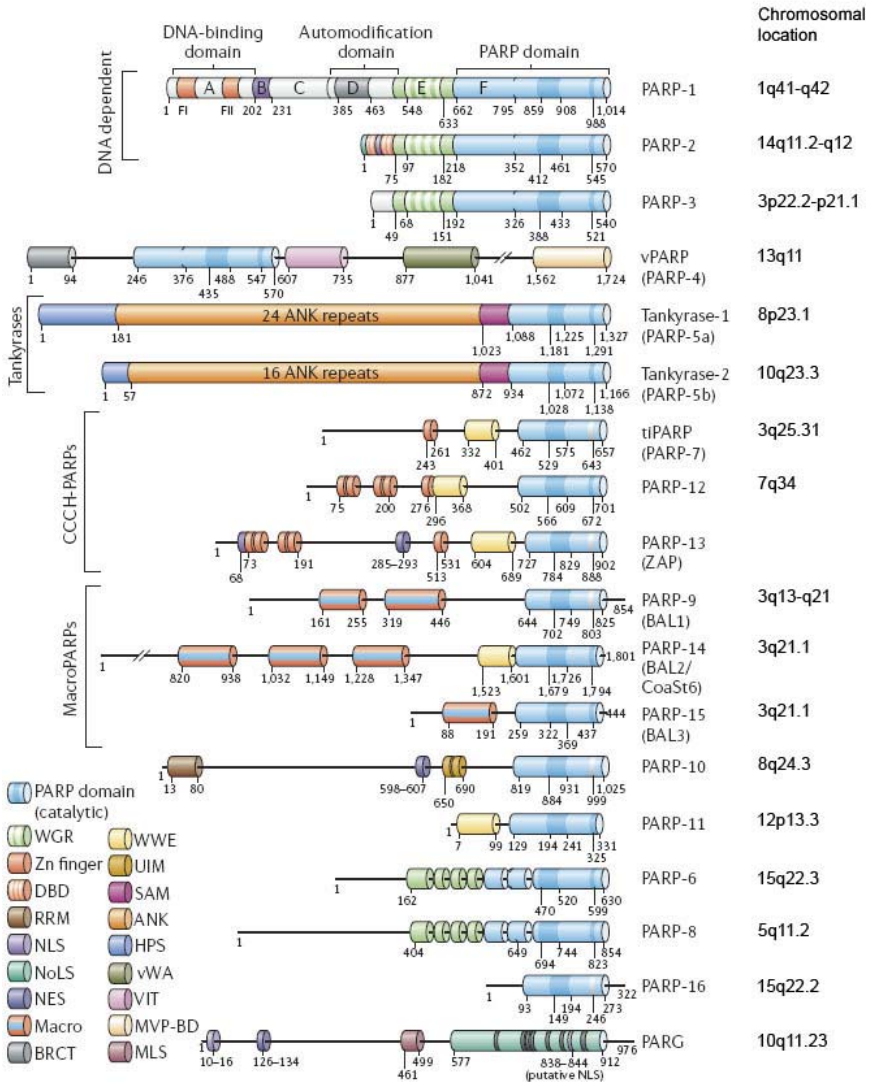


Figure 1. The domain architecture and chromosomal location of the 17 members of the PARP superfamily and of poly (ADP-ribose) glycohydrolase (PARG) (3).

Protein domains that are illustrated by colored boxes were defined according to the Pfam 19.0 or CCD v2.06 (National Center for Biotechnology Information) databases. Within each putative PARP domain, the region that is homologous to the PARP signature (residues 859–908 of PARP-1) as well as the equivalent of the PARP-1 Glu988 catalytic residue (when present) are darkened.

Domain	***Function***
WGR	*Unknown*
Zn fingers	*DNA-binding/ putative RNA-binding*
DBD	*DNA-binding*
RRM	*RNA-binding motif*
Macro	*ADP, ADP-ribose, poly(ADP-ribose) or O-acetyl-ADP-ribose binding*
BRCT (BRCA1 C terminus)	*Protein–protein interaction*
WWE	*Protein–protein interaction*
UIM	*Ubiquitin-interaction*
SAM (sterile α-motif)	*Protein-interaction*
ANK (ankyrin)	*Protein-interaction*
HPS	*Unknown*
VWA (von Willebrand factor type A)	*A metal-ion-dependent site for binding*
VIT (vault inter-α-trypsin)	*Unknown*
MVP-BD	*Binding site for major vault protein (MVP)*
MLS	*Mitochondrial localization signal*
NES	*Nuclear export signal*
NoLS	*Nucleolar localization signal*
NLS	*Nuclear localization signal*

Although several members of the PARP family display splicing variants (for example, PARP-3, PARP-9/BAL1, PARP-14/BAL2/CoaSt6 and PARP-15/BAL3, only one variant is illustrated.

1.1.2. Other PARPs

While tankyrase (TRF-1 (telomeric-repeat binding factor) interacting, ankyrin-related ADP-ribose polymerase) was first described as a partner of TRF-1 (10), PARP-3 was identified as a core component of the centrosome (3). vPARP/PARP-4 was

discovered in association with vault particles (3). PARP-10 is known to be the partner of the proto-oncoprotein cMyc (1). (Table 1.)

Table 1. Location, protein partners, function and regulation of Tankyrases, PARP-3, vPARP/PARP-4, PARP-10.

Name	**Location**	**Protein partners**	**Function/ regulation**	**Ref.**
Tankyrases				
Tankyrase-1	Nuclear pores Golgi Mitotic centromeres	TRF1 insulin-responsive aminopeptidase (IRAP) Mcl1 proteins (members of the Bcl2 family) Epstein–Barr nuclear antigen-1 (EBNA1) nuclear and mitotic apparatus (NuMA) protein cytoplasmic and heterochromatic factor TAB182	Regulating telomere homeostasis by modifying the negative regulator of telomere length (TRF1) Essential function in mitotic segregation (telomere segregation) Possible functions related to partner proteins Phosphorylation upon insulin signal increases activity	(1, 3, 6, 10-12)
Tankyrase-2	Similar to Tankyrase-1	Similar to Tankyrase-1	Similar to Tankyrase-1 Not essential for mitotic segregation In case of overexpression, it induces caspase-independent cell death (loss of mitochondrial potential)	(1, 13, 14)

Name	Location	Protein partners	Function/ regulation	Ref.
PARP-3	Daughter centriole in centrosomes Splicing variant in nucleus		Maturation of the daughter centriole until the G1–S restriction point.	(1, 15)
vPARP/ PARP-4	Vault particles Nucleolus Mitotic spindle	Major vault protein (MVP) Telomerase-associated protein (TEP1) Untraslated vault RNA (VRNA)	Multidrog resistance Intracellular transport	(3, 16)
PARP-10	Cytoplasm Nucleus Nucleolus	c-Myc	Accumulates in the nucleolus where it acquires a cycline-dependent kinase (CDK2)-dependent phosphorylation during late-G1–S phase and during prometaphase to cytokinesis Potent inhibitor of the cell transformation mediated by c-Myc in the presence of Ha-Ras (PARP activity is not required)	(1, 17, 18)
		PAR-ylates histone H2A	Potential role in chromatin regulation	

CCCH-type zinc-finger PARPs have a similar domain organization that contains $CX_8CX_5CX_3$-like zing fingers. MacroPARPs link 1-3 macro domains to the PARP domain (1). (Table 2.)

Table 2. Name, function and regulation of CCCH-type zinc-finger PARPs and MacroPARPs

Name	Function/regulation	Ref.
CCCH-type zinc-finger PARPs.		
tiPARP	Gene expression is induced by 2,3,7,8-tetrachlorodi benzo-p-dioxin (TCDD), under the control of the dioxin-bound aryl hydrocarbon receptor (AHR) Possible contribution to the detrimental effects of TCDD on neurological functions (behavior, learning, memory)	(1, 19)
PARP-13	The rat protein lacking PARP domain (ZAP) provides resistance to retroviral infection	(20)
PARP-12	Closely related to PARP-13	(1)
MacroPARPs	Transcription cofactors	
PARP-9/ BAL1	Repress activity of STAT6 (signal transducer and activator of transcription-6)-mediated transcription activation in interleukin (IL)-4-stimulated T cells Overexpressed in aggressive diffuse large B-cell lymphomas	(1, 21)
PARP-14/ BAL2/ CoaSt6	Activates STAT6-mediated transcription activation in IL-4-stimulated T cells	(22)
PARP-15/ BAL3		(1)

1.2. PARP-1

The most significant member of the PARP family is PARP-1. It was shown that PARP-1 is responsible for the 90% of PARylation activity due to DNA damage in mouse embryonic and 3T3 cells (23). The enzyme is located in the nucleus of eukaryotic cells with the exemption of yeast and neutrophil granulocytes. The 113-kDa protein consists of four main domains:

domain A: the N-terminal DNA-binding domain containing two zinc fingers, that is responsible for the interaction with DNA breaks

domain B: contains a nuclear homing signal (NLS) and a caspase-3 cleavage site

domain D: automodification domain, containing BRCT motif (major protein interface to various nuclear partners)

domain F: terminal catalytic domain (3).

Its catalytic activity is stimulated more than 500-fold on binding to DNA breaks. The resulting PAR production has three major roles: 1: PARylation of histone H1 and H2B contributes to the relaxation of chromatin and increases the access to breaks. 2: indication of the occurrence and severity of DNA-damage (consequent DNA-repair or cell death), 3: recruitment of single-strand break repair (SSBR)/ base-excision repair (BER) factors to the damaged site (1).

Concerning the consequences of PARP-1 activity, cells exposed to DNA-damaging agents can enter three pathways based on the intensity of stimuli. In case of mild DNA-damage, PARP-1 activation results in relaxation of chromatin structure, recruitment of repair enzymes and consequent DNA-repair (1). More severe DNA-damage induces apoptotic cell death, during which caspases inactivate PARP-1 by cleaving it into two fragments (24). Blocking PARP-1 is essential for apoptosis. It prevents PARP-1 activation by DNA-fragmentation, thereby preserves cellular energy for ATP (adenosine triphosphate)-requiring steps of apoptosis (25). It is also necessary for the release of the human Ca^{2+} and Mg^{2+} dependent endonuclease (DNAS1L3) from PARylation mediated inhibition (26). Extensive DNA-breakage usually triggered by a massive oxidative or nitrosative stress leads to the overactivation of PARP-1 that results in the rapid depletion of cellular NAD^{+} and consequently APT, leading to energy depletion and necrotic cell death (27).

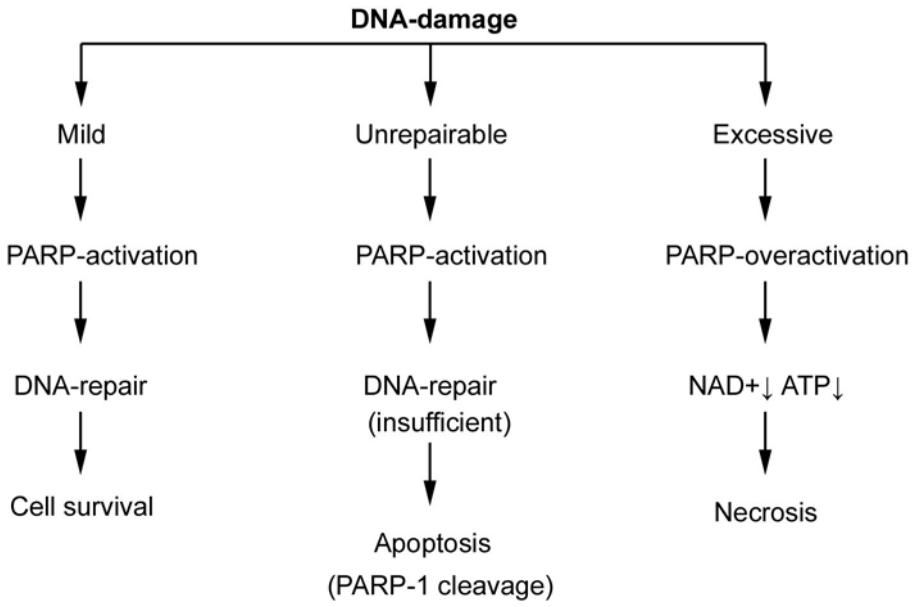


Figure 2: Three pathways of PARP-1 activation according to the severity of DNA-damage

PARP-1 also plays a central role in the caspase-independent apoptosis mediated by apoptosis inducing factor (AIF) (28). PARP activation, more precisely PAR polymers are necessary for the translocation of AIF from the mitochondria to the nucleus (29).

PARP-1 also regulates the expression of various proteins at the transcriptional level. Generally PARP-1 has stimulatory effects on transcriptional factors and cofactors (e.g. PAX6, activator protein-2 (AP-2), b-Myb, transcriptional enhancer factor-1 (TEF-1)) (30, 31). PARP-1 interacts with and stimulates NF-κB (nuclear factor- κB), the transcriptional factor that regulates immune and inflammatory-response genes (such as macrophage inflammatory protein-2 (MIP-2), inducible nitric oxide synthase (iNOS)). These processes do not necessarily require PAR formation (32-34).

1.2.1. Regulation of PARP-1 activity

Originally PARP was thought to be primary regulated by the recognition of damaged DNA. The triggers of DNA breakage such as ionizing radiation, genotoxic

compounds, endogenous oxidants and free radicals are ultimate activators of PARP (35).

Conditions, which can produce intracellular oxidants and free radicals, are also initiators of PARP activation. For example hypoxia-reoxygenation was shown to induce PARP activation among others in cardiac myoblast cells (36). The PARP activating effect of elevated glucose concentration was described in murine and human endothelial cells (37, 38). In mouse fibroblast cells the role of intracellular Ca^{2+} was also established (39). PARP activation in angiotensin II-mediated endothelial dysfunction was assessed in mouse and rat endothelial cells (40).

1.2.1.1. Endogenous regulators

Auto-PARylation of PARP-1 represents a major regulatory mechanism. While auto-PARylation is necessary for the recruitment of DNA-repair enzymes (41), by electrostatic repulsion it inhibits DNA-binding (42) and down-regulates enzyme activity (27). It has been shown that Mg^{2+}, Ca^{2+}, polyamines, ATP and histones H1 and H3 are allosteric regulators of auto-PARylation (43).

The activity of PARP-1 can be also modified by phosphorylation. In case of DNA-damage phosphorylation by the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase2 (ERK2) seems to be necessary for maximal PARP activation (44). Calmodulin-dependent protein kinase (CaMK)IIδ also activates PARP-1 by phosphorylation.

The NAD^+ derivate nicotinamide, which is the product of PARP, has inhibitory effect on the enzyme (27).

Thyroid hormones were shown to have PARP-1 regulatory effect; administration of L-triiodothyronine inhibits PARP-1 activity in cardiomyocytes (45). It was also demonstrated that hepatic PARP-activity in rats is controlled by thyroid hormones (46).

The active form of vitamin D3, 1,25-dihydroxyvitamin D3 was shown to inhibit PARP-1 activation in cell-free PARP assay, in RAW 264.7 macrophage cells and in human keratinocytes (HaCaT) (47). As evidenced in cell-free PARP assay and in macrophage cells ATP and certain purines, including hypoxantine and inosine, are also endogenous inhibitors of PARP-1 (48).

1.2.1.2. Exogenous regulators

Xanthine derivates, such as certain caffeine metabolites (1-methylxanthine, 1,7-dimetilxanthine) are even more potent PARP-1 inhibitors than the endogenous hypoxanthine (49). Theophylline also has PARP inhibitory effect in human pulmonary epithelial cells (50).

Recent studies demonstrated that common antibiotics of tetracycline class, such as doxycycline and minocycline exert PARP inhibitory effect. The mode of this inhibition is competitive and occurs by preventing NAD^+ from binding to the active center of the enzyme (51).

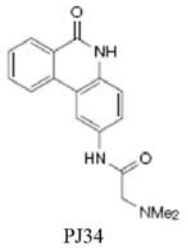
1.2.1.3. Pharmacological inhibition

The classic pharmacological inhibitors of PARP are nicotinamide and 3-aminobenzamide (3-AB). These compounds have low inhibitory potential, limited cell uptake and cellular residence time, and exert non-specific effects (27).

The better understanding of the molecular details of the active site of the enzyme led to the discovery of more potent PARP inhibitors. The majority of PARP inhibitors work similarly to benzamide pharmacophore, which mimics the nicotinamide moiety of NAD^+ and binds to the donor site of the protein (8).

One group of inhibitors is derived from the classical PARP scaffolds (benzamide or cyclic lactams). Cyclizing an open benzamide structure or creating a further ring system on the existing cyclic amide (lactam) may lead to more powerful PARP inhibition. The highly potent tricyclic PARP-1 inhibitor INO-1001 and also 5-amino-isoquinolinone (5-AIQ) and 1,5-dihydroxy-isoquinoline (ISO) are examples for this structure (8).

An alternative way to inhibit PARP-1 activation is using nucleic acid and nucleoside derivates. PJ34 is one member of this family (Figure 3.) (8).



PJ34

Figure 3: Structure of PJ34

1.3. Catabolism of PAR

PARylation is a dynamic process, which is indicated by the short half-life of the polymer. Two enzymes are involved in the catabolism of PAR: poly (ADP-ribose) glycohydrolase (PARG), which cleaves the ribose-ribose binds, and ADP-ribosyl protein lyase, which removes the proximal ADP-ribose monomer from the protein (52).

While there are 18 genes encoding PARP isoforms, only one gene is known to encode PARG proteins. These proteins are located in various cellular compartments. Beside the predominant isoforms - the very active nuclear PARG protein (110 kDa), and the short mitochondrial isoform (65 kDa) – several splicing variants have been described (1).

