Raising cellular NAD^+ levels in human cells by nicotinic acid supplementation:

Biological consequences related to PARP-1 mediated reactions

DISSERTATION

zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften (Dr. rer. nat.) des Fachbereichs Biologie

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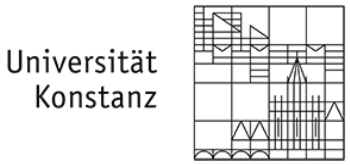

vorgelegt von

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SUMMARY/ZUSAMMENFASSUNG

The cellular response to DNA damage includes a number of mechanisms to detect and repair various types of DNA damage in order to preserve the integrity and stability of the genome. One reaction involved is the poly(ADP-ribosyl)ation (PARylation) of proteins, a modification performed by nuclear poly(ADP-ribose)polymerase-1 or -2 (PARP-1 and PARP-2) immediately after DNA damage infliction. PARPs covalently attach ADP-ribose units in a sequential fashion to target proteins including themselves, synthesizing a negatively charged polymer by using nicotinamide adenine dinucleotide (NAD^+) as substrate. According to the level of DNA damage and intracellular NAD^+ status, the most active enzyme PARP-1 and its product PAR mediate the recruitment of DNA repair factors to sites of lesions, facilitate DNA repair and thus maintain genomic integrity under conditions of moderate stress. In this scenario a tolerable amount of total cellular NAD^+ is used for polymer synthesis. In contrast, the critical expenditure of NAD^+ due to massive activation of PARP-1 under severe stress conditions can lead to cell death thus influencing deleterious or health-enhancing processes, as is apparent in inflammatory diseases or neurodegenerative disorders.

One important parameter determining the cellular response to stresses is the level of available NAD^+, which is crucial for adequate PAR synthesis and other NAD^+ dependent processes, as the energy metabolism or sirtuin functions. Sirtuins can act as deacetylases in terms of response to cellular damage and to metabolic imbalances thus regulating fundamental processes as well.

In order to analyse the biological consequences of elevated NAD^+ levels in respect to PARP-1 mediated reactions and to address the question, if modulated NAD^+ and/or PAR levels contribute to physiological or pathophysiological outcomes, distinct end points were investigated.

Human blood peripheral mononuclear cells (NAD^+) were *ex vivo* supplemented with the NAD^+ precursor nicotinic aid (NA), which significantly raised intracellular nucleotide pools and led to an intensified PAR formation in response to genotoxic stimuli.

It was observed that NA supplementation reduces cell death after genotoxic stress and shifts the residual fraction from necrosis to apoptosis, which is less harmful in regard to tissue integrity. To investigate, if this is a result of improved DNA repair, strand break formation and subsequent repair within the first 40 minutes was accessed. Interestingly, we observed that

strand break rejoining is tightly regulated but was positively affected in NA supplemented cells under massive DNA damaging conditions and to a minor extent also under mild genotoxic stress. Furthermore, enhanced NAD^+ levels seem to be beneficial in context of genomic integrity, as supplemented cells displayed reduced frequencies of micronucleus formation. In addition to the impact on already mentioned cellular functions, it was monitored if higher NAD^+ levels favour also sirtuin activities. The SIRT-1 target p53 is acetylated in response to DNA damage. Preliminary results give evidence that the DNA damaged induced p53 acetylation is reduced in nicotinic acid supplemented cells, potentially due to increased deacetylation rate.

In summary, increased NAD^+ levels by NA supplementation can be beneficial for a cellular system, most notably in context of massive damage, but also in situations of minor damage. So far no adverse effects were observed for the investigated parameters. Based on the current results, supplying nicotinic acid seems to be a valuable approach to augment NAD^+ levels especially in the case of acute damage or to correct suboptimal NAD^+ levels to prevent negative consequences, often linked with excessive or elevated PARP-1 activation.

Die zelluläre Antwort infolge von Schädigung beinhaltet etliche Mechanismen, um DNA Schäden unterschiedlichster Art zu detektieren und zu reparieren, die dazu dienen die Integrität und Stabilität des Genoms zu erhalten. Eine der beteiligten Reaktionen ist die Poly(ADP-ribosyl)ierung (PARylierung), eine Modifikation die unmittelbar nach der Schadensentstehung von Poly(ADP-ribose)polymerasen im Zellkern ausgeführt wird. PARP-1 sowie PARP-2 binden unter der Verwendung von dem Substrat Nikotinamidadenindinukleotid (NAD^+) schrittweise ADP-Ribose Einheiten kovalent an Zielproteine, einschließlich sich selbst, wobei ein negativ geladenes Polymer entsteht. Entsprechend des Levels des DNA Schadens und des intrazellulären NAD^+ Status vermittelt hauptsächlich PARP-1 und das erzeugte PAR Produkt die Rekrutierung von Reparaturfaktoren zur Schadensstelle, begünstigt dadurch die Reparatur der DNA und folglich den Erhalt der genomischen Stabilität unter moderaten Stressbedingungen. In dieser Situation wird ein zu tolerierender Anteil von NAD^+ zur Polymersynthese verwendet. Im Gegensatz dazu kann der bedenklich hohe Verbrauch an NAD^+ durch eine starke PARP-1 Aktivierung bei massiver Schädigung zum Zelltod führen, wodurch gesundheitsfördernde oder schädigende Prozesse beeinflusst werden, was erkennbar bei entzündlichen oder neurodegenerativen Erkrankungen ist.