As there are no specific and selective inhibitors of PARG, PAR catabolism can be investigated by gene-disruption strategies.

Targeted deletion of exon 4 leads to the complete depletion of all PARG isoforms. Homozygous mice die at embryonic day 3.5 (53).

Mice that are homozygous for targeted deletion of exon 2 and 3 are viable and show normal phenotype and normal PAR metabolism. This deletion results in the depletion of the full length (110 kDa) PARG, but in a 3.3 fold accumulation of the 65 kDa mitochondrial version, which is probably due to an alternative translational site at exon 4 (1, 54).

1.4. Inflammation and PARP

Inflammation is characterized by a complex series of events, including leukocyte migration and proinflammatory cytokine production. Proinflammatory cytokines trigger free-radical formation by a variety of mechanisms, such as stimulation of xanthine oxidase and recruitment of neutrophils that express NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. The activation and increased expression of iNOS causes augmented NO (nitric oxide) synthesis. As a consequence, the very potent oxidant peroxynitrite is formed from the reaction of superoxide and NO. Oxidative/nitrosative stress generates DNA single strand breakage that activates PARP-1, which in turn potentiates NFκB activation and AP-1 expression, resulting in the greater expression of AP-1- and NFκB-dependent genes, such as iNOS, ICAM-1 (intracellular adhesion molecule-1), TNF-α (tumor necrosis factor- α), MIP1-α, and C3

(caspase3). The increased endothelial expression of ICAM-1 recruits a greater number of activated leukocytes that produce even more oxidative stress. PARP activation can also accelerate this positive feedback cycle by preventing the energy dependent reduction of oxidized glutathione (27).

PARP activation may also lead to caspase independent apoptosis by the translocation of AIF from the mitochondria to the nucleus (29).

However PARP-mediated processes can be beneficial by helping to eliminate parenchymal cells having severely damaged DNA caused by the nearby occurring neutrophil attack and by recruiting mononuclear cells to the inflammatory site, the above mentioned positive feedback cycles can amplify themselves beyond the desired or controllable level (Figure 4.) (27).

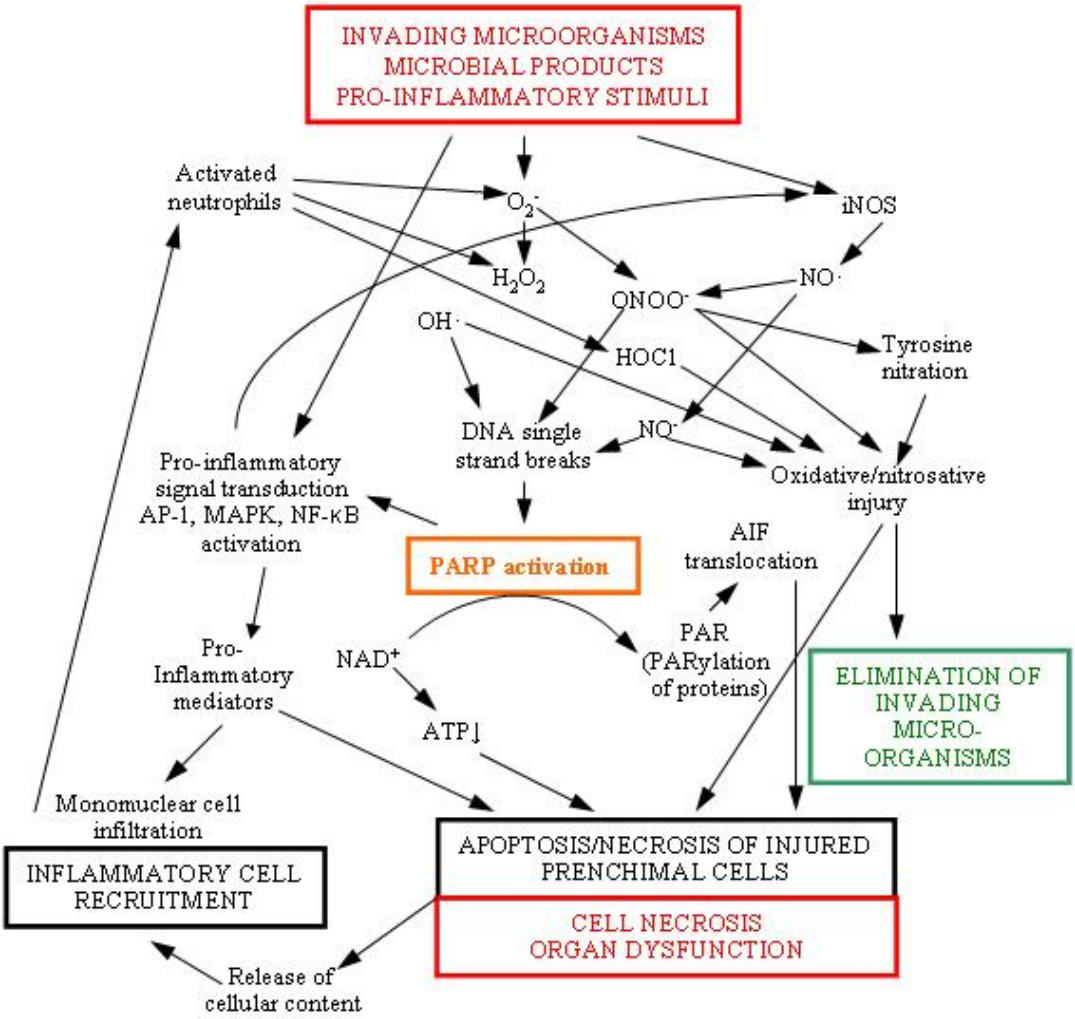


Figure 4: Proposed physiological/pathological role of PARP-1 activation during the inflammatory process.

The beneficial effects of PARP inhibition or deficiency have been demonstrated in various models of inflammation. The main effects of PARP inhibition or deficiency in animal models include: a) increased survival, b) reduced organ inflammation, c) improvement of organ function, d) reduced inflammatory mediator expression; e) attenuation of neutrophil recruitment. (Table 3.).

Table 3. Effect of PARP inhibition or deficiency in various models of inflammation

Model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	Ref.
Mouse	High-dose LPS (20mg/kg)	PARP -/-	Increased survival (100% vs. 40% in wild type animals) Reduced necrotic liver damage	(55)
	High-dose LPS (40mg/kg)	PARP -/-	Increased survival (90% vs. 10% in wild type animals) Reduced TNF-α and IFN-γ production	(56)
	LPS	PJ34	Reduced liver and kidney dysfunction Reduced TNF-α and IL-1β production	(57)
	LPS	PARP -/-	Reduced leukocyte recruitment within pulmonary arterioles, capillaries, and venules Reduction of pulmonary capillary leakage and oedema formation Reduced expression of ICAM-1	(58)
	Cecal ligation and puncture (CLP)	PARP -/-	Reduced TNF-α and IL-6, IL-10 production Reduced degree of organ inflammation, indicated by decreased myeloperoxidase (MPO) activity in the gut and lung Increased survival	(59)
	Mild form of CLP (93% survival in vehicle treated animals)	3-AB	No effect on the biological signs of sepsis-induced organ dysfunction or tissue leukocyte infiltration	(60)

Model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	Ref.
Mouse	Lung injury induced by intra-thoracal administration of zymosan activated plasma	5-amino-isoquinolinone (5-AIQ)	Reduced expression of P-selectin and ICAM-1 Reduced neutrophil recruitment	(61)
	Zymosan peritonitis/multi-organ failure	3-AB PARP-/-	Reduced migration of polymorpho-nuclear cells Reduced exudates volumes Reduced MPO activity	(62)
	Acute pancreatitis caused by cerulein	3-AB	Reduced degree of pancreatic inflammation and tissue injury Reduced upregulation/formation of ICAM-1 and P-selectin Reduced neutrophil infiltration Reduced TGF (transforming growth factor) beta and VEGF (vascular endothelial growth factor) expression	(63)
Rat	*Esherichia coli* Endoxotin (LPS)	Nicotinamide	Prevented the decrease in mitochondrial respiration and intracellular NAD^+ and ATP levels in peritoneal macrophages. Improved the contractility of the thoracic aorta *ex vivo*.	(64, 65)
	LPS	3-AB Nicotinamide	No effect on liver injury	(66)
		1,5-dihydroxy-isoquinoline (ISO)	Small protective effect in liver injury (possibly due to the antioxidant effect of its vehicle dimethyl sulphoxide)	
		PJ34	Increased survival Improved the contractility and endothelium dependent relaxation of the thoracic aorta *ex vivo*.	(57)
Rat	Carrageenan-induced pleurisy	3-AB	Reduced pleural exudation Reduced migration of polymorpho-nuclear cells Reduced MPO activity and NT staining of the lung	(67)

Model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	Ref.
Rat	Zymosane	3-AB	Reduced peroxynitrite formation Improved the contractility and endothelium dependent relaxation of the thoracic aorta *ex vivo*	(68)
Rabbit	LPS	3-AB	Reduced leukocyte recruitment in the within pulmonary arterioles, capillaries, and venules Reduction of pulmonary capillary leakage and oedema formation Reduced expression of ICAM-1	(58)
Pig	LPS	(3-AB)	Eliminated the LPS-induced increase in pulmonary and total respiratory resistance.	(69)
	Intraperitoneal implantation of Escherichia coli clots	PJ34	Increased survival (12% vs. 83% in wild type animals at 24hrs) Attenuated the increase in pulmonary and total vascular resistance. Reduced TNF-α production	(70)
Sheep	Burn/smoke inhalation injury	INO-1001	Reduced lung edema formation Reduced deterioration of gas exchange Reduced airway blood flow Reduced airway pressure Reduced lung histological injury Reduced systemic vascular leakage. Reduced MPO activity in the lung	(71)
	Smoke inhalation and *Pseudomonas aeruginosa* instillation	INO-1001	Reduced peak airway pressure Reduced pulmonary shunt fraction Reduced pulmonary histological injury	(72)

On the other hand at a higher concentration hydrogen peroxide, peroxynitrite and hypochlorous acid actually inactivates PARP (27, 73). This means that in case of extreme oxidative/nitrosative stress PARP-independent pathways of cell injury take over, and the effects of both peroxynitrite and hydrogen peroxide is no longer completely inhibitable with PARP inhibitors or deficiency (74, 75).

1.4. Myocardial ischemia-reperfusion injury and PARP

Ischemia-reperfusion injury is caused by the transient disruption of normal blood supply followed by reperfusion. In case of the heart various conditions such as myocardial infarction, cardiopulmonary bypass, aortic reconstructive surgeries, and circulatory shock lead to ischemia-reperfusion injury. Although in these conditions the therapy for the ischemic episode is providing reperfusion, reperfusion itself leads to additional tissue damage mediated by a multitude of factors including reactive oxygen and nitrogen species and pro-inflammatory signal transduction activation (76). These processes lead to the so-called "respiratory burst": recruitment and activation of polymorphonuclear leukocytes and consequent amplification of the initial inflammatory response and ROS generation (77).

During ischemia the reduction of oxygen supply alters mitochondrial functions that lead to reactive oxygen species production. The reaction between superoxide and NO (derived from the increased endothelial NOS and iNOS activity) results in the formation of the very potent oxidant peroxynitrite. Hydroxyl radical and peroxynitrite causes DNA-damage, which in turn activates PARP. PARP activation leads to the rapid depletion of cellular NAD^+ and ATP pools, leading to energy exhaustion and cellular dysfunction. This maintains mitochondria in reduced state allowing further ROS production at reperfusion. Depletion of NADPH leads to reduced endothelial NO production. Cellular dysfunction is further enhanced by the pro-inflammatory signal transduction activation by PARP. Oxidant-induced pro-inflammatory mediator expression and endothelial dysfunction induce neutrophil recruitment and activation, which initiates a positive feedback cycle of ROS generation, PARP activation and cellular injury (78). (Figure 5.)

In experimental models of myocardial infarction and cardiopulmonary bypass, there is a marked PARP activation in the reperfused myocardium, which parallels with the contractile dysfunction and the depletion of cellular NAD^+ and ATP pools (76).

The beneficial effects of PARP inhibitors, as evidenced by cell culture studies, perfused heart studies, rodent and large animal studies of myocardial infarction and cardiopulmonary bypass include: a) reduction in myocyte necrosis; b) improvement of cardiac contractility; c) improvement in myocardial intracellular energy charge; d) reduction in inflammatory mediator expression; and e) attenuation of neutrophil

infiltration into the reperfused myocardium (Table 4). Recent work has also demonstrated that local insults, such as myocardial infarction can lead to a systemic activation of PARP, as evidenced in studies of circulating leukocytes in rats subjected to acute myocardial infarction (79).

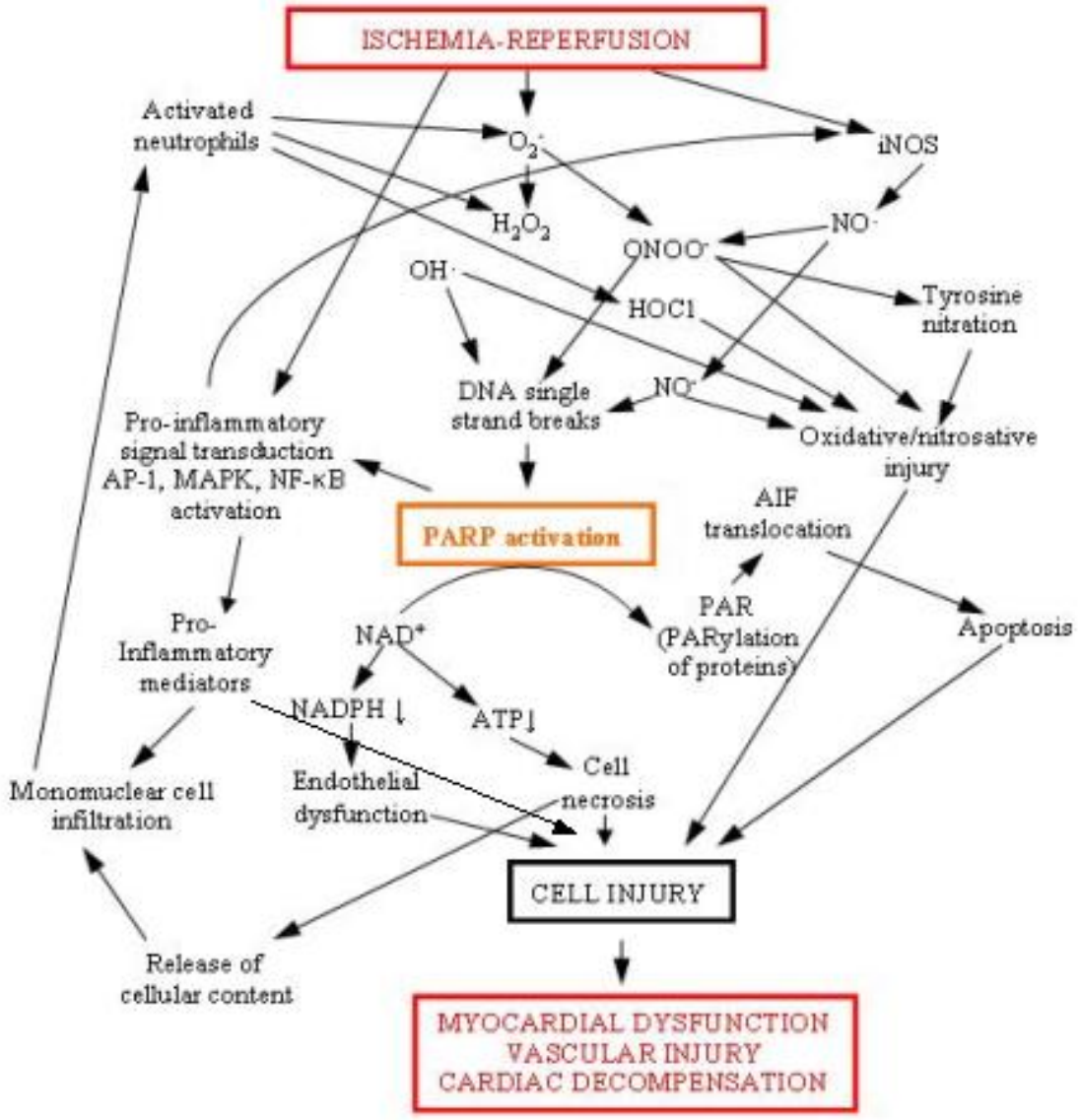


Figure 5: Proposed role of PARP-1 activation during myocardial ischemia-reperfusion injury.

Table 4: Protective effects of PARP inhibitors in experimental models of acute myocardial infarction and cardiopulmonary bypass in preclinical models.