Ein wichtiger Parameter, der die zelluläre Antwort beeinflusst, ist der verfügbare intrazelluläre NAD^+ Gehalt, der entscheidend für eine ausreichende PAR Synthese und weitere NAD^+ abhängige Prozesse, wie der Energiehaushalt oder Sirtuin-abhängige Reaktionen ist. Sirtuine agieren als Deacetylasen bei Zellschäden und metabolischem Ungleichwicht, wodurch fundamentale Prozesse reguliert werden.

Um die biologischen Auswirkungen eines gesteigerten NAD^+ Levels in Bezug auf PARP-1 vermittelte Reaktionen zu untersuchen und die Frage zu beantworten, ob veränderte NAD^+, beziehungsweise PAR Levels, physiologische oder pathophysiologische Folgen haben, wurden bestimmte Endpunkte betrachtet.

Humane periphere mononukleäre Blutzellen (PBMC) wurden *ex vivo* mit dem NAD^+ Vorläufer Nikotinsäure (NA) behandelt, wodurch der intrazellulären Nukleotidpool signifikant erhöht wurde und eine gesteigerte Synthese von PAR infolge genotoxischer Stimulierung ermöglicht wurde.

Es wurde beobachtet, dass die Supplementaton mit NA den Zelltod nach genotoxischen Stress vermindert und der Anteil des nekrotischen Zelltods Richtung Apoptose verschoben wird. Dies ist in Bezug auf die Gewebeintegrität weniger schädlich. Um zu untersuchen, ob der

verminderte Zelltod mit einer verbesserten DNA Reparatur einhergeht, wurde die Bildung von Strangbrüchen und deren Reparatur innerhalb der ersten 40 Minuten verfolgt. Interessanterweise zeigte sich, dass die Reparatur zwar streng reguliert ist, aber dennoch eine positive Auswirkung der NA Supplementation bei extremer, und zu einem Teil auch bei geringerer Schädigung, erkennbar ist. Darüber hinaus scheint ein erhöhter NAD^+ Level im Zusammenhang mit genomischer Integrität von Vorteil zu sein, da supplementierte Zellen weniger Mikrokerne aufwiesen.

Neben den Auswirkungen auf die eben genannten zellulären Funktionen, wurde untersucht ob ein höherer NAD^+ Level die Aktivität von Sirtuinen ebenfalls begünstigt. Nach Schädigung wird das SIRT-1 Zielprotein p53 acetyliert. Vorläufige Ergebnisse zeigten, dass die schadensinduzierte Acetylierung von p53 in NA supplementierten Zellen reduziert ist, das möglicherweise durch eine erhöhte Deacetylationrate bedingt sein kann.

Zusammenfassend zeigte sich, dass ein erhöhter NAD^+ Level durch Supplementation mit NA einem zellulären System von Vorteil sein kann, vor allem in Zusammenhang mit massiver und mittlerer Schadensinduktion. In den bisherigen Untersuchungen wurden keine negativen Einflüsse auf die beobachteten Parameter verzeichnet. Aufgrund der gegenwärtigen Ergebnisse könnte die Zugabe von Nikotinsäure ein sinnvoller Ansatz sein, um den Anteil an vorhandenem NAD^+ bei akuter Schadensinduktion zu stärken, um vor negativen Konsequenzen einer übermäßigen oder erhöhten PARP-1 Aktivierung zu schützen.

GENERAL INTRODUCTION

DNA damaging events lead to a diversified cellular response involving multitude of proteins and pathways, supporting the repair process to maintain chromosomal integrity and survival. Immoderate damage or inadequate repair however can alter the genetic integrity, consequently driving genomic instability, a potential source of cancer and cell death. One immediate response to genotoxic insults is the activation of poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear protein involved in recognizing DNA distortions, participating in the DNA repair processes and chromatin remodelling by synthesis of negatively charged poly(ADP-ribose) (PAR) using nicotinamide adenine dinucleotide (NAD^+) as substrate. Moreover PARP-1, its product PAR and the level of NAD^+ are linked to pathophysiological outcomes, making modulation of its activity a clinical target.

In the following introducing chapters these factors and their interplay in biological activities, as well as medical aspects, will be discussed.

PARPs and poly(ADP-ribosyl)ation

In response to genotoxic stress PARP-1, a highly conserved 113 kDa nuclear protein composed of 1014 amino acids [1, 2] - and to a lesser extent also PARP-2 (68 kDa) [3] - is activated and attaches ADP-ribose units to target proteins including itself [4] in a sequential and processive way forming a negatively charged polymer using NAD^+ as substrate [5] (Figure 1). The modification of glutamate, aspartate or lysine residues has been reported [6, 7].

The ADP-ribose units are linked via glycosidic ribose-ribose bonds to each other, resulting in linear or branched polymers containing up to 200 units [8-10]. PAR is a rapidly appearing but highly transient modification of short half life [11]. The breakdown is performed by poly(ADP-ribose) glycohydrolase [12], which possesses exoglycosidic activity to hydrolyze terminal ADP-ribose units from PAR polymers and is capable of removing short fragments of oligo(ADP-ribose) via endoglycosidic cleavage [13], creating free ADP-ribose as well as PAR. Both are messenger molecules involved in cell death signalling [14, 15]. Other enzymatic activities, namely poly- and mono(ADP-ribosyl) protein hydrolase or mono(ADP-ribosyl) protein lyase,

may also act to remove PAR polymers and ADP-ribose monomers from target proteins [16, 17].