Experimental model	Effect of PARP inhibition	Reference
Rat and human cardiac myoblasts	PARP inhibitors (3-aminobenzamide, nicotinamide) reduce myocardial necrosis, and improve mitochondrial respiration	(80-84)
Global ischemia-reperfusion in isolated mouse heart	In $PARP^{-/-}$ phenotype reduction of NAD^{+} consumption and left ventricular (LV) dysfunction is observable.	(85-87)
Regional or global ischemia- reperfusion in isolated rat and rabbit heart	PARP inhibitors (3-aminobenzamide, nicotinamide, BGP-15, 1,5 dihydroxyisoquinoline) reduce NAD^{+} and ATP catabolism, creatine phosphokinase (CPK) and glucotamic-oxaloacetic transaminase (GOT) LV dysfunction, myocardial damage and infarct size.	(83, 88-92)
Regional ischemia-reperfusion injury in mouse	$PARP^{-/-}$ phenotype is resulted in decrease of infarct size, neutrophil infiltration, circulating IL-10, TNF-α and nitrate, in reduction of P-selectin and ICAM-1 expression.	(93-95)
Regional ischemia-reperfusion injury in rat	PARP inhibitors (3-aminobenzamide, GP16150) decrease infarct size, reduce LV dysfunction, neutrophil infiltration and preserve the myocardial ATP stores. In diabetic rats inhibition of PARP (INO-1001) provides a similar degree of myocardial protective effect as in non-diabetic animals, prevent apoptosis inducing factor translocation, reduces infarct size, and improves myocardial contractility.	(85, 96, 97) (98)
Regional ischemia-reperfusion injury in rabbit	Pharmacological inhibition of PARP (3-aminobenzamide, nicotinamide, 1,5 dihydr-oxyisoquinoline) decreases infarct size.	(91)
Regional ischemia-reperfusion injury in pig	PARP inhibitors reduce infarct size and improve left ventricular function.	(81, 99)
Ischemic preconditioning (IPC) in mouse	In $PARP^{-/-}$ phenotype the protective effect of ischemic preconditioning is absent.	(100)
Ischemic preconditioning in rat	PARP inhibitors suppress the effect of preconditioning.	(100)
Cardiopulmonary bypass, hypothermic cardiac arrest in dog	PARP inhibitors (PJ-43, INO-1001) improve recovery of left and right ventricular function and endothelial function, improve coronary blood flow and pulmonary function.	(101-104)

Experimental model	Effect of PARP inhibition	Reference
Heterotopic rat heart transplantation	PARP inhibitors (PJ34, 5-aminoisoquinoline) improve the myocardial contractility and relaxation, coronary blood flow and endothelial function of the graft.	(105)
Murine chronic heart failure as a consequence of permanent coronary artery ligation.	$PARP^{-/-}$ phenotype and PARP inhibition prevent cardiac remodelling.	(95, 106-108)

2. Aims/hypothesis

2.1. Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of PARP activation

However PARP was originally thought to be primary regulated by the recognition of damaged DNA, recent studies identified several endogenous modulators such as thyroid hormones (45, 46), 1,25-dihydroxyvitamin D3 (47), ATP and certain purines, including hypoxantine and inosine (48).

Recent studies have confirmed the earlier observations that PARP inhibition or PARP deficiency is protective in stroke. However it was found that PARP inhibition and PARP-1 deficiency confer their protection only in male mice, it has no benefit or it has even deleterious effects in female mice (109-111).

It is also known that female gender has protective effect in sepsis. In a CLP induced sepsis model it was shown that female gender and intramuscular estrogen + progesterone administration to male rats reduced the inflammation induced damage of the lung and the liver (112). In another set of experiments female gender resulted in decreased mortality, TNF-α and IL-6 production in case of hemorrhage followed by CLP (113).

Our aim was to test whether gender difference exists in the endotoxin induced inflammatory and vascular response and to test, whether PARP inhibitors gender-preferentially modulate this response.

2.2. Treatment with insulin inhibits PARP activation in a rat model of endotoxemia

Many conditions of critically ill patients are associated with hyperglycemia. Insulin therapy, in order to maintain tight glycemic control, has been demonstrated to provide significant benefit: in a prospective, randomized, controlled study involving adults admitted to surgical intensive care units and receiving mechanical ventilation, intensive insulin therapy substantially reduced mortality and morbidity. Intensive insulin treatment reduced the number of deaths from multiple organ failure with sepsis. Markers of inflammation were found to be less frequently abnormal in the intensive insulin treatment group than in the conventional treatment group (114, 115). Although a

number of outstanding issues remain to be addressed with this concept, and the degree of benefit that normalization of circulating glucose offers may be different in different groups of patients (116-118), intensive glucose control emerges as a potential therapeutic tool for critically ill patients.

In a rat model of endotoxemia it was shown that insulin therapy attenuated systemic inflammatory response; reduced TNF-α production (119).

The aim of the current study was to test whether there is a link between hyperglycemia and PARP activation in endotoxin shock. We have tested, therefore, in a rat model of endotoxemia, whether insulin therapy affects PARP activation and TNF-α production. In addition, the potential direct effect of insulin on the PARP activation of mononuclear cells and human umbilical vein endothelial cells (HUVEC) was also tested *in vitro*.

2.3. Activation of PARP by myocardial ischemia and coronary reperfusion in human circulating leukocytes

Recently it was reported in a rat model that free radical–mediated activation of PARP is not limited to the myocardium: in response to myocardial ischemia, in circulating leukocytes—similarly to myocytes—significant PARP activation occurs after reperfusion. This phenomenon—which is most likely caused by free radicals as the circulating cells pass through the reperfused myocardial tissue—has been proposed to serve as a potential marker of myocardial oxidative and peroxidative injury (120).

While the above experimental data demonstrated the role of PARP in myocardial reperfusion injury in animal models, the presence of PARP activation in patients with myocardial infarction has not yet been confirmed.

The main objective of the study was to examine PARP activity changes in circulating peripheral leukocytes isolated from cardiovascular patients with STEMI (ST-elevation myocardial infarction) followed by primary PCI (percutaneous coronary intervention). Tyrosine nitration (a marker of nitrosative stress) and nuclear translocation of AIF (a downstream event of PARP activation) was also studied in circulating leukocytes. We also examined the presence of direct DNA-damage, indicated by increased levels of serum 8OHdG (8-hydroxy-2′-deoxyguanosine) and the

degree of systemic oxidative stress estimated by the measurement of plasma hydrogenperoxide level.

2.4. PARP activation in circulating leukocytes

Pharmacological inhibitors of PARP move toward clinical testing for a variety of indications, including cardioprotection, malignant tumors. Some of the compounds are already in clinical trials. (Table 5.)

Table 5: Developmental phase of some PARP inhibitors (121)

Company	Product	Status	Indication
Inotek Pharmaceuticals (Beverly, Massachusetts)	INO-1001	Phase 2	Cardiovascular indications
Pfizer (New York)	AG014699	Phase 2	Malignant melanoma
Inotek/Genentech (S. San Francisco)	INO-1001	Phase 1b	Malignant melanoma
BiPar Sciences (Brisbane, California)	BS-201	Phase 1	Cancer
AstraZeneca (London)	AZD2281	Phase 1	Breast cancer
BiPar Sciences	BS-401	Preclinical	Pancreatic cancer
MGI Pharma (Bloomington, Minnesota)	n/a	Preclinical	Radiation / chemotherapy sensitizer

During the development of these compounds, especially in clinical trials a sentinel test - that besides reflecting the degree of PARP activation and the efficiency of PARP inhibition requires easily accessible material for analysis – is of utmost importance.

Our aim was to test also in animal models and in a human study weather measuring the PARP activity in circulating leukocytes is suitable for this role.

3. Materials and Methods

All procedures were approved by institutional and regional ethics review committee. In case of human study written informed consent was obtained from all participating patients before enrollment.

3.1. Study protocols

3.1.1. Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of PARP activation

3.1.1.1. Mouse model

To induce systemic inflammatory mediator production without mortality, *Escherichia coli* endotoxin (lipopolysaccharide (LPS)) was injected into 5 groups of animals intraperitoneally (i.p.) at a dose of 1 mg/kg, followed by the measurement of TNF-α at 90 min. To inhibit the catalytic activity of PARP *in vivo*, the phenanthridinone-based PARP inhibitor PJ34 at 10 mg/kg was given i.p. as a 30-min pretreatment prior to the injection of LPS. In another set of experiments (in female mice), the dose of LPS was increased to 30 mg/kg to induce a more robust TNF-α production (to make it comparable with the TNF-α response seen in male animals). (Figure 6.)

3.1.1.2. Rat model

Male or female Wistar rats (350-400 g) - either pretreated with 30mg/kg intravenous (i.v.). PJ34 (Inotek Pharmaceutical Co. Beverly, MA, USA) or its vehicle, saline for 30 min - were given LPS injection (10 mg/kg i.v., Sigma/Aldrich St. Louis, MO, USA). Thoracic aortae were obtained 3 h later, and endothelium-dependent relaxant responses to acetylcholine were recorded in isolated thoracic aortic rings. Poly (ADP-ribose) polymerase activation in circulating leukocytes was measured by flow cytometric method. (Figure 7.)

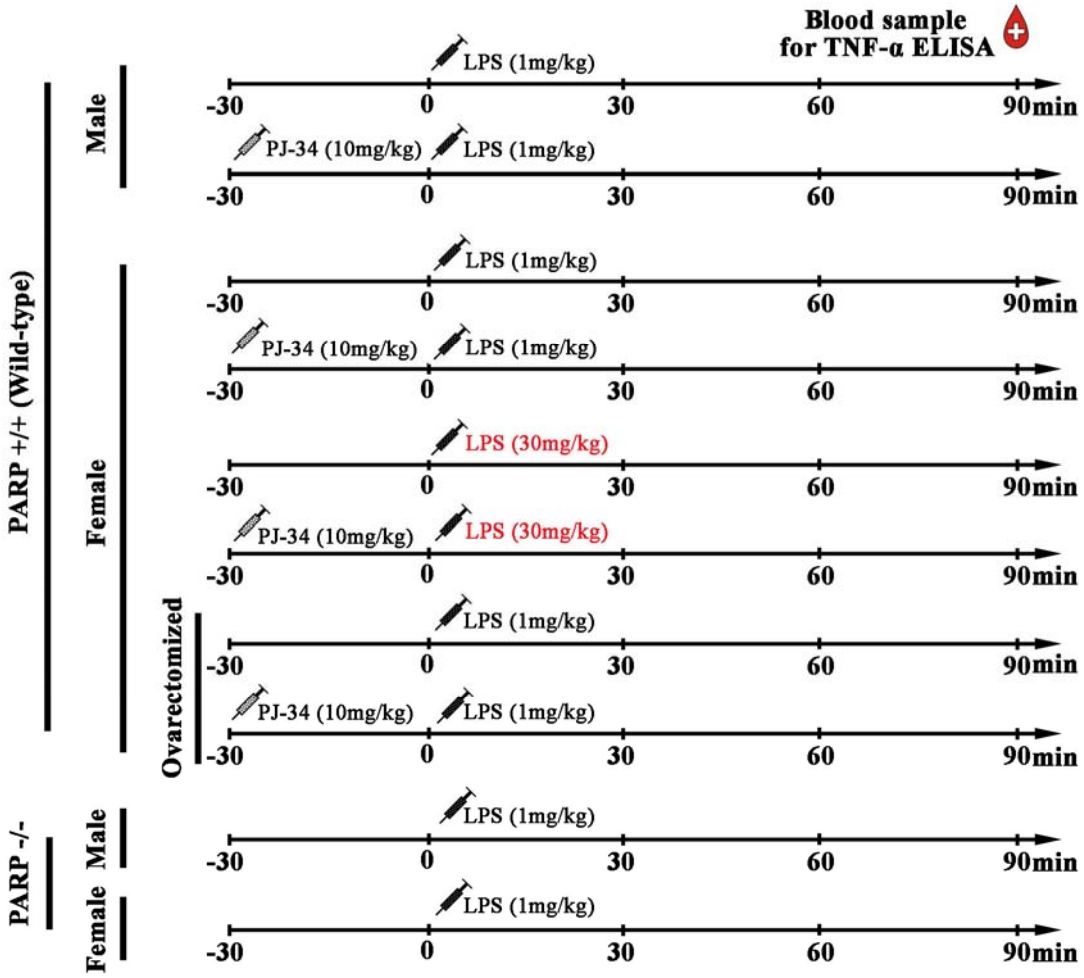


Figure 6: Time course of mouse model

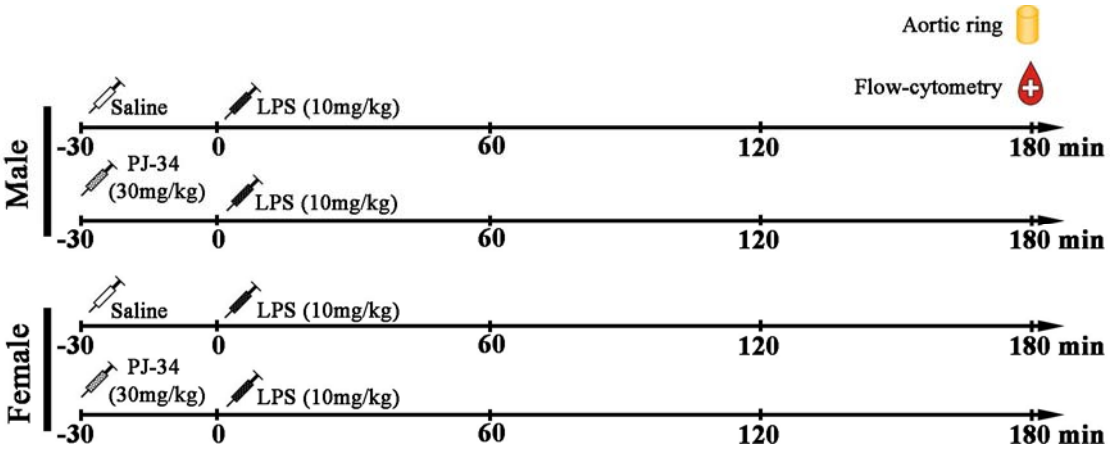


Figure 7: Time course of rat model

3.1.2. Treatment with insulin inhibits PARP activation in a rat model of endotoxemia

3.1.2.1. Rat model

Age-matched male Wistar rats (n=16, weighing 350–400 g) were treated with a single dose of 15 mg/kg LPS (Sigma/Aldrich), followed by treatment with vehicle (n=8) or 5 units of rapid insulin subcutaneously (Novorapid, Novo Nordisk) (n=8). The control group (n=8) remained untreated. Blood glucose levels of the animals were periodically monitored (Accu-Check). Sample collection started immediately before LPS administration and was repeated every 10 min in the first hour, every 30 min in the second hour and at 3 h. At 3 h, animals were anesthetized with N_2O–Narcothan mixture (Sigma/Aldrich). Peripheral blood leukocytes were isolated for the measurement of PARP activation, and serum was collected for TNF-α measurement. (Figure 8.)

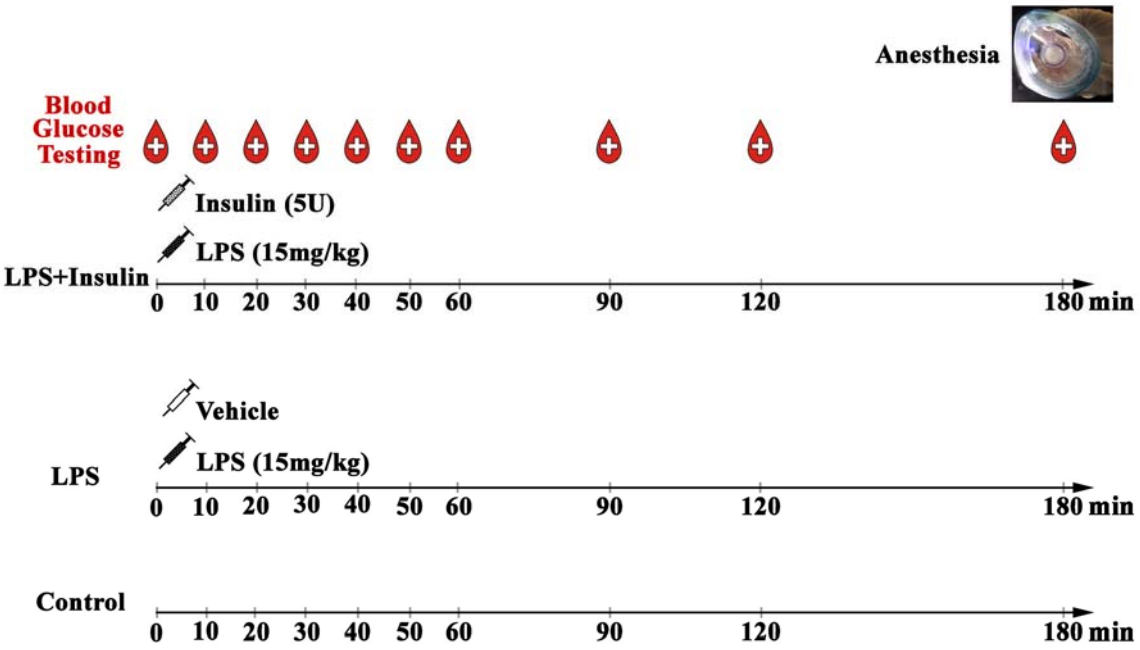


Figure 8: Time course of endotoxemic rat model

3.1.2.2. *In vitro* experiments

Cell culture experiments were conducted to test the *in vitro* effect of insulin treatment on PARP activation. Whole blood collected from male Wistar rats (300–350 g) was diluted 1:10 in RPMI medium containing 30 mM glucose with or without insulin (10 nM, Sigma/Aldrich) and incubated for 24 h in 37 °C (5% CO2). Mononuclear cells were isolated using Histopaque-1083.

Human umbilical endothelial cells (HUVEC, Cambrex, East Rutherford, NJ) were cultured in Endothelial Basal Medium containing 2% FBS, recombinant human EGF 0.1%, bovine brain extract 0.4%, gentamycin 0.1%, hydrocortisone 0.1% and 30 mM glucose with or without insulin (10 nM) for 24 h.