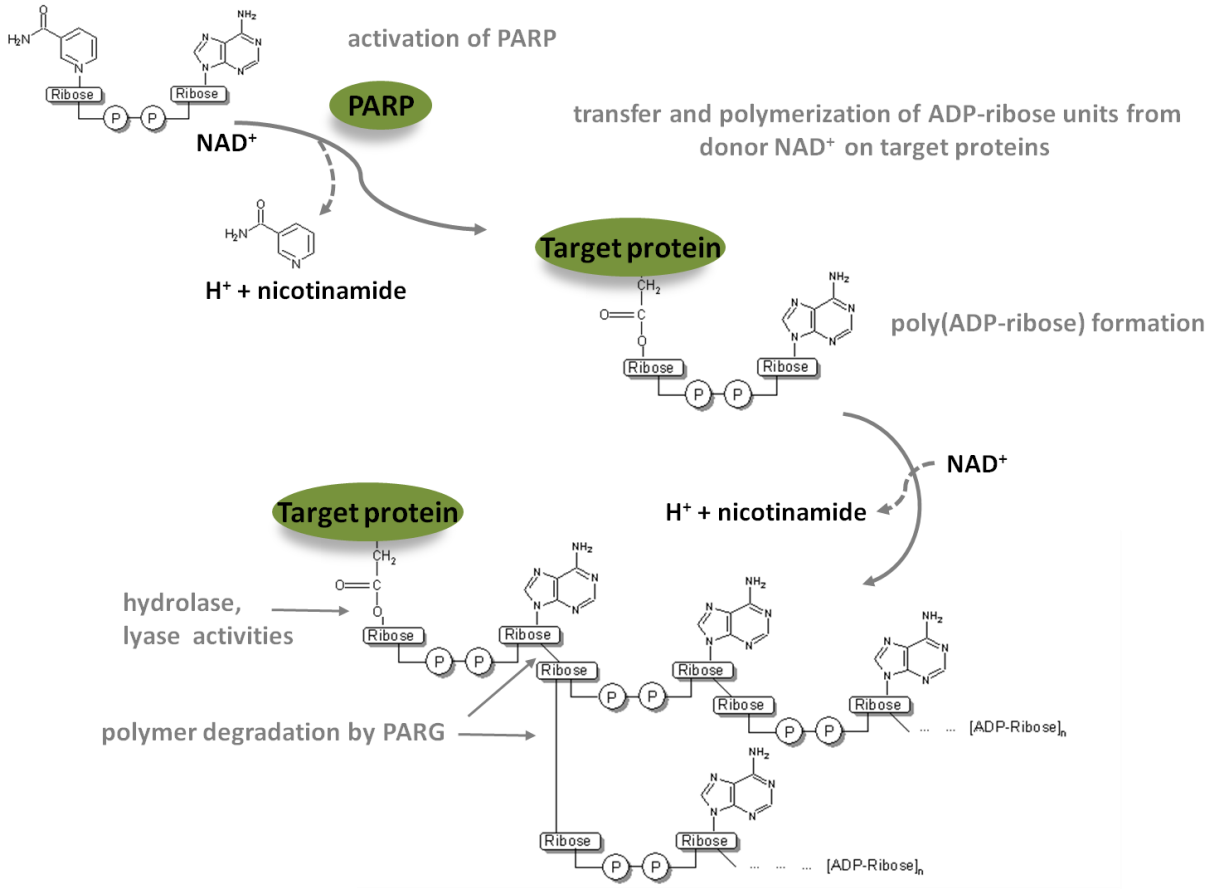

Figure 1: Poly(ADP-ribosyl)ation reaction.

Cellular poly(ADP-ribose) (PAR) is produced by activated PARPs, which catalyze the polymerization of ADP-ribose units from NAD^+ molecules on itself (automodification) or other target proteins (transmodification), thereby releasing nicotinamide and one proton (H^+). The resulting ADP-ribose units are linked to each other via glycosidic ribose-ribose bonds, creating linear or branched PAR polymers. Rapidly after synthesis PAR is degraded by poly(ADP-ribose)glycohydrolases (PARG) or cleaved from the target protein by hydrolases or lyases.

The majority of the damage-induced PAR is predominantly formed by PARP-1, making this member of ADP-ribosyltransferases the most active polymer producing enzyme and probably also the most important PARP in DNA damage responses [18]. PARP-1 functions as a dimer or interacts with PARP-2 as heterodimer. Aside from PARP-1/2 and its role in DNA damage detection and repair, the human genome contains 17 genes encoding for proteins of the PARP family, functioning in diverse cellular features in the manner of transcriptional regulation,

chromatin remodelling, trafficking or telomere maintenance, but also centromeric organization, making not only PARP-1 a molecule of interest [19, 20].

Relevant for the enzymatic and functional activity is the structural characteristic of PARP-1. It is a multidomain protein, consisting of (i) a DNA binding domain localized on the N-terminus with two zinc fingers and a zinc-ribbon motif, implicated in binding to DNA and strand breaks as well as dimerization, respectively [21], (ii) automodification domain, which provides acceptor sites for the auto-ribosylation and contains a BRCT (BRCA-1 C- terminus) motif, and (iii) a C-terminal catalytic domain essential for the conversion of NAD^+ in ADP-ribose [22, 23].

PARP-1 and PAR-mediated responses in the context of DNA damage

The presence of a DNA lesion leads to a complex signalling network comprising the recognition by sensors, the recruitment of mediators and the transduction of the signal by effector proteins [24]. Among others, PARP-1 and poly(ADP-ribosy)ation is implicated via particular mechanisms in a variety of these pathways which will be highlighted below.

Single strand-, as well as double strand breaks, but also other interruptions in the DNA strand caused by genotoxic stimuli *e.g.* ionising radiation, oxidative stress or alkylation exposure, represent the common substrates that are recognized by PARP-1. The synthesized polymer can act in different ways in the DNA damage response cascade (Figure 2).

(i) Due to its negatively charged character the polymer relaxes locally the chromatin architecture by poly(ADP-ribosyl)ation of histones [25, 26], making the lesion site accessible for repair and transcriptional factors.

(ii) Furthermore it facilitates the recruitment of selected proteins of the repair machinery to certain sites or complexes through direct interaction in a non-covalent manner or by covalent poly(ADP-ribosyl)ation. This comprises particularly those proteins implicated in strand break repair pathways to restore the original DNA sequence (see also DNA repair pathways), but also initiates signalling pathways as cell cycle arrest and apoptosis (e.g. via transcription factor p53) or inflammation (e.g. transcription factor NF-kB) [27, 28].

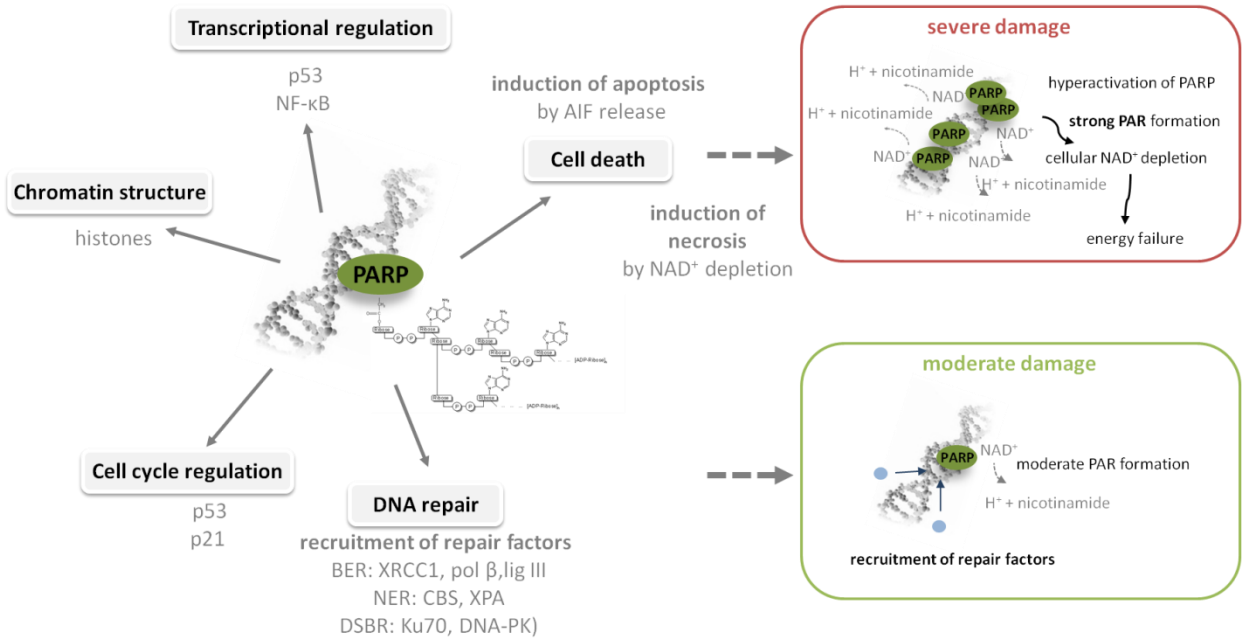

Figure 2: Implication of PARP-1 and its products PAR in cellular key functions in the context of DNA damage

PARP-1 interacts with and/or PARylates proteins involved in DNA repair, transcription and regulation of chromatin structure to control physiological and pathological outcomes. Note that the magnitude of damage (right boxes) dictates PARP activity, polymer synthesis and thereby critically influences NAD^+ levels, which influences the balance between prevention and aggravation of disease processes.

Functions in chromatin organization

A central role of PARP-1 at the site of damage is the local relaxation of chromatin structure, enabling the formation of DNA repair complexes to accomplish repair. This includes on the one hand the covalent poly(ADP-ribosyl)ation of the linker histone H1 and the histones H2A, H2B, H3 and H4 [29], as well as the non-covalent interactions with polymer attached to PARP-1 [30, 31]. Both interactions lead to the interruption of the condensed nucleosomal structure, as histones arte trapped in PARP-1-linked polymer or are repelled from DNA after covalent way modification [32].

Functions in DNA damage signalling

The DNA dependent protein kinase ataxia telangiectasia mutated (ATM) is a key signalling components of the DNA damage response. It was shown that PARP-1 interplays with ATM [33], and PAR formation at sites of DNA damage contributes to the mobilization of ATM

assuring phosphorylation of its downstream targets as p53 [34], enabling repair by the activation of checkpoint pathways that temporarily arrest cell cycle progression [35].

Functions in DNA repair

So far, PARP-1 and partly PARP-2 have been implicated in at least three distinct DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER) and double-strand break (DSB) repair. Thus, the next chapters will specifically address biological functions of PAR and PARPs in DNA damage recognition and repair with the focus on individual repair pathways and proteins.