3.3. Activation of PARP by myocardial ischemia and coronary reperfusion in human circulating leukocytes

15 cardiovascular patients were enrolled with acute ST-segment elevation myocardial infarction referred to the National Health Center, Budapest for primary percutaneous coronary intervention between October 2004 and February 2005. Age-matched patients with stable angina pectoris undergoing elective coronary angiography (n = 6) and elective percutaneous intervention (n = 9) were tested in parallel as negative controls.

Resting venous blood was taken into native and heparin- and EDTA (ethylenediaminetetraacetic acid)-containing tubes from STEMI and elective PCI patients at 4 different time points: (1) before the angiography, (2) within 15 min after opening of the infarct-related or target coronary artery, (3) 24 ± 4 h after the PCI, and (4) 96 ± 4 h after the PCI. Peripheral leukocytes were prepared. After isolation 100-µl aliquot of mononuclear cells was used to prepare peripheral smears for immunohistochemistry. Remaining leukocytes were pelleted and kept at –80 °C until Western blot analysis. Plasma hydrogen peroxide and serum 8OHdG (8-hydroxy-2′-deoxyguanosine) concentration was also measured.

3.4. Measurement of vascular reactivity on isolated aortic rings of rats

The method to determine endothelium-dependent vascular relaxation in thoracic aortic rings from rats was described previously in Pacher et al., 2002.(122). Briefly, the thoracic aorta was cleared from periadventitial fat and cut into 3-4 mm width rings, mounted in organ baths filled with warmed (37 °C) and oxygenated (95% O2, 5% CO2) Krebs' solution ($CaCl_2$ 1.6 mM; $MgSO_4$ 1.17 mM; NaCl 130 mM; $NaHCO_3$ 14.9 mM; KCl 4.7 mM; KH_2PO_4 1.18 mM; Glucose 11 mM). Isometric tension was measured with isometric transducers (Kent Scientific), digitized, and stored and displayed on an Apple computer. A tension of 1.5gram was applied and the rings were equilibrated for 60 minutes, followed by precontraction with epinephrine (10^{-7} M) and concentration-dependent relaxation to acetylcholine (10^{-9} to 3 * 10^{-4} M). Experiments were conducted in 5–6 pairs of rings in each experimental group.

3.5. Immunohistochemistry

Leukocytes were isolated from blood samples using Histopaque-1083 in case of animal studies and Histopaque-1077 in case of human subjects (Sigma/Aldrich) according to the users' manual. From the cell suspension methanol fixed smears were made. Anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) (1:80, 4°C, overnight) was used to stain 3-nitro-tyrosine, the marker of tyrosine nitration. Poly (ADP-ribose) detection was performed using the mouse monoclonal anti-PAR antibody (Calbiochem, San Diego, CA USA/Tulip Biolabs, West Point, PA, USA) (1:1000/1:100, 4°C, overnight) after antigen retrieval (0.1M citrate buffer, pH 3 , cooked in microwave oven for 15 min). Anti-AIF rabbit polyclonal antibody (Chemicon International, Temecula, CA, USA) (1:100, 4°C, overnight) was used to label AIF. A specific labeling was avoided by incubating the smears in 15% normal goat/horse serum for 1 h at room temperature.

Secondary labeling was achieved using biotinylated anti-mouse immunoglobulin horse or anti-rabbit immunoglobulin goat antibody (Vector Laboratories, Burlingame, CA, USA) (30 min, room temperature). Horseradish peroxidase–conjugated avidin (30 min, room temperature) and nickel-enhanced diaminobenzidine (6 min, room temperature, brown/black color) was used to visualize the labeling (Vector

Laboratories). Smears were counter-stained with nuclear fast red (NFR) or hematoxiline.

To determine the proportion of NT- and AIF-positive cells at least 300 cells were counted in each smear and ratio was given by the percentage of all cells. To evaluate PAR-stained slides, semi-quantitative PAR-positivity score was established from 1 to 10. (Table 6.) All these procedures were done by a blinded investigator.

Table 6: PAR-positivity score

1	no staining
2	light cytoplasmic staining
3	strong cytoplasmic staining
4	cytoplasmic staining with a few positive nuclei
5	approximately 50% of the nuclei positive
6	approximately 75% of the nuclei positive
7	general nuclear staining with a few negative cells
8	all nuclei positive
9	strong nuclear staining in all cells
10	very strong general nuclear staining in all cells

3.6. Flow-cytometry

PARP activation in circulating leukocytes was measured by a flow cytometric method based on the immunohistochemical detection of the product of the enzyme, poly (ADP-ribose) (PAR). Circulating leukocytes were isolated from whole blood using Histopaque-1083 in case of animal studies and Histopaque-1077 in case of human subjects according to the users manual (Sigma/Aldrich). After the fixation and permeabilization of the cells with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Becton-Dickinson), monoclonal mouse anti-PAR antibody was used as primary antibody to stain intracellular PAR (Tulip Biolabs, West Point, PA). All procedures were performed in Cytoperm solution after the fixation of the cells. We used purified mouse IgG3κ isotype control (anti-KLH) antibody (Beckton Dickinson). As secondary antibody FITC-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody was used (multiple adsorption) (Becton Dickinson). Flow cytometric

measurements were performed on single cell suspension of rat leukocytes using FACSCalibur (Becton Dickinson, San Jose, CA). Region 1 (R1) was defined to contain cells having typical Forward Scatter and Side Scatter properties of lymphocytes. Isotype control stained cells served as negative control for each sample. Fluorescence data were collected using logarithmic amplification until we reached 10,000 counts of R1 cells. On the PAR histograms the gate was R1. (Figure 9.) The histograms were further analyzed by CellQuest Pro software, mean fluorescence intensity was calculated.

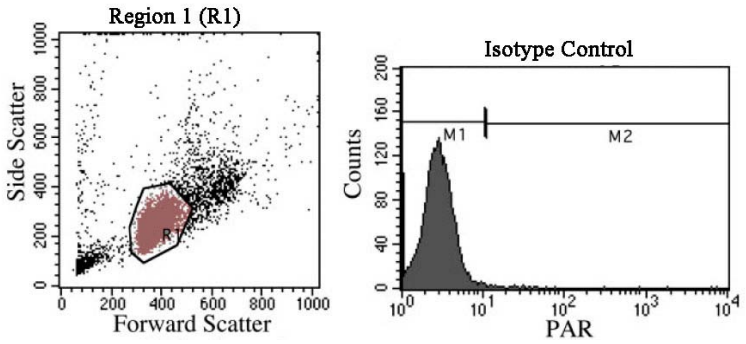


Figure 9: *Region 1 (R1) contains cells having typical Forward Scatter and Side Scatter properties of lymphocytes. FACSCalibur was calibrated using isotype control stained cells; the fluorescence of these cells were set to be in the negative M1 region.*

3.7. Western-blot

HUVECs and mononuclear cells were lysated in hot lysis buffer (1% TRIS, 1% SDS). The lysates were centrifuged and the supernatant were used for western blot analysis. 10 μg protein was loaded in each well of 4–12% Bis–Tris Gel (Invitrogen, Carlsbad, CA). After electrophoresis proteins were transferred to nitrocellulose membrane (Invitrogen). Unspecific labeling was prevented by incubating the membranes in 10% milk (1 h, room temperature). Anti-PAR monoclonal mouse antibody was used as primary antibody (1:8000, 3 h, room temperature, Calbiochem), secondary antibody was goat horseradish peroxidase linked anti-mouse IgG antibody (1:5000, 1 h, room temperature, Cell Signaling, Danvers, MA). The blots were then developed using chemiluminescence method (Amersham, Arlington Heights, IL).

3.8. Biochemical analysis

3.8.1. TNF-α

Serum TNF-α levels were measured using sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay) method (R&D Systems).

3.8.2. Plasma hydrogen peroxide

Using the OxyStat assay (Biomedica Gruppe, Wien, Austria), total hydrogen peroxide concentration was determined in patient EDTA plasma samples (detection limit 7 μmol/L).

3.8.3. Serum 8OhdG

Serum levels of 8OHdG were measured using a competitive ELISA based on a 8OHdG monoclonal antibody (Gentaur, Brussels, Belgium). Serum samples were purified using an ultrafilter according to the instructions of the manufacturer.

3.9. Statistical analysis

Results are expressed as mean ± SEM (standard error of the mean), in case of box plots mean ± SEM and SD (standard deviation). Analysis of variance with Bonferroni's correction/Tukey's post hoc test or Student's t test was used to compare mean values, as appropriate. Where data were not normally distributed nonparametric statistic tests were performed using Statistica 6.0 software (Stat Soft, Tulsa, OK, USA). Mann-Whitney U test was conducted to investigate the association between independent parameters of different patient groups. To analyze dependent variables, the Wilcoxon matchedpairs test was used. Differences were considered significant when $P < 0.05$.

4. Results

4.1. Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of PARP activation

4.1.1. Inflammatory response

We found that female animals produced less TNF-α than male mice. Inhibition of the catalytic activity of PARP by PJ34 reduced TNF-α production in male animals but did not further reduce TNF-α production in female animals (Figure 10.). PARP inhibition was unable to significantly reduce TNF-α production in female mice even when the dose of LPS was increased to produce a higher level of baseline TNF-α production to make it comparable with the level seen in LPS-treated male animals. (Figure 10.)

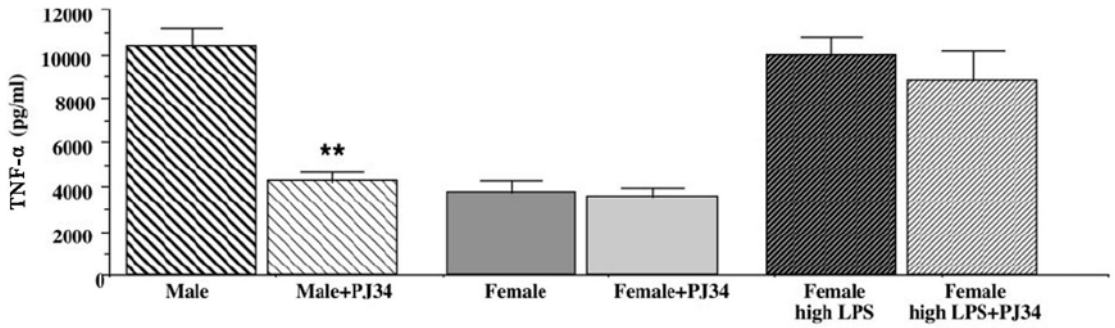


***Figure 10**: Female animals produce less TNF-α in response to i.p. LPS (1 mg/kg) than age-matched males. Although in male animals the PARP inhibitor PJ34 (10 mg/kg) significantly (**: $p < 0.01$) inhibits LPS-elicited TNF-α production (as measured at 90 min after LPS injection) in female animals, the effect of the PARP inhibitor is minimal. In female mice PARP inhibition failed to reduce LPS-induced TNF-α production, even when LPS dose was increased to 30 mg/kg. (n=6 in each experimental group)*

In addition, PARP-1-deficient male mice were resistant to LPS-induced TNF-α production, whereas in female mice (which were already resistant to these responses), genetic inactivation of PARP-1 failed to produce additional benefit. (Figure 11.)

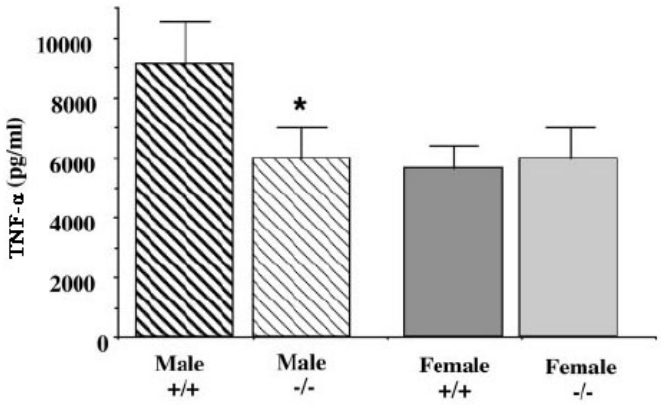


Figure 11: *Both PARP-deficient phenotype (in males) and female gender protect against LPS induced TNF-α production. In female animals PARP-deficient phenotype do not ensure further protection. (n=6 in each experimental group)*

In ovariectomized female mice, LPS induced higher levels of TNF-α compared with regular control females (8582 ± 1187 vs. 5504 ± 806 pg/ml, $n = 5$). Furthermore, in ovariectomized animals, a restoration of the sensitivity of the animals to inhibition of TNF-α production by PARP inhibitors was seen. Pharmacological inhibition of PARP reduced LPS-induced TNF-α production to 4668 ± 1187 pg/ml ($n = 5$, $P < 0.05$). (Figure 12.)

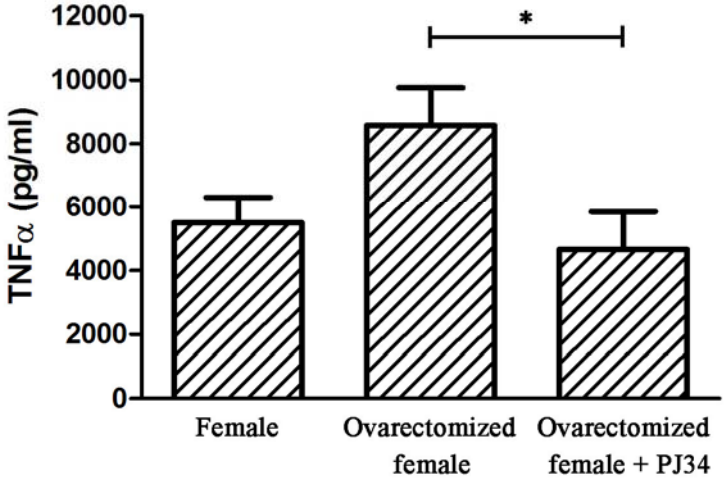
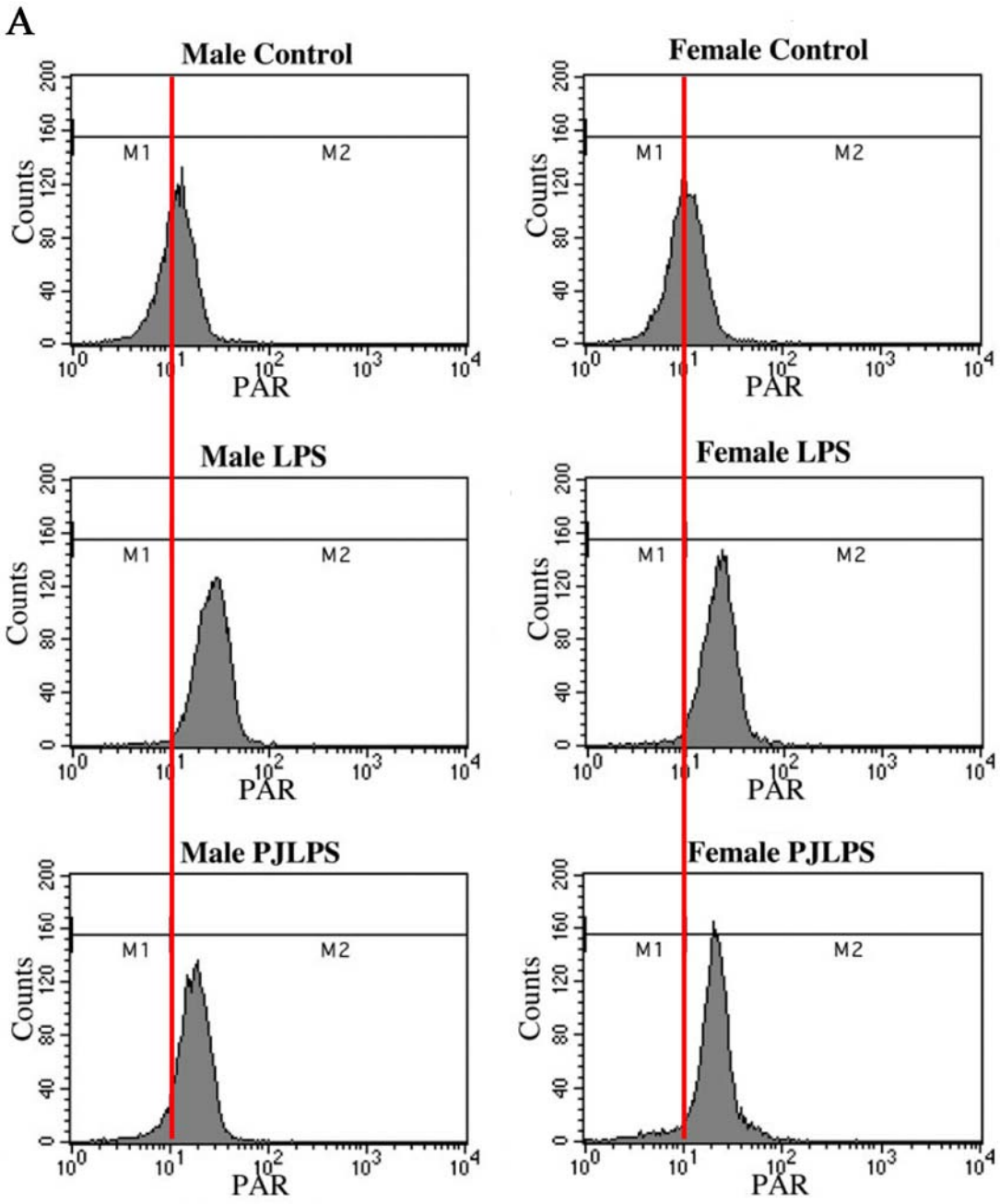


Figure 12*: Ovarectomized mice are sensitive to PARP inhibition in case of LPS-induced TNF-α production. n=5 in each experimental group)*

4.1.2. PARP activation in circulating leukocytes

There was no difference between male and female animals in basal PARP activity, as detected in circulating leukocytes by flow cytometry. LPS stimulation induced significant increases in PARP activation both in male and female animals. However, pharmacological inhibition of PARP with PJ34 only reduced PARP activity in male animals, but not in females. (Figure 13.)



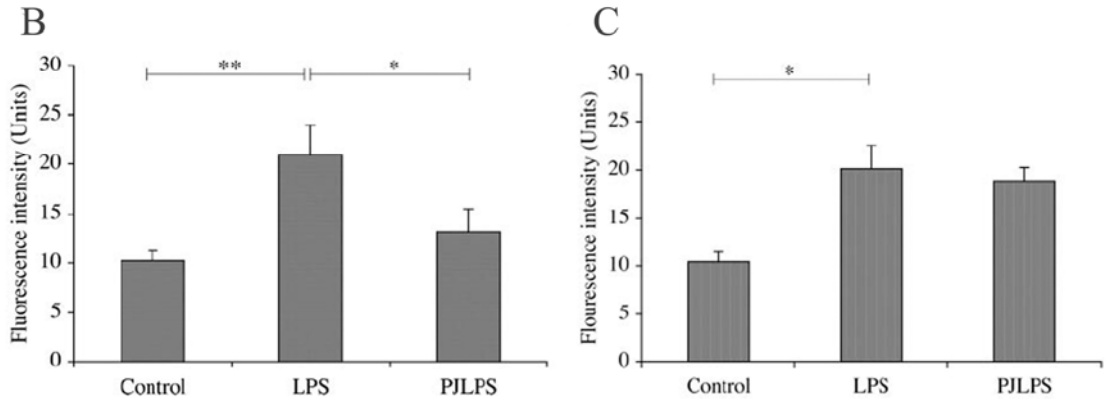


Figure 13: Gender differences in PARP activation in circulating leukocytes in response to LPS and in the effect of PARP inhibition*. **Panel A: Representative flow cytometry plots of PAR-stained leukocytes.** Male or female Wistar rats (either pretreated with 30 mg/kg i.v. PJ34 for 30 min or its vehicle saline) were given LPS injection (10 mg/kg i.v.). Leukocytes were prepared 3 h later. Cells having typical forward scatter and side-scatter properties of lymphocytes were defined as R1. For each sample, isotype control-stained cells served as negative control. On the PAR histograms, R1 was set as gate. **Panel B: Effect of LPS and PARP inhibition with PJ34 on PARP activation in circulating leukocytes in male rats.** Mean fluorescence intensity of R1 cells (lymphocytes) stained with anti-PAR antibody in male rats. LPS treatment of the animals resulted in significant increase of the PAR content of these cells (**: $P < 0.001$). In the case of male rats, PJ34 pretreatment significantly reduced this effect of LPS (*: $P < 0.05$). **Panel C: Effect of LPS and PARP inhibition with PJ34 on PARP activation in circulating leukocytes in female rats.** Mean fluorescence intensity of R1 cells (lymphocytes) stained with anti-PAR antibody in female rats. LPS treatment of the animals resulted in significant increase of the PAR content of these cells (*: $P < 0.05$). In the case of female rats, PJ34 pretreatment failed to alter this effect of LPS. (n=6-8 in each experimental group)*

4.1.3. Vascular reactivity

There was a significant degree of reduction in the endothelium-dependent relaxant ability of the vascular rings in response to LPS treatment in male animals but not in female animals. (Figure 14. A) Pretreatment with the PARP inhibitor PJ34 prevented the development of this LPS-induced endothelial dysfunction in the male rats, whereas it tended to attenuate the relaxant response in LPS-treated female animals. (Figure 14.B)

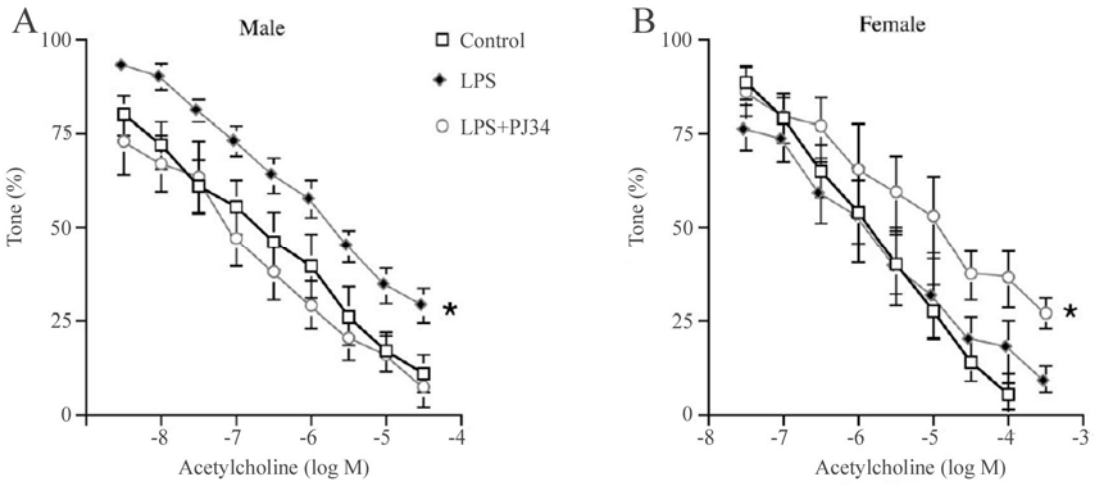


***Figure 14: Gender differences in vascular effect of LPS: effect of PARP inhibition**. **Panel A:** In male animals, LPS treatment induces a significant loss of acetylcholine-induced vascular relaxations, which is evidenced by both a reduction of the relaxant response to acetylcholine (*: $p < 0.05$) and by a significant ($p < 0.01$) shift to the right in the EC50 value of acetylcholine from $3.6 \pm 1.5 \times 10^{-7}$ to $26 \pm 13 \times 10^{-7}$ M. Inhibition of PARP with PJ34 prevents these changes. The relaxation curve is restored (*: $p < 0.05$), and sensitivity to acetylcholine is improved, the EC50 being $1.1 \pm 0.4 \times 10^{-7}$ ($p < 0.01$). **Panel B:** Female animals produce no endothelial dysfunction in response to LPS challenge and no change in the EC50 value for acetylcholine-induced relaxation ($6.2 \pm 2.0 \times 10^{-7}$ versus $5.5 \pm 4.1 \times 10^{-7}$ before and after LPS). Pharmacological inhibition of PARP attenuates the maximal endothelium-dependent relaxations in female animals challenged with LPS (*:$p < 0.05$) and shifts the EC50 value for acetylcholine-induced relaxations to the right to $41 \pm 17 \times 10^{-7}$ ($p < 0.01$). Values shown represent mean ± SEM of n = 10 to 12 determinations.*

4.2. Treatment with insulin inhibits PARP activation in a rat model of endotoxemia

4.2.1. Stress induced hyperglycemia

LPS treatment caused significant hyperglycemia for the first 120 min. The highest blood glucose level was measured 60 min after LPS injection. In the second hour the blood glucose levels of the animals gradually decreased and returned to the normal even hypoglycemic range by 180 min after LPS treatment. (Figure 15.) Insulin co-treatment prevented the LPS-induced hyperglycemia (Figure 15.).

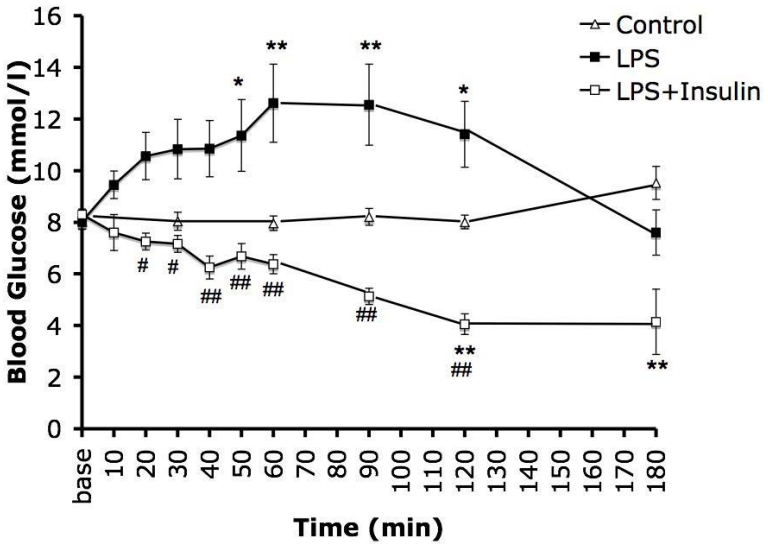


Figure 15: Blood glucose profiles of rats injected with E. coli LPS (15 mg/kg i.v.) with or without insulin treatment (5 U s.c.). *Endotoxin induced a significant degree of hyperglycemia, which was normalized by insulin treatment. In the control group, a slight decline of glucose levels occurred over time, perhaps due to a spontaneous restitution of a slight degree of hyperglycemia associated with the surgical procedure itself. Values are mean±SEM. *: p≤0.05 vs. Control, **: p≤0.01 vs. Control, #: p≤0.05 Insulin+LPS vs. LPS alone, ##: p≤0.01 Insulin+LPS vs. LPS alone. (n=8 animals per experimental group)*

4.2.2. PARP activation in circulating leukocytes

The PAR content of circulating lymphocytes – representing their PARP activity – was significantly increased in response to LPS. This activation of PARP was prevented by the administration of insulin. (Figure 16. A, B)

4.2.3. TNF-α production

Serum TNF-α levels also showed a significant increase, as measured at 3 h after LPS injection, which was prevented by insulin treatment. (Figure 16. C) It is noteworthy that while the inhibition of PARP activation by insulin in the circulating cells was a complete response, the inhibition of TNF-α production was a partial one. These findings may suggest that the promotion of TNF-α release into the serum during endotoxemia is triggered by multiple factors, PARP activation being one of these stimuli.

4.2.4. Glucose independent effect of insulin

In vitro experiments using Western blot analysis showed that insulin slightly, but significantly decreased PARylation of proteins in HUVECs and mononuclear cells cultured in medium containing 30 mM glucose, indicating that insulin has a slight, but significant direct effect on PARP activation, independent from its role in altering extracellular glucose concentration *in vivo*. (Figure 17.)

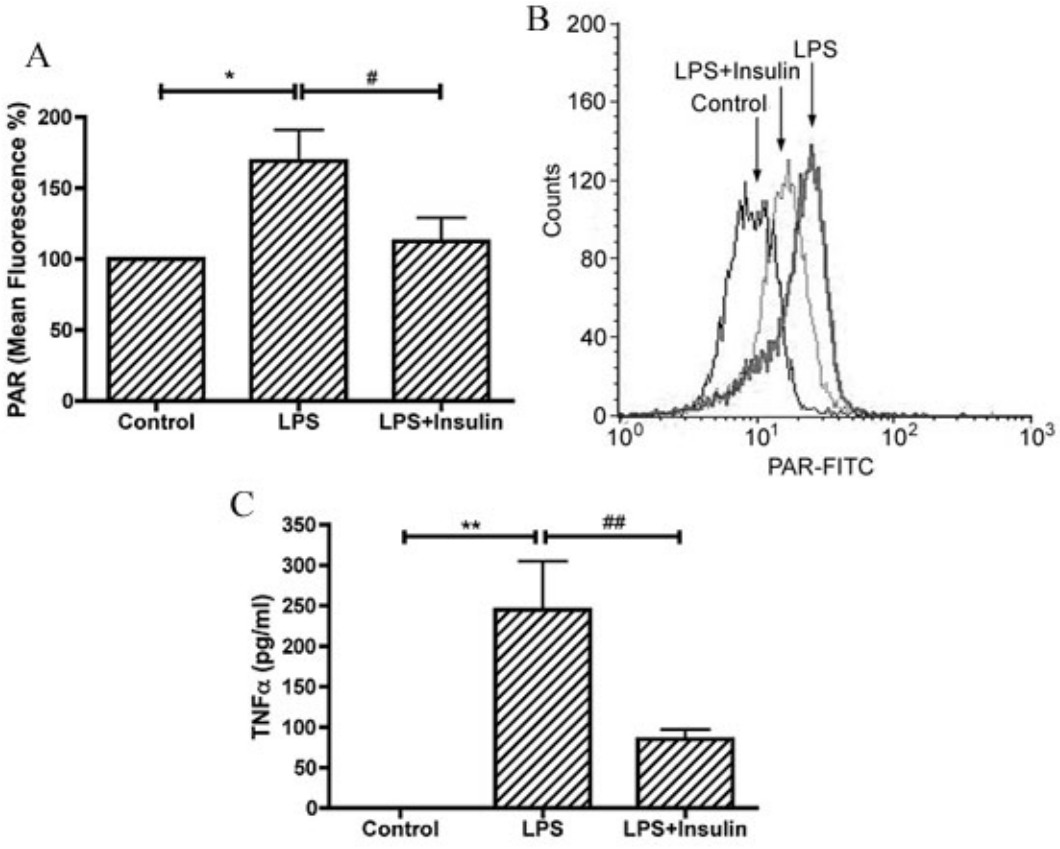


Figure 16: Panel A. Differences in PAR content of circulating lymphocytes in response to lipopolysaccharide and insulin treatment*. Mean fluorescence intensity of R1 cells (lymphocytes) stained with anti-PAR antibody. Endotoxin caused a significant increase of the PAR content of these cells, which was attenuated by insulin treatment. Values are mean±SEM. *: p≤0.05 vs. Control, #: p≤0.05 Insulin+LPS vs. LPS alone.* ***Panel B. Representative flow cytometric measurement****. PAR-FITC shows the amount of fluorescent labeling in the cells****. Panel C. Serum TNF-α levels at 180 min after LPS injection with or without insulin treatment.*** *Endotoxin caused a marked increase in serum TNF-α levels, which was abolished by insulin treatment. Values are mean±SEM. **: p≤0.01 vs. Control, ##: p≤0.01 Insulin+LPS vs. LPS alone. (n=4–6 determinations per experimental group).*

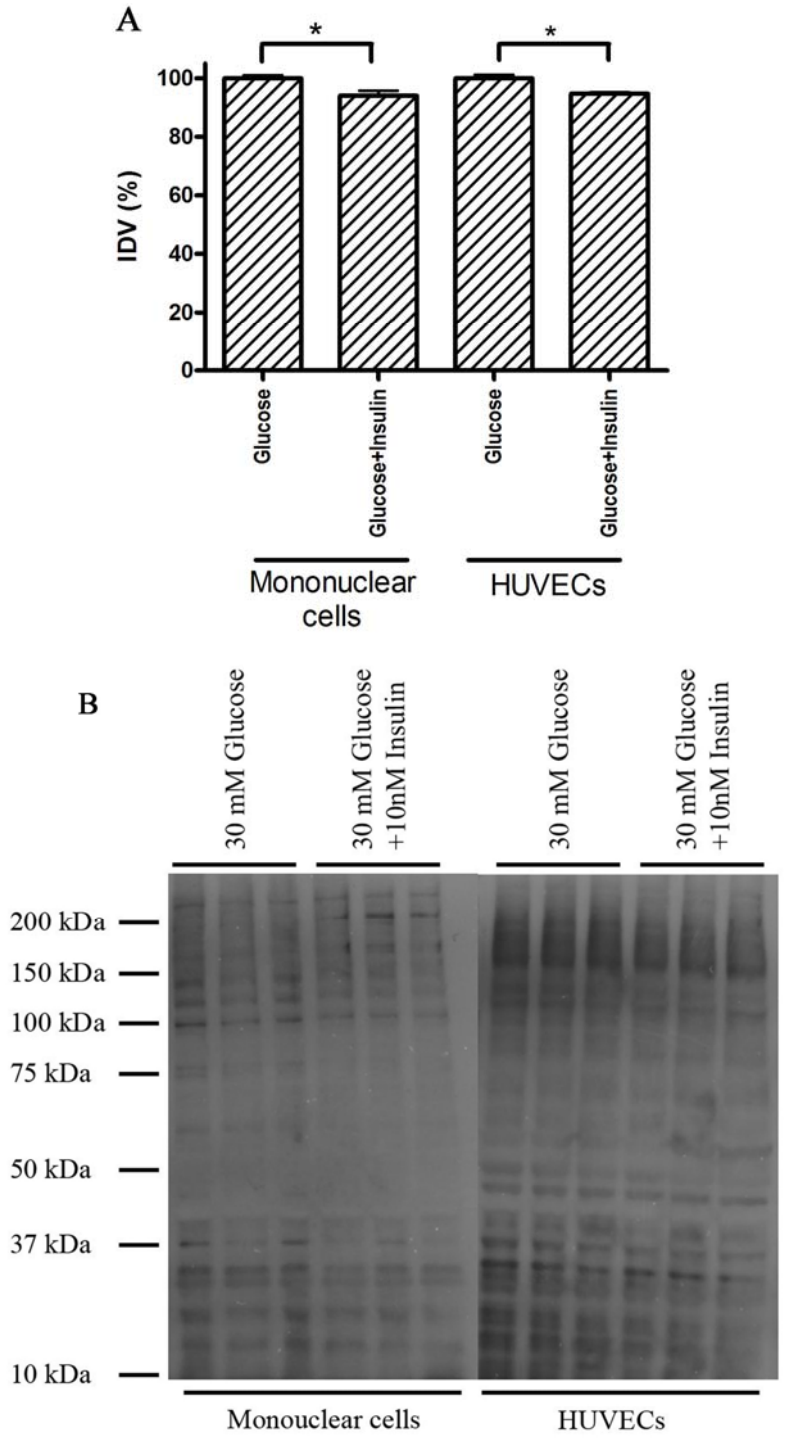


Figure 17: Panel A. Densitometric analysis of Western blots. Insulin treatment slightly decreased the degree of protein PARylation in both cell types. N=3 determinations per experimental group. (IDV: integrated density value) **Panel B. Representative Western blot image.** In case of mononuclear cells two PAR positive bands that can be seen in the untreated group around 60 and 75 kDa disappear due to insulin treatment.