Base excision repair (BER)

Small base lesions derived from oxidation or alkylation damages are processed by the base excision repair pathway. At first lesion-specific DNA glycosylases bind and remove the modified bases, generating an apurinic or apurimidinic site (AP), which is substrate for the endonuclease APE 1 leading to strand incision, forming a single strand intermediate. The process is completed by one of the two sub-pathways: the short-patch BER replaces one nucleotide [36], whereas in the long-patch BER 2-13 nucleotides are displaced [37]. Each sub-pathway is based on the assembly of distinct multiprotein complexes - including PARP-1- at the site of the DNA lesion and facilitate coordinated repair by gap filling and ligation. PARP-1 binds at BER intermediates via zinc finger II [21], synthesizes PAR and therefore enables the recruitment of short-patch BER components including X-ray cross-complementing protein-1 (XRCC1) [38, 39], polymerase β (Pol β) [40] and ligase III (Lig III) [41] to the lesion site. PARP-2 was also found to interact with these proteins [42]. In addition, the involvement of PARP-1 in long-patch BER sub-pathway [40] via cooperation with the flap endonuclease FEN-1 was also described [43]. Interestingly, the repair of abasic sites was shown to be dependent on NAD^+, as in the absence of NAD^+ PARP-1 persists at the strand break and repair will be stalled [44].

Nucleotide excision repair (NER)

Bulky DNA lesions caused by UV irradiation, mutagenic chemicals or chemotherapeutic compounds are repaired via the excision of a single stranded DNA stretch containing the modified nucleotides, involving a multitude of proteins. A role of PARP-1 was suggested in both NER sub-pathways, the global genome coupled (GGC) and transcription coupled repair

pathway (TC) [45]. Specific interactions have been described for the cockayne syndrome B protein (CSB), essential for the recognition and initiation of TC-NER, a functional and physical interaction with PARP-1 was identified [46]. Furthermore the xeroderma pigmentosum-A protein XPA was identified as a PAR- binding protein [47].

Double strand break repair

Double strand breaks induced by ionizing radiation, chemicals or during replication represent highly toxic intermediates. They can be repaired by error-prone non homologous end joining (NHEJ), which is the predominant pathway in mammalian cells, or homologous recombination (HR). Double strand breaks generated in G_1 phase of the cell cycle are processed by the non-homologous DNA end joining pathway, where two-ended breaks are ligated together, involving DNA-dependent protein kinase, consisting of the catalytic subunit DNA-PKcs and the Ku70/Ku80 heterodimer, as well as the XRCC4-DNA ligase IV complex with DNA break resealing activity [48]. PARP-1 has been shown to interact with DNA-PK_{CS} [49] and Ku70/Ku80 proteins [50] in NHEJ and to catalyze their poly(ADP-ribosyl)ation. It is furthermore suggested that PARP-1 operates in an alternative pathway of NHEJ that functions as backup to the classical pathway. In that complementary pathway, PARP-1 acts together with a XRCC-1/DNA ligase III complex independent of the classical NHEJ proteins [51, 52].

The HR pathway operates during S/G_2 phase of the cell cycle using sister chromatid sequence as the template to mediate faithful repair. It is thought that PARP-1 has a regulatory function in HR and protects this pathway from interference by NHEJ [53], but the precise mechanism is very complex.

Functions in cell cycle regulation and transcription

As mentioned, PARP-1 activation and poly(ADP-ribosyl)ation initiates a series of events designed to control processes as DNA repair, but also cell cycle arrest and, if necessary, cell death. One part of this signalling includes the regulation of the tumor suppressor protein p53, which can be covalently poly(ADP-ribosyl)ated or bind polymer in a non-covalent way [54]. p53 preserves genomic integrity by delaying cell cycle progression or induction of apoptotic cell death via the transcription of p53-responsive genes [55].

PARP-1 regulates gene expression via poly(ADP-ribosyl)ation of p53 [56], but can also act as transcriptional cofactor with other transcription-related factors [57]. One is the interaction with the transcription factor nuclear factor kappa B (NF-κB), which is activated by oxidant stress, leading to increased expression of inflammatory cytokines [58], linking PARP-1 with pathological processes such as a variety of inflammatory diseases [59].

Functions in genomic stability

The versatile implication in the regulation of chromatin structure, repair pathways and cell cycle control directly links PARP-1 with the maintenance of genomic integrity.
As a consequence of inhibition or abrogation of PARP-1 activity, an increase in strand break formation or chromosomal aberrations was observed after genotoxic exposure, resulting in sister chromatide exchanges (SCE) or higher micronucleus frequencies [60-62]. Conversely, the over-expression of PARP-1 suppressed alkylation-induced SCE formation [63]. Together these data suggest that a fundamental function of PARP-1 is to maintain the stable genomic replication by limiting chromosomal damage thus safeguarding the genome.

Functions in cell death

Apart from the function in damage sensing and DNA repair, PARP-1 is involved in processes of cell death. The basal PAR level in unstressed cells is usually quite low, but can be intensified immediately after genotoxic insults as described above. Depending on the magnitude of DNA damage, PARP-1 and its product PAR acts as a survival factor and therefore maintain genomic integrity under moderate stress conditions. In this scenario a bearable amount of NAD^+ is used for polymer synthesis. In contrast, drastic NAD^+ depletion [64] after hyperactivation of PARP-1 can lead to cell death under severe stress conditions as concomitantly cellular ATP stores are diminished leading to energetic catastrophe, ultimately resulting in necrotic cell death [65]. This mode of action is apparent in inflammatory diseases or neurodegenerative disorders [66, 67] and directly links PARP-1 with pathophysiology.
Aside from the attendance of PARP-1 in necrotic cell death, it appears to be also involved in aspects of apoptosis. On one hand by release of apoptosis inducing factor (AIF) from mitochondria to the nucleus, which induces peripheral chromatin condensation, DNA

fragmentation and cytotoxicity [68]. PAR and free ADP-ribose are suggested to be the executors of this cell death program mediated by PARP-1. On the other hand PARP-1 is cleaved during apoptotic process by caspases under severe conditions [69], thus preventing PARP-1 overactivation and energy crisis.

Sirtuins

The sirtuins are a family of NAD^+-dependent enzymes, related to yeast silent information regulator 2 (Sir2) [70], which is implicated in suppressing senescence and ageing by transcriptional regulation [71].

In general, sirtuins remove acetyl groups at lysines from targets proteins under consumption of NAD^+, thereby releasing nicotinamide (NAM) and O-acetyl-ribose and the deacetylated substrate [72] (Figure 3). However, some family members, like SIRT-6, also possess ADP-ribosylation activities.

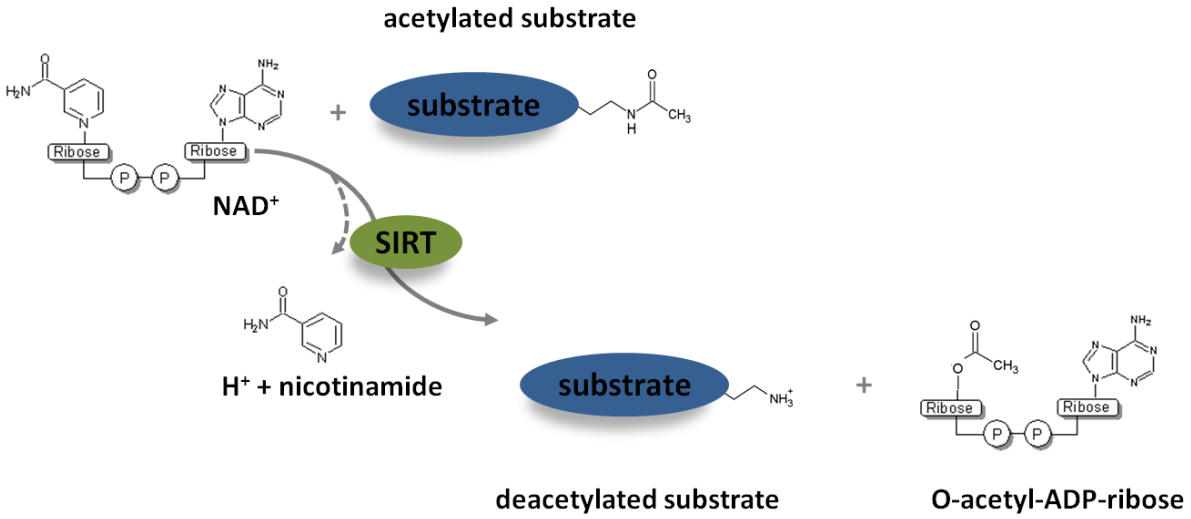

Figure 3: SIRT mediated deacetylation reaction.

Sirtuins act as deacetylases using NAD^+ to cleave acetyl groups from acetyl lysine residues of target proteins generating NAM, the deacetylated substrate and 2-O-acetyl-ADP-ribose.

In mammalians there are seven homologues, SIRT-1 to SIRT-7, which are differently localized in the cell, target multiple substrates and affect a broad range of cellular functions. In the context of NAD^+ depletion by PARPs the nuclear sirtuins SIRT-1, SIRT-6 and SIRT-7 are of significance, as they are predominantly affected by modulated NAD^+ levels resulting from

polymer formation. SIRT-1 was shown to be activated in presence of high levels of NAD^+, whereas high concentrations of NAM and/or NADH tend to inhibit the activity [73].

Functions of sirtuins

SIRT-1 can modulate the chromatin functions through direct deacetylation of histones, thus promoting chromatin silencing by compaction and the repression of transcription [70, 74]. In addition, SIRT-1 can interact and deacetylate a broad range of transcription factors and coregulators, thereby regulating target gene expression both positively and negatively.
One SIRT-1 target proteins is p53 [75]. The deacetylation of p53 at lysine 382 leads to the inactivation of its transcriptional activity which attenuates stress induced apoptosis [76]. Furthermore, it promotes cell survival by initiating cell cycle arrest and DNA repair through Forkhead box class O(FOXO) transcription factor [77] and Ku70 [78] or by suppressing NF-κB-dependent inflammatory responses [79]. Additionally, peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) is deacetylated, thereby controlling mitochondrial function, fatty acid oxidation and glucogenesis [80, 81].
In summary, SIRT-1 is involved in response to cellular damage and to metabolic imbalances triggered by stress, fasting, exercise, nutritional deficiencies and caloric restriction.

Another nuclear sirtuin, which is implicated in DNA repair, is SIRT-6, which acts as ADP-ribose transferase [82]. Recently it was published, that SIRT-6 is recruited to DSBs and thereby stimulates PARP-1, finally promoting DNA repair [83]. SIRT-6 knockout cells exhibit genomic instability and are hypersensitive to DNA damage [84], making this enzyme another NAD^+-dependent protein controlling DNA damage-mediated responses apart from PARP-1/2 and SIRT-1.