4.3. Activation of PARP by myocardial ischemia and coronary reperfusion in human circulating leukocytes

4.3.1. Clinical data

Detailed patient demographics, clinical parameters, angiography and laboratory test results are summarized in Tables 7 and 8. The enrolled STEMI patients were predominantly men, with multiple cardiovascular risk factors in their medical history. All of them had permanent chest pain (maximum 12 h) and showed ST elevation (≥ 2 mm) in at least 2 consecutive ECG (electrocardiogram) leads on admission. In each case, coronary angiography revealed subtotal or total coronary artery occlusion, and successful recanalization was confirmed by high TIMI (trombolysis in myocardial infarction) flow rate values after PCI. Definitive acute myocardial damage was demonstrated by elevated creatine kinase (CK)/CK MB values.

Table7: Patient baseline characteristics.

	Acute ST-segment elevation myocardial infarction	Stabile angina pectoris	
		Coronarography	Elective PCI
n	15	6	9
Age	68.13 ± 2.93	57.0 ± 2.47	61.22 ± 3.6
Sex M/F	9/6	2/3	5/4
Body mass index, kg/m^2	28.11 ± 0.86	30.83 ± 1.32	30.3 ± 1.28
Risk factors			
Family history of IHD	6 (40)	4 (67)	4 (66)
History of tobacco use	7 (46.7)	5 (83.3)	9 (100)
Hypertension	15 (100)	4 (67)	7 (77)
Diabetes mellitus	5 (33)	2 (33.3)	4 (44)
Previous evidence of IHD	7 (46.7)	5 (83.3)	9 (100)
Angina 1-6 days before admission	4 (26.7)	4 (67)	6 (66)
Previous PCI	0	1 (17)	8 (88)
Diagnosis on admission			
Stable angina pectoris	—	5 (83.3)	9 (100)
Acute myocardial infarction		—	—
Inferior	3 (20)		
Infero-posterior	6 (40)		
Anterior	2 (13.4)		
Extensive anterior	3 (20)		
Posterior	1 (6.7)		

	Acute ST-segment elevation myocardial infarction	Stabile angina pectoris	
		Coronarography	Elective PCI
Time from event to balloon			
<3 hrs	3 (20)		
3-6 hrs	8 (53.3)		
>6 hrs	4 (26.6)		
Coronography results			
LM stenosis/occlusion	3/0	1/0	1/0
LAD stenosis/occlusion	9/4	3/1	7/1
RCA stenosis/occlusion	2/10	2/2	6/0
LCx stenosis/occlusion	8/1	2/0	2/0
R. diagonalis stenosis/occlusion	6/0	1/0	2/0
RRV stenosis/occlusion	1/1	0/0	0/0
OM stenosis/occlusion	6/0	0/0	1/0
Number of coronary stenoses per patient	2.27 ± 0.32	1.8 ± 0.58	2.11 ± 0.2
Number of coronary occlusions per patient	1.06 ± 0.12	0.6 ± 0.24	0.11
Number of implanted stents per patient	1.53 ± 0.27	—	0.88 ± 0.26
TIMI flow grade of the target vessel after PCI	2.77 ± 0.12	—	2.75 ± 0.16
Complications			
Atrial flutter/fibrillation	2 (13.3)	—	—
Ventricular tachycardia/fibrillation	4 (26.6)	—	—
AV block	3 (20)	—	—
Pacemaker therapy	2 (13.3)	—	—
Cardiogenic shock	3 (20)	—	—
IABP	2 (13.3)	—	—
Gastrointestinal/femoral	4 (26.6)	—	—
Exithus lethalis	2 (13.3)	—	—

Values are mean ± SEM or n (%). Percentages are compared to the entire patient population. [a]Time between the onset of persisting chest pain and the beginning of the PCI. IHD indicates ischemic heart disease; PCI, percutaneous coronary intervention; LM, left main coronary artery; LAD, left anterior descending artery; RCA, right

coronary artery; LCx, left circumflex artery; RRV, right retroventricularis; OM, obtuse marginal; TIMI, thrombolysis in myocardial infarction; IAB, intra-aortic balloon pump.

Table 8: Laboratory results in the STEMI group

	Post-PCI	Post-PCI	After 24 h	After 96 h
CK, U/L	230.6 ± 64.9	2152.8 ± 914.3	854.2 ± 194.42	275.5 ± 58.7
CKMB, U/ L	53.3 ± 17.1	254.4 ± 108.7	121.1 ± 35.46	33.25 ± 6.3
LDH, U/L	366.1 ± 35.8	—	1339.21 ± 207.2	1703 ± 488.6
AST, U/L	40.13 ± 8.29	—	174.1 ± 35.84	157.63 ± 91.53
ALT, U/L.	27.2 ± 5.51	—	48.07 ± 9.61 56	18 ± 16.03

Values are mean ± SEM. CK indicates creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

4.3.2. Determination of the oxidative imbalance: plasma total peroxide concentration

Total plasma hydrogen peroxide concentration as an established marker of oxidative injury and lipid peroxidation was determined. As shown in Figure 18. Panel A, total hydrogen peroxide concentration was not affected by coronary reperfusion directly; pre- and post-PCI values were not different statistically. A significant increase was observed in total peroxide levels 24 and 96 hrs after the acute cardiovascular event. Baseline values in patients with stable angina pectoris and STEMI were not different. Similar to the primary PCI group, in control stable angina patients hydrogen peroxide concentrations were identical before and after coronarography (420 ± 90 vs. 431 ± 104 μmol/L, respectively, n = 5, data not shown).

4.3.3. Verification of PCI-related DNA-damage: serum 8OHdG level measurements

In contrast to peroxide levels, primary PCI, representing a successful myocardial reperfusion, led to an explicit, rapid 8OHdG level increase ($P < 0.005$), indicating systemic, immediate, reperfusion-related DNA-damage in the STEMI patients in response to PCI (Figure 18. B). Furthermore, unlike peroxide concentrations, 8OHdG

levels were normalized within 96 hrs. Serum 8OHdG concentrations in control stable angina patients were not different statistically from pre-PCI STEMI values.

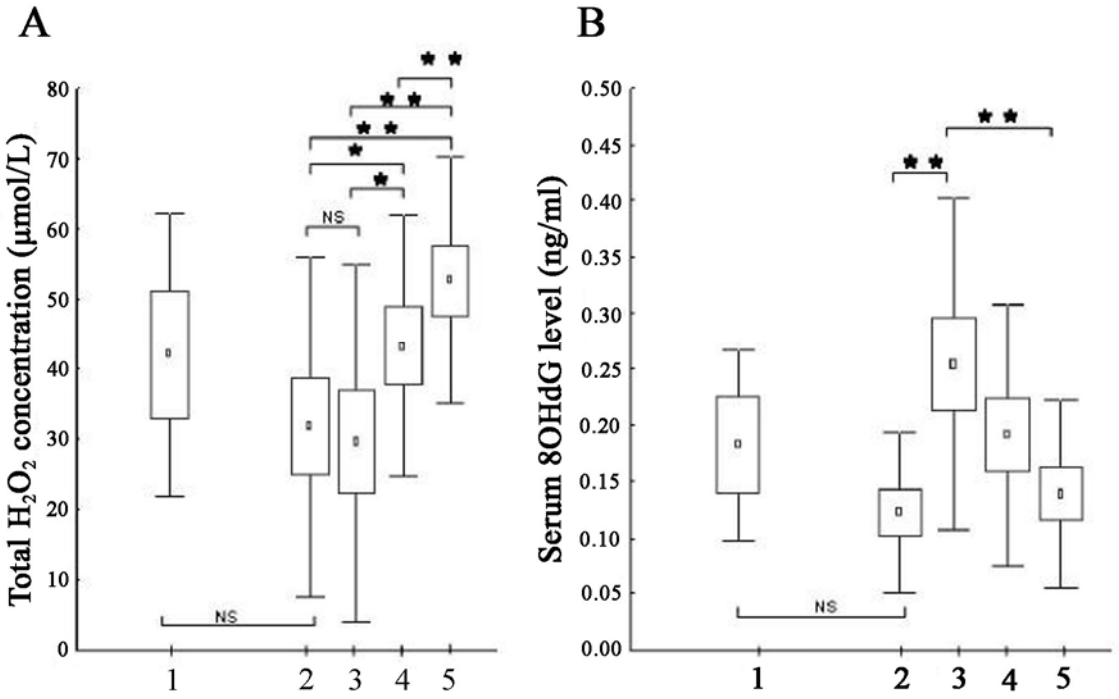
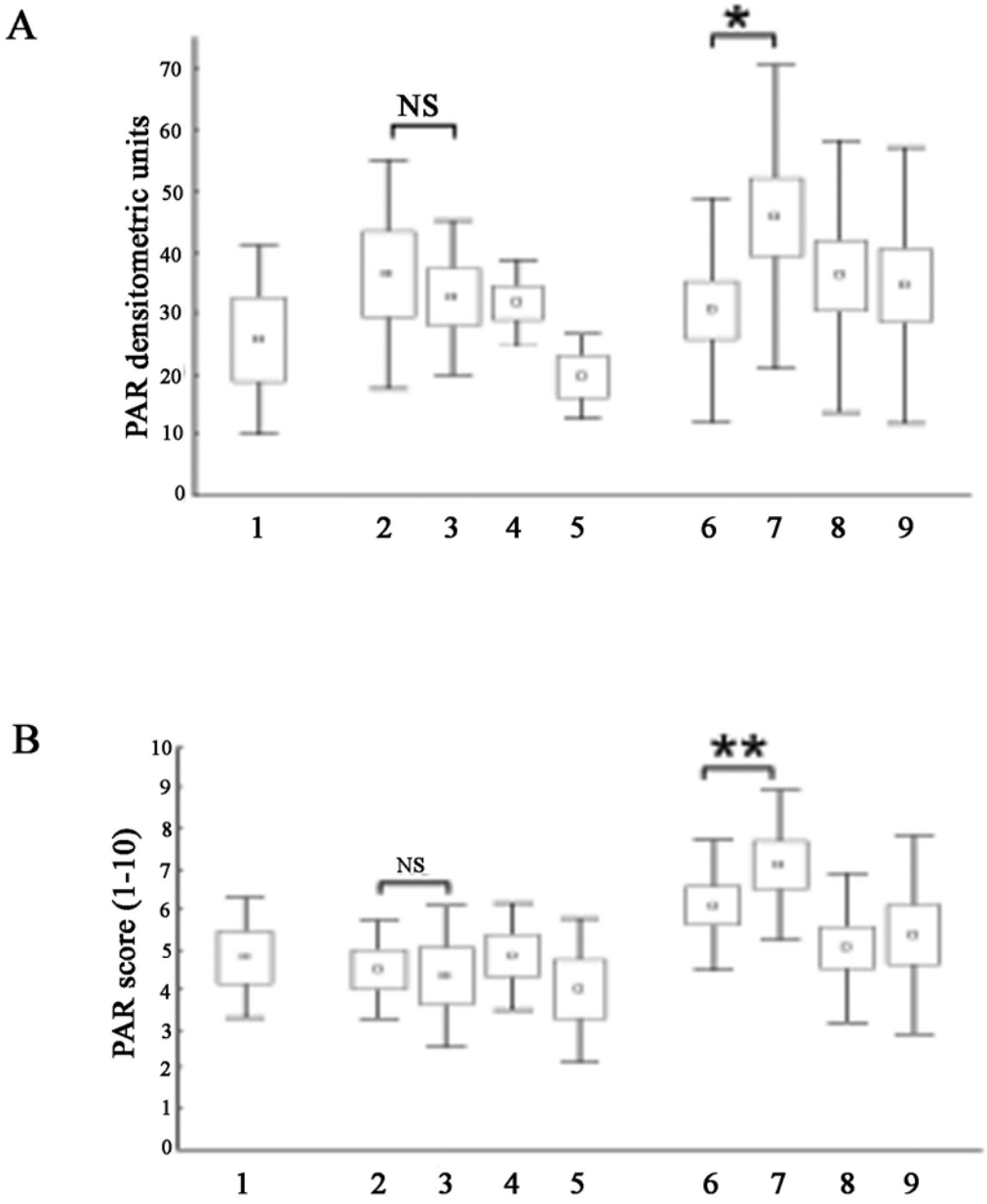


Figure 18: Determination of the oxidative imbalance in patients with stable angina pectoris and acute ST-segment elevation myocardial infarction. Panel A: Total plasma hydrogen peroxide concentration measurements. Panel B: Serum 8OHdG level measurements. *Results are expressed as mean (represented by squares) ± SEM (represented by boxes) and ± SD (represented by bars). Lane 1 indicates peroxide levels in stable angina patients; lanes 2-5 show peroxide concentration in patients with acute myocardial infarction before coronarography (lane 2), just after the successful primary PCI (lane 3), 24 ± 4 hrs after reperfusion of the ischemic myocardium (lane 4), and 96 ± 4 hrs after PCI (lane 5). Primary PCI itself did not affect total peroxide levels; gradual increase of hydrogen peroxide concentration was observed at 24- and 96-h time points after myocardial infarction. In contrast, serum 8OHdG levels showed a significant, rapid increase after the primary PCI, and were normalized by 96 h. *P < 0.05, **P < 0.005; NS, nonsignificant.*

4.3.4. PARP activation in circulating leukocytes

Immunohistochemistry and densitometry analysis of the buffy-coat cell pellet Western blots (n = 15) confirmed a significant immediate PARP activation in

circulating leukocytes due to myocardial reperfusion achieved by primary PCI (Figure 19, 21.). Similar to the kinetics of 8OhdG serum levels, the increase of PARP activity in isolated leukocytes occurred rapidly after myocardial reperfusion and decreased over time. Importantly, in patients undergoing elective percutaneous intervention (n = 9), no PARP activation occurred after the PCI. These results indicate that significant myocardial ischemia is required before reperfusion to induce PARP activation. Baseline initial PARP activity in stable angina and STEMI patients was comparable.



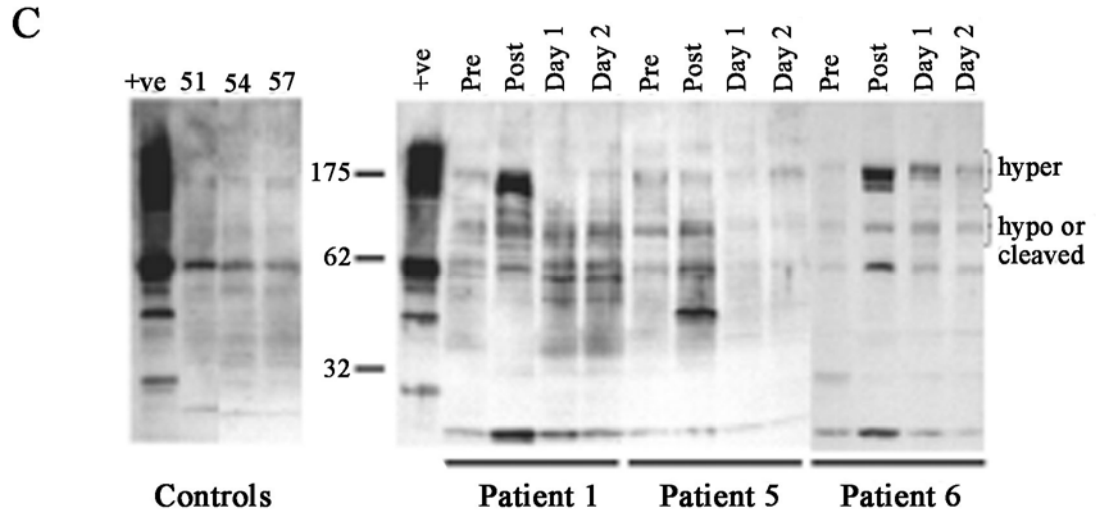


Figure 19: Rapid activation of PARP-1 in peripheral leukocytes induced after recanalization of the infarct-related coronary artery by primary PCI. Panel A: Densitometry analysis of PAR Western blots performed on patient leukocytes. Panel B: Immunohistochemical PAR-score analysis of patient leukocytes. *In both figures, lane 1 indicates densitometry units (A) or PAR-score values (B) in stable angina patients before coronarography; lanes 2-5 show PAR content in control patients with elective PCI before coronarography (lane 2), immediately after the successful PCI (lane 3), 24 ± 4 h after (lane 4), and 96 ± 4 h after PCI (lane 5); lanes 6-9 indicate PAR content in patients with acute myocardial infarction before coronarography (lane 6), immediately after the successful PCI (lane 7), 24 ± 4 hrs after reperfusion of the ischemic myocardium (lane 8), and 96 ± 4 hrs after PCI (lane 9). Primary PCI leads to a significant increase in leukocyte cellular PAR content, reflecting rapid activation of PARP during reperfusion. A gradual decrease of PARP activity can be observed at 24- and 96-h time points after myocardial infarction. PARP activity is not affected during elective PCI. Results are expressed as mean (represented by squares) ± SEM (represented by boxes) and ± SD (represented by bars).* ***Panel C: Representative examples of PAR Western blots from 3 stable angina patient leukocytes as controls (first panel) and 3 STEMI patients (second panel).*** *Time points are indicated. Commercially available PARP enzyme served as a positive control. *P < 0.05, NS, non-significant.*

4.3.5. Nitrotyrosine production and translocation of AIF

Immunohistochemical staining demonstrated that tyrosine nitration of the isolated cells significantly increased after PCI, compared with pre-PCI values (Figures 20. A and 21.). Again, when nitrotyrosine-positive cells were counted, rapid kinetics was observed: tyrosine nitration was maximal just after PCI and decreased by 96 h (Figure 20. A). The number of nitrotyrosine-positive cells did not differ between control and STEMI patients prior to surgery (Figure 20. A). In contrast to these parameters, translocation of AIF to the nuclei showed a gradual tendency to increase (Figures 20. B and 21.), a difference that became significant compared with the pre-PCI values by day 4 (23 ± 2% vs. 33 ± 5% positive cells, $P < 0.05$, $n = 8$, Figure 20. B).

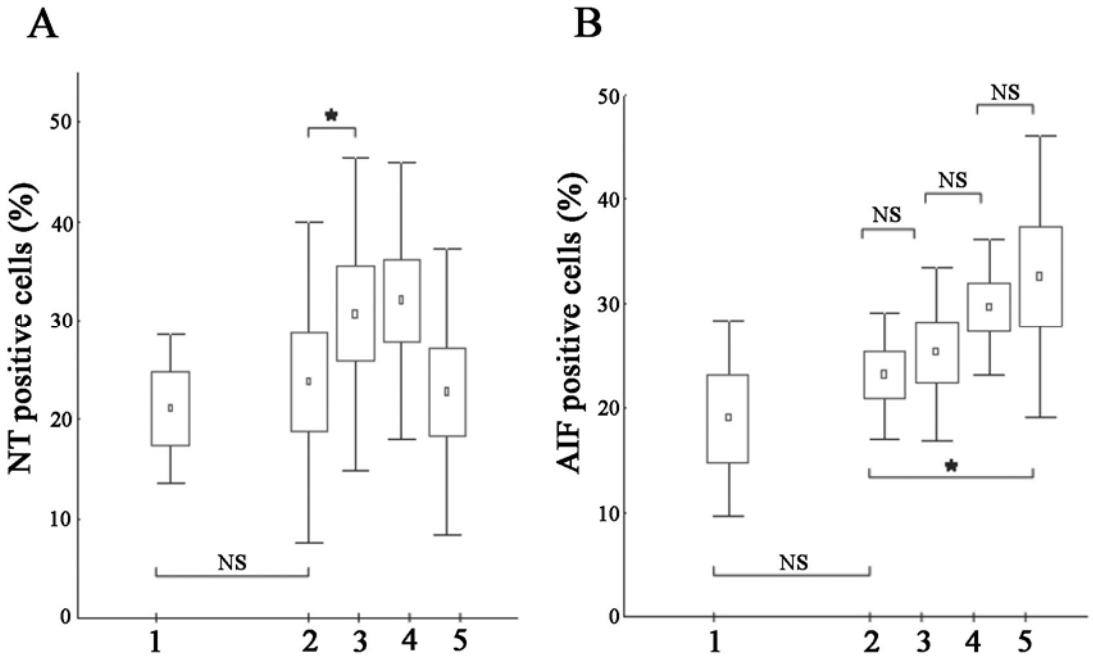


Figure 20: Tyrosine nitration (Panel A) and AIF translocation (Panel B) determined by immunohistochemistry*. Results are expressed as mean (represented by squares) ± SEM (represented by boxes) and ± SD (represented by bars). Lane 1 indicates control samples from stable angina patients; lanes 2-5 show NT-positive cell counts or AIF translocation–positive cell counts in patients with acute myocardial infarction before coronarography (lane 2), immediately after the successful primary PCI (lane 3), 24 ± 4 hrs after reperfusion of the ischemic myocardium (lane 4), and 96 ± 4 h after PCI (lane 5). Primary PCI induced an immediate increase in tyrosine nitration, whereas a gradual increase of AIF translocation was observed at 24- and 96-h time points after reoxygenation of the ischemic myocardium. *P < 0.05; NS, nonsignificant.*

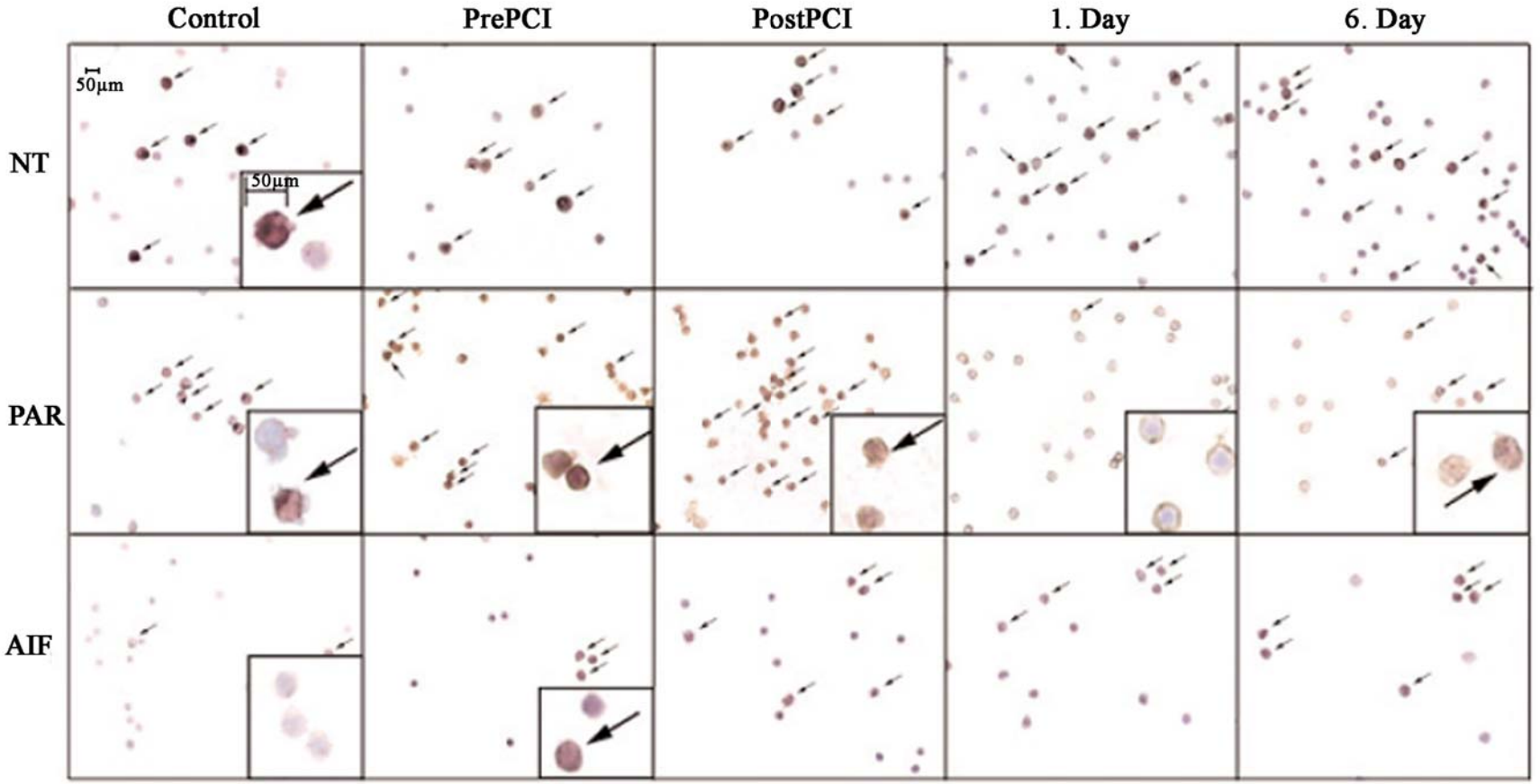

Control
PrePCI
PostPCI
1. Day
6. Day
NT
PAR
AIF
50µm
50µm

Figure 21: Immunohistochemical analysis of tyrosine nitration, PAR content, and AIF translocation. *Representative examples indicating gradual increase of the NT positive cell numbers (arrows) in peripheral leukocyte preparations after STEMI. The second row demonstrates increased PAR content in leukocytes immediately after the primary PCI. In the third row, AIF staining was performed and positive cells are depicted by arrows; AIF translocation increased by 96 h. Leukocytes from a stable angina patient are shown as negative controls in the first column.*

5. Discussion

5.1. Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of PARP activation

Our results showed the production of the inflammatory mediator TNF-α and the development of LPS-induced endothelial dysfunction were all markedly attenuated in female mice/rats, and pharmacological inhibition of PARP failed to provide further protection in female animals. On the other hand, in male mice/rats, pharmacological inhibition reduced TNF-α and prevented the development of endothelial dysfunction. PARP inhibition in male animals and female gender provided a comparable degree of protection in case of the various inflammatory/cardiovascular parameters investigated in the current study. Consistent with these findings, we observed that in circulating leukocytes, the pharmacological PARP inhibitor PJ34 only inhibited LPS-induced PARP activation in males, but not in females.

The observation that the gender difference to the LPS-induced TNF-α production is partially diminished in ovariectomized animals and other findings of our group that PARylation is attenuated by estrogen in male animals challenged with LPS *in vivo*, there is a difference in the degree of PARP activation between cells incubated in male versus female rat serum, and 17-α-estradiol pretreatment in male animals protects against LPS-induced mortality and PARP activation (123) all point to the potential involvement of the main female sex hormone, 17-α-estradiol, in the observed effects.

The finding that estrogen does not directly inhibit the catalytic activity of PARP in a cell-free assay (123) implicates an indirect mode of action. Although estrogen can act as an antioxidant, this effect generally occurs at fairly high concentrations *in vitro* (124, 125). Nevertheless, the contribution of an antioxidant effect of estrogen to our findings cannot be excluded.

ERα (estrogen receptor α) is a well-known potent activator of transcription (126, 127). ERα modulates transcription through its interaction with components of basal transcription machinery, chromatin modifiers, and regulatory proteins. In the absence of ligand, ERα binds to the corepressor complex containing histone deacetylases and remains inactive. However, in the presence of estrogen ligand, ERα associates with coactivator complex containing histone acetylases and activate transcription.

Furthermore, the MAPK-dependent phosphorylation of ERα serine residues within the AF-1 domain also recruits coactivators and activates transcription through ligand-independent mechanism.

Using an *in vitro* gel shift assay, it was shown that PARP and ERα cooperatively interact with the DNA, and these interactions are further reinforced by the presence of estrogen (123). One can, therefore, propose a model of interaction between PARP and ERα (Figure 22.).

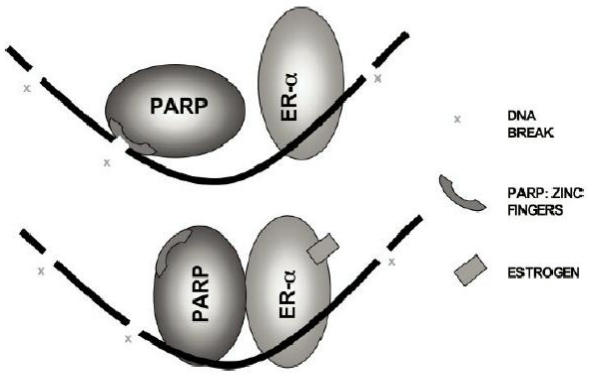


Figure 22: Proposed model for the interaction of estrogen, estrogen receptor, and PARP in conjunction with DNA single-strand breakage and PARP activation. *Top, the situation in the absence of estrogen (PARP and ERα interact weakly, and PARP can recognize DNA strand breaks and can become catalytically activated). In the presence of estrogen (bottom), PARP and ERα interact strongly, which may anchor PARP to the DNA and reduce its ability to recognize DNA single-strand breaks and thereby may prevent its activation.*

PARP or ERα or PARP and ERα (on their own) interact with DNA, and these interactions are weak and reversible. In addition, PARP-ERα complex is as active as PARP alone and moves freely on the DNA and repairs the DNA-damage sites. However, the presence of estrogen ligand alters the conformation of ERα and forms a more stable ERα-PARP-DNA tercenary complex. Such a stable complex may sequester PARP to specific regions on the DNA, making it difficult for its zinc fingers to access and recognize DNA breakpoints (without which its activation would be inhibited). Such a model would be consistent with our findings that estrogen is not a direct inhibitor of

the enzymatic activity of the purified PARP enzyme but is a potent inhibitor of the activation of PARP *in vivo* in estrogen-pretreated animals.

However, we must note that even the estrogen receptor/PARP interaction was demonstrated *in vitro* there is no direct evidence that such interaction also occurs in intact cells or in *in vivo* systems. Further work is required to link the *in vitro* findings to cell-based and *in vivo* mechanisms. It is possible that the actions of estrogen or gender in modulating PARP activation involve more than one regulatory mechanism. It is also possible that in different cell types, different regulatory mechanisms may be involved. Likewise, the identification of the specific domains of PARP and ERα involved in recognizing each other remains a subject of further investigation.

As in the current study only TNF-α production and vascular reactivity was measured, it would be interesting to study additional parameters such as other proinflammatory mediators (e.g., the expression of the inducible isoform of NO synthase by measuring plasma nitrite/nitrate levels at later time points after LPS), anti-inflammatory cytokines such as interleukin-10 or other inflammatory factors and processes (e.g., infiltration of mononuclear cells into tissues) to determine the broader applicability of the current findings.

It is interesting to note that many cell-based experiments are being conducted in tissue culture medium containing various concentrations (typically 10%) fetal calf serum, which contains detectable amounts of maternal estrogen. Based on the present data, one may wonder whether the results derived from such studies reflect artificial conditions in which estrogen receptors are engaged and PARP may be partially inhibited.

It has to be also mentioned that PARP inhibitors are not always ineffective in female animals. In female nonobese diabetic (NOD) mice that by autoimmune β-cell loss develop type-1 diabetes-like disease PARP inhibitors are of major therapeutic benefit (128, 129). PARP inhibition is also protective in female sheep subjected to shock, or burn and smoke inhalation injury (130). Thus, further investigation is needed to establish the limits and applicability of gender specific PARP actions.

Pharmacological inhibitors of PARP move toward clinical testing for a variety of indications for example myocardial infarction and various tumors. It is known that women are protected against cardiovascular disease, and this protection disappears after

menopause (131). It remains to be tested whether this protection is related to the inhibitory potential of female sex hormones. However as myocardial infarction predominantly develop in men or in postmenopausal women, gender differences in PARP actions do not discourage the clinical testing of the therapeutic effect of PARP inhibitors in both males and females. The sensitivity of cancer cells for PARP inhibition is usually the characteristic of the tumor. Nevertheless careful analysis should be conducted and potential gender differences in the upcoming clinical trials should be examined.

5.2. Treatment with insulin inhibits PARP activation in a rat model of endotoxemia

However, the role of PARP activation in endotoxin shock has been wildly studied, the potential role of hyperglycemia in triggering oxidative and nitrosative stress and PARP activation in endotoxin shock has not been investigated yet.

The importance of hyperglycemia in the context of critically ill patients has been highlighted by experimental and clinical studies demonstrating that tight glycemic control with insulin provides significant protective effects in patients in medical and surgical intensive care units (114-116, 132).

There is a separate field of investigation, in connection with the pathomechanisms of diabetic complications that is involved in studying the mechanisms of free radical production and cellular alterations in conjunction with elevated extracellular glucose concentrations. This line of investigation demonstrated that elevated extracellular glucose levels induce the overproduction of mitochondrial oxidants and free radicals, which, in turn, results in the activation of PARP, and the modulation of various downstream pathways of inflammation and cell response (128, 133, 134). By linking the findings of these studies with the results of our experiments, we propose a possible mechanism whereby endotoxin shock induced PARP activation involves hyperglycemia, subsequent leak of oxidants and free radicals from the mitochondria, followed by DNA strand breaks. The exact source of the reactive species during hyperglycemia is a subject of on-going investigations; there is evidence, in various experimental models, for a role for mitochondrial reactive oxidant species, as well as for NADPH oxidase, myeloperoxidase and aldose reductase in the production of reactive

oxygen and nitrogen species in the vascular endothelium (134-139). Whatever the source of reactive species is, it appears that this oxidative stress is sufficient to induce the activation of PARP in circulating leukocytes. Further work is required to determine whether PARP activation in circulating leukocytes serves as a 'damage signal' that precedes the death of these cells, or possibly PARP activation might act as an enhancer of leukocyte-derived inflammatory responses, thereby perpetuating the systemic inflammatory response.

The finding that insulin therapy reduces TNF-α production (which has been previously reported by other groups as well (119)) can also be integrated with the PARP concept, as PARP is known to upregulate a variety of proinflammatory genes and PARP inhibitors have previously been shown to suppress the endotoxin-induced production of TNF-α in multiple experimental systems (140).

Insulin exerts a variety of cellular and biological effects additional to its effect on glucose control. In HUVECs insulin stimulates adenosine and L-arginine transport and nitric oxide (NO) synthesis (141). Likewise, recent data indicate that PARP can be regulated by alternative pathways that do not involve free radical generation or DNA-damage (142). Our *in vitro* experiments showed that insulin has a slight, but noticeable direct inhibitory effect on PARP activity in HUVECs and also in mononuclear leukocytes.