Features about NAD^+

The intracellular NAD^+ pool serves as energy metabolite in redox reactions, but is also a direct substrate for mono/poly(ADP-ribosyl)ation and NAD^+-dependent deacetylases, thus is consequently linked to their respective functions. In addition to protein modifications, it

serves as substrate for signalling molecules in cADP-ribosylation reactions implicated in calcium signalling [85] (Figure 4).

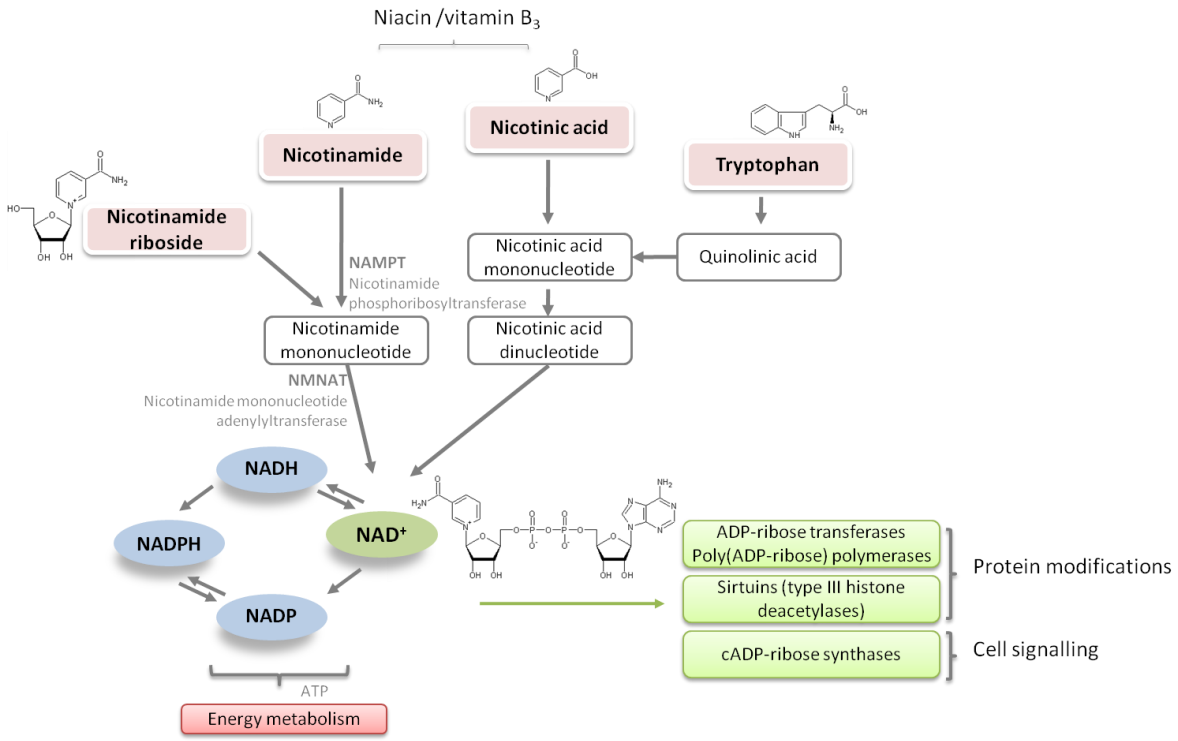

Figure 4: NAD$^+$ biosynthesis.

Simplified schematic view of the reactions involved in NAD$^+$ biosynthesis. NAD$^+$ can be synthesis from different precursors, de novo from tryptophan or through a salvage pathway and is used as substrate for diverse cellular reactions.

The physiological concentration in human cells was reported to range from 200-700 µM [86], but dramatically decreases upon genotoxic stimulation [87], correlating with polymer formation, as PARP-1 is the main NAD$^+$ consumer under stress conditions.

Although the synthesis occurs in several cellular compartments, the most relevant NAD$^+$ pool for PARP-1 and SIRT-1 is the nuclear one [88].

Nicotinic acid (NA) and nicotinamide (NAM) - collectively termed vitamin B3 or niacin - are together with nicotinamide riboside (NR) [89] and the amino acid tryptophan precursors for NAD$^+$ synthesis. Via different intermediates these precursors are metabolized *de novo* or via a salvage pathway and integrated in the pyridine nucleotide pool. In the final step nicotinamide

mononucleotide is converted into NAD^+ via the enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT), which regulates PARP-1 [90] as well as SIRT-1 [91] activity, supporting an interplay between all tree pathways. The rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) is also considered an important regulatory enzyme with respect to SIRT-1 [91, 92].

NAD^+ can be converted to its reduced form NADH or phosphorylated to NADP/NADPH, respectively. But only the oxidized NAD^+ is by PARPs or sirtuins as substrate. The accessible part of the cellular NAD^+ for nuclear proteins is about 30% and exchangeable between the nucleus and cytosol, however the main part is stored in mitochondria [93] serving there in energy metabolism.

SIRT-1, PARP-1 and their interplay with NAD^+

Based on the fact that both PARP-1 and SIRT-1 utilize NAD^+ for their activity and act in many common pathways, a cross talk between these proteins has been suggested [94]. The increased activity of one enzyme might interfere with the activity of the other when both proteins compete for the same substrate.