In the context of critical care illnesses, the glucose-mediated therapeutic effects of insulin appear to be predominant over glycemic control-independent actions (114, 143). Our observation that insulin decreases the degree of protein PARylation in vitro only by approximately 5% may also support this hypothesis and is consistent with the hypothesis that insulin reduces PARP activation in the current model of endotoxemia by normalizing plasma glucose levels, and hence reducing the amount of reactive oxygen and nitrogen species capable of PARP activation via DNA injury.

5.3. Activation of PARP by myocardial ischemia and coronary reperfusion in human circulating leukocytes

Despite the fact that myocardial reperfusion therapy is often called a double-edged sword (144), the aim of the clinician is to reperfuse the ischemic myocardium as soon as possible to recover contractile function and to avoid irreversible myocardial damage.

Previous studies have demonstrated oxidative DNA injury and consequent PARP activation in cardiomyocytes, endothelial cells, and circulating peripheral leukocytes in animal models of acute myocardial infarction (8).

The current study investigated multiple aspects of human myocardial ischemia/reperfusion-related pathologies by analyzing serum, plasma, and isolated peripheral leukocyte samples from cardiovascular patients with acute ST segment elevation myocardial infarction and successful primary PCI. Our results provide evidence for (1) general oxidative/peroxidative imbalance (elevated total plasma peroxide concentration and augmented nitrotyrosine production), (2) direct, PCI-generated DNA-damage (evidenced by increased levels of serum 8OHdG), (3) rapid post-PCI activation of PARP in circulating human peripheral leukocytes (shown by immunohistochemistry and Western blotting), and (4) translocation of AIF from mitochondria to nuclei (which may be a downstream signaling event triggered by PARP activation).

These results provide the first clinical evidence for PARP activation in patients with myocardial infarction and are consistent with the concept that local myocardial hypoxia/reperfusion triggered by percutaneous interventions in acute myocardial infarction is able to trigger systemic oxidative responses in humans.

Although the pathomechanism of reperfusion injury has been extensively studied, relatively limited therapeutic applications have been developed to date (145, 146). So far, therapeutic attempts to prevent reperfusion injury are limited to relatively small-scale studies testing the administration of vitamin E (147), calcium antagonists (148), early use of ACE (angiotensin converting enzyme) inhibitors, sulfhydrylrich reagents such as N-acetylcysteine, magnesium, and various free radical scavengers (149). However, accelerated myocyte necrosis and destruction related to reoxygenation of the ischemic myocardium continues to represent a clinically relevant question. It is generally accepted that the combination of the reopening of the coronary artery with therapeutic approaches that protect the reperfused myocardium may improve the outcome of the PCI. As demonstrated by analysis of circulating leukocytes passing through the reperfused myocardium, consequent PARP activation with abrupt kinetics also occurs. Taken together with previous data from experimental myocardial ischemia

models (8), one can speculate that, as with leukocytes, myocyte PARP activation is likely to develop in human reperfusion injury, leading to myocyte necrotic cell death.

The present observations may also have direct therapeutic implications. Potent novel PARP inhibitors have been developed in recent years, and these agents were shown to be beneficial in *in vitro* and *in vivo* ischemia/reperfusion models as attenuating reperfusion injury by acting at several levels (prevention of energetic failure, inflammatory mediator production, neutrophil infiltration, and endothelial dysfunction) (8, 150, 151). Therefore, in theory—as is supported by our human data confirming rapid PARP activation due to primary PCI—PARP inhibition before the planned reperfusion might provide multiple benefits, most importantly, myocyte salvage and therefore improves survival (8). In this context, analysis of PARP activity in human circulating leukocytes during PARP inhibitor treatment might serve as a useful marker and provide evidence for the ability of PARP inhibitors to block the activation of the target enzyme in clinical trials *in vivo*.

Besides acute myocardial infarction, various degrees of myocardial reperfusion injury may occur under such common clinical conditions as elective percutaneous coronary interventions. Our data indicate that in the case of elective PCI without complications, PARP activation did not develop in circulating leukocytes. It would be important to test whether in the case of periprocedural myocardial necrosis – which is a negative prognostic factor in patients with elective PCI (152, 153) – the oxidative/nitrosative balance triggers the PARP pathway.

What, then, is the molecular trigger of PARP activation in human myocardial infarction? Based on animal studies, oxidative and nitrosative stress (namely, hydrogen peroxide, peroxynitrite, and hydroxyl radical) are pathophysiologically relevant triggers of PARP activation (154). In the present study, tyrosine nitration (a relatively specific marker of peroxynitrite production) but not hydrogen peroxide levels showed a close correlation with the degree of PARP activation, possibly implicating the role of reactive nitrogen species, such as peroxynitrite. We must point out, nevertheless, that the likely trigger of PARP activation is within the reperfused myocardial tissue, and peripheral blood parameters may not necessarily correlate with the changes within the myocardial tissue itself.

It is interesting to note that the current study demonstrated that AIF translocation occurs 4 days after PCI during myocardial infarction. The translocation of AIF can be triggered by multiple factors, one of them being PARP activation. It is unlikely that PARP is the only contributor of AIF translocation in the current study, as the time course of PAR staining and AIF translocation is quite different.

Although our observations provide evidence for primary PCI-related oxidative injury and subsequent DNA-damage–induced PARP activation in a human cardiovascular patient cohort, our case numbers are limited. A subsequent large-scale study would be needed to link and correlate these biochemical markers to patient outcome and clinical parameters, such as major cardiac events or cardiovascular death. Analysis of 8OHdG or PARP activity levels in context to serum troponin, CK MB values, or ejection fraction would be particularly important. Also, in future clinical studies with PARP inhibitors, peripheral leukocyte PAR content or PAR immunostaining may serve as useful sentinel markers for the efficacy of the PARP inhibitor to block its enzymatic target *in vivo*. In future studies, using antioxidants or PARP inhibitors, one may also be able to assess whether oxidant stress contributes to AIF translocation, whether there are alterations in the viability and lifetime of peripheral blood cells, and whether these changes are related to oxidant stress and PARP activation.

5.4. PARP activation in circulating leukocytes

PARP activity of circulating leukocytes was measured in rat model of endotoxemia, and in patients with STEMI followed by reperfusion. In all studies PARP activity reflected the pathological condition and the efficiency of PARP inhibition therapy.

Three different methods were used to estimate PARP activity, two of them were based on the measurement of cellular PAR content (immunohistochemistry, flow-cytometry), one determined the level of auto-PARylation (Western-blot). Another possibility is to directly measure PARP activity using cell-based enzyme activity assay.

In a recent study it has been shown that in acute myocardial infarction PARP activity of circulating mononuclear cells measured by cell-based enzyme activity assay positively correlates with plasma TNF-α and IL-10 levels and negatively correlates with

left ventricular ejection fraction and left ventricular fractional shortening (155). On the other hand the observation that PARG mutant mice are more sensitive to LPS induce endotoxin shock (increased mortality and TNF-α production) (156) points out that determination of PAR content of cells may provide better estimation of the actual pathological state.

Each method has other advantages and disadvantages summarized in table 9.

Table 9: Basic characteristics of methods for PARP activity measurement

Method	Measured parameter	Evaluation	Sample preparation
Immunohistochemistry	PAR content of cells	Semi-quantitative score Opportunity to separate cell types Partially subjective	Methanol fixed smears (long tenability)
Flow-cytometry	PAR content of cells	Computer based Opportunity to separate cell types Objective	Requires fresh blood
Western-blot	Auto-PARylation	Densitometry Objective	Frozen isolated cells (long tenability)
Cell-based activity measurement	PARP activity	Colorimetry Objective	Frozen isolated cells (long tenability)

As neutrophil and eosinophil granulocytes do not have PARP-1, in all methods isolating mononuclear cells provide increased accuracy. (The distribution of white blood cells does not alter the results.) However this isolation is usually based on gradient centrifugation and especially in pathological conditions where the size and density of cells change, the resulted cell suspension contains not only mononuclear cells, but other cell types and debris. While immunohistochemistry and flow-cytometry provides further opportunity to separate debris and different cell types, Western-blot and cell-based assay do not have this advantage.

The evaluation of immunohistochemistry is only partially objective, as it is done by a blinded experimenter using a semi-quantitative score. Using special software would increase accuracy; however the complexity of PAR staining (location and intensity) would cause difficulties.

However flow-cytometry has the advantage that it measures PAR content, objective and makes it possible to separate cell types, freshly acquired blood is necessary for the procedure, which makes it difficult to use it in a clinical setting.

All four techniques may serve as potential sentinel in pharmacological development and clinical trials. However further investigation is required to validate these methods, and to determine the most appropriate and accurate method.

6. Conclusion

Our results demonstrate that there is an interrelated regulation of the endotoxin-induced inflammatory and vascular responses by gender and PARP. Our observations together with the finding that the estrogen sensitive cooperative interaction of PARP, ERα and DNA may prevent recognition of DNA breakpoints by PARP indicate that estrogen in a novel endogenous inhibitor of PARP.

In our study we demonstrated that insulin therapy in a rat model of endotoxemia blocks PARP activation and prevents inflammatory mediator production. Thus, the beneficial effects of insulin therapy in critical illness may be related, at least in part, to an inhibition of the pathophysiological consequences of the excessive overactivation of PARP.

Our data provide evidence for PARP activation for the first time in humans suffering from myocardial infarction. In the present population of cardiovascular patients with ST-segment elevation myocardial infarction, primary percutaneous intervention is accompanied by significant systemic DNA-damage, PARP-1 activation, and consequent AIF translocation. On the other hand additional investigation would be necessary to link and correlate these biochemical markers to patient outcome and clinical parameters, such as serum troponin, CK MB values, ejection fraction, major cardiac events or cardiovascular death.

PARP activity of circulating leukocytes was measured in rat model of endotoxemia, and in patients with STEMI followed by reperfusion by three different technique; immunohistochemistry, flow-cytometry and Western-blot. In all studies PARP activity of mononuclear cells reflected the pathological condition and the efficiency of PARP inhibition therapy.

Gender differences and PARP-inhibitory effect of insulin therapy has to be considered in pharmacological development and upcoming clinical trials of PARP inhibitors. Measuring the PARP activity of circulating leukocytes may serve as potential sentinel in these studies.

7. Summary

PARP activation significantly contributes to the pathogenesis of various conditions, such as endotoxin shock and myocardial infarction. Pharmacological inhibitors of PARP move toward clinical testing for a variety of indications including cardioprotection and malignant tumors. Our aim was to identify possible novel modulators of PARP, that may influence the outcome of these studies and to test weather measuring PARP activity in circulating leukocytes may serve as a sentinel test reflecting the degree of PARP activation and the efficiency of PARP inhibition.

Our results showed that in LPS treated female mice/rats LPS-induced TNF-α production and endothelial dysfunction were markedly attenuated, and in contrast to male mice/rats, pharmacological inhibition of PARP failed to provide further protection. The gender difference in TNF-α production is partially diminished by ovariectomy. In circulating leukocytes, the PARP inhibitor PJ34 only inhibited LPS-induced PARP activation in males. Our observations demonstrate that there is an interrelated regulation of the endotoxin-induced inflammatory and vascular responses by gender and PARP.

We demonstrated that insulin therapy in a rat model of endotoxemia blocks PARP activation and prevents inflammatory mediator production. Insulin treatment prevented LPS-induced hyperglycemic response, blocked PARP activation in circulating leukocytes and blunted LPS-induced TNF-α response. Insulin treatment caused a slight reduction in PARP activity of mononuclear cells and HUVECs in elevated glucose conditions *in vitro*.

In the examined population of cardiovascular patients STEMI followed by PCI is accompanied by increased nitrosative stress, PARP activation, and consequent AIF translocation in circulating leukocytes. These data provide evidence for PARP activation for the first time in humans suffering from myocardial infarction.

In all studies PARP activity of mononuclear cells reflected the pathological condition and the efficiency of PARP inhibition therapy.

Our observations indicate that estrogen is a novel endogenous inhibitor of PARP. Gender differences and PARP-inhibitory effect of insulin therapy has to be considered in pharmacological development and upcoming clinical trials of PARP inhibitors. Measuring the PARP activity of circulating leukocytes may serve as potential sentinel in these studies.

8. Összefoglalás

A PARP aktivációja jelentős szerepet játszik számos betegség, többek között az endotoxin sokk és a miokardiális infarktus patogenezisében. A PARP farmakológiai gátlószerei különböző indikációkban – például kardioprotekció, malignus tumorok – vannak az emberi kipróbálás közelében. Célunk volt új, a PARP működését befolyásoló anyagok azonosítása, melyek befolyásolhatják ezen vizsgálatok eredményét, illetve annak tesztelése, hogy a keringő leukociták PARP aktivációjának mérése megfelelően tükrözi-e a PARP aktiváció mértékét és a PARP-gátlás hatékonyságát.

Eredményeink azt mutatták, hogy LPS-sel kezelt nőstény egerekben/patkányokban az LPS-inukált TNF-α termelés és endotélium diszfunkció kialakulása kifejezetten csökkent mértékű, és a hímekkel ellentétben, a PARP farmakológiai gátlása vagy nem biztosít további védelmet. A nembeli különbség ovariectomia hatására részlegesen megszűnt. Keringő leukocitákban a PARP gátló PJ34 csak hím egyedekben gátolta az LPS indukálta PARP aktivációt. Vizsgálataink alapján a nem és a PARP egymással kölcsönhatásban szabályozza az endotoxin által indukált gyulladásos és érválaszt.

Patkány endotoxaemia modellben kimutattuk, hogy az inzulin kezelés gátolja a PARP aktivációját és csökkenti a gyulladásos mediátorok felszabadulását. Az inzulin kezelés meggátolta az LPS-indukálta hiperglikaemiát, megakadályozta a keringő leukocták PARP aktivációját és gyengítette a TNF-α választ. Inzulin hatására kis mértékben csökken a mononucleáris és HUVEC sejtek PARP aktivációja magas glukóz koncentrációjú tápoldatban *in vitro*.

A vizsgált beteg populációban a STEMI-t követő PCI-t a keringő leukocitákban megfigyelhető emelkedett nitrozatív stressz, PARP aktiváció és AIF transzlokáció kíséri. Ezek az első adatok, melyek bizonyítják a PARP aktiváció jelenlétét miokardiális infarktusban szenvedő betegekben.

A mononukleáris leukociták PARP akivációja mindegyik vizsgálatban tükrözte a patológiás állapotot és a PARP gátlás hatását.

Megfigyeléseink arra utalnak, hogy az az ösztrogén a PARP egy újonnan azonosított endogén gátlószere. A nemi különbségeket és az inzulin PARP gátló hatását a jövőben figyelembe kell venni a PARP gátlók farmakológiai fejlesztése és a klinikai vizsgálatok során. A keringő leukociták PARP aktivitásának mérése e vizsgálatokban ellenőrző markerként szolgálhat.

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Publications

Publications closely related to the thesis

1. **Horvath E. M.,** Benko R., Gero D., Kiss L.,Szabo C. Treatment with insulin inhibits poly(ADP-ribose)polymerase activation in a rat model of endotoxemia. (2008) *Life Sci.* 82(3-4):205-209. IF: 2.389

2. **Horvath E. M.,**Szabo C. Poly(ADP-ribose) polymerase as a drug target for cardiovascular disease and cancer: an update. (2007) *Drug News.Perspect.* 20(3):171-181. Review. IF: 2.721

3. Toth-Zsamboki E., **Horvath E.,** Vargova K., Pankotai E., Murthy K., Zsengeller Z., Barany T., Pek T., Fekete K., Kiss R. G., Preda I., Lacza Z., Gero D.,Szabo C. Activation of poly(ADP-ribose) polymerase by myocardial ischemia and coronary reperfusion in human circulating leukocytes. (2006) *Mol.Med.* 12(9-10):221-228. IF: 2.708 (*Toth-Zsamboki E.and Horvath E. contributed equally to this work***)**

4. Mabley J. G., **Horvath E. M.,** Murthy K. G., Zsengeller Z., Vaslin A., Benko R., Kollai M.,Szabo C. Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of poly(ADP-ribose) polymerase activation. (2005) *J.Pharmacol.Exp.Ther.* 315(2):812-820. IF: 4.098

Other publications

1. Hamahata A., Enkhbaatar P., Kraft E.R., Lange M., Leonard S.W., Traber M.G., Cox R.A., Schmalstieg F.C., Hawkins H.K., Whorton E.B., **Horvath E.M**., Szabo C., Traber L.D., Herndon D.N., Traber D.L. gamma-Tocopherol nebulization by a lipid aerosolization device improves pulmonary function in sheep with burn and smoke inhalation injury. (2008) *Free. Radic. Biol. Med*. 15;45(4):425-33. IF: 4.813

2. Esechie A., Kiss L., Olah G., **Horvath E. M.,** Hawkins H. K., Szabo C.,Traber D. L. Protective effect of hydrogen sulfide in a murine model of combined burn and smoke inhalation-induced acute lung injury. (2008) *Clin.Sci.(Lond).* 115(3):91-7. IF: 3.9

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5. Beller C. J., **Horvath E.,** Kosse J., Becker A., Radovits T., Krempien R., Berger I., Hagl S., Szabo C.,Szabo G. Opposite effects of vascular irradiation on inflammatory response and apoptosis induction in the vessel wall layers via the peroxynitrite-poly(ADP-ribose) polymerase pathway. (2007) *Clin.Res.Cardiol.* 96(1):8-16. IF: 1.442 (*Beller C. J and Horvath E. contributed equally to this work***)**

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