The affinity of PARP-1 for NAD^+ is high, the reported *in vitro* K_m values ranging from 20-60 µM [95] and for SIRT-1 between 100-200 µM [96], indicating that PARP-1 has a 5 to 10 fold higher affinity for NAD^+. As steady state levels in unstressed cells are usually much higher than the respective K_m values, the activities of both enzyme are not affected, but under stress conditions potentially compromised. It was shown that PARP-1 overactivation suppresses the activity of SIRT-1 by depleting cellular NAD^+ levels, thus triggering cell death. [97].
It is conceivable that upon genotoxic treatment PARP-1 is activated and incorporates the ADP-ribose unit from NAD^+ in polymer, decreasing the NAD^+ to 10-20 % of their normal levels [87, 98]. During that reaction nicotinamide and a proton is released and NAD^+ concomitantly reduced, therefore transiently limiting the deacetylase activity of SIRT-1 by diminishing the substrate availability. Chromatin relaxation will be supported by poly(ADP-ribosyl)ation and temporarily sirtuin activity will be further inhibited due to increased NAM levels. Of note,

nicotinamide is also an inhibitor for PARP-1, but of low potency, suggesting a negative feedback due to product-inhibition after prolonged activity.

PARP-2 binds upon genotoxic stress to the SIRT-1 promoter, thereby inhibiting its transcription and reducing SIRT-1 levels [99]. Together, PARP-1 and PARP-2, when activated upon damage, collectively decrease SIRT-1 activity through different means. Recently it was reported that the deletion of either the PARP-1 or -2 gene in mice enhances SIRT-1 activity, leading to a increased mitochondrial biogenesis [100].

However, there is also evidence, that SIRT-1 negatively influences PARP-1 activity by deacetylation, as reported for cardiomyocytes [101], suggesting that both are able to counterbalance each other's activity.

Furthermore, both enzymes modulate common target proteins, including histones or p53, via their respective activities.

In a simplified context, PARP-1 and SIRT-1 regulate fundamental cellular and physiological processes that are thought to be involved in sustaining cellular healthiness and delaying the pathogenesis of age-associated complications [102-104]. Of importance seems to be the adequate NAD^+ level, which is crucial for adequate PAR synthesis and sirtuin activity, respectively (Figure 5).

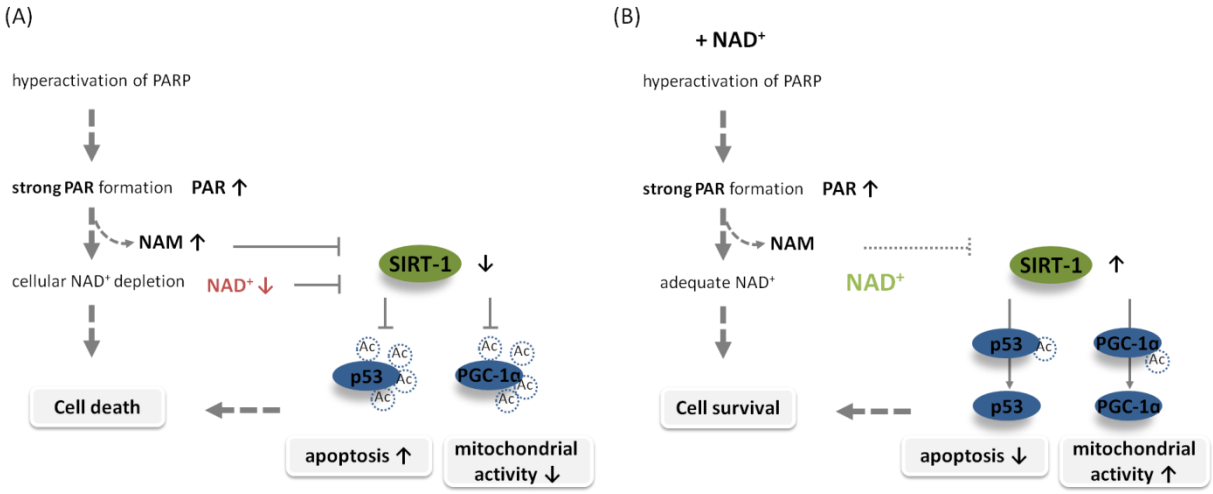

Figure 5: Schematic model of PARP-1 activation and the influence on SIRT-1 functions according to NAD^+ availability.

(A) Upon severe DNA damage, PARP-1 is massively activated producing enormous amounts of PAR. SIRT-1 activity is robustly reduced, as PARP-1 activation limits NAD^+ bioavailability and increases inhibitory NAM levels. Attenuation of SIRT-1 activity results in hyperacetylation of target proteins as pro-apoptotic factor p53 or PGC-1, which strongly impairs metabolic activity, consequently tilting the balance toward cell death. (B) Augmentation of cellular NAD^+ pools may help to maintain adequate NAD^+ levels even under circumstances of massive PAR formation, thus increasing deacetylase activity of SIRT-1 and cellular survival.

Therapeutic potential of NAD^+

The importance of NAD^+ in health and disease is indicated by studies showing the consequences of niacin deficiency or pathophysiological alterations in the NAD^+ metabolism. Nutritional deficits occurring from malnutrition, alcoholism or during cancer therapy can lead to diarrhea, dermatitis, dementia or even death, also classical symptoms of pellagra [105]. Restoring the physiological NAD^+ pool by supplementation with NAM or NA is therefore often used as therapeutic tool.

This strategy is also beneficial to mitigate the NAD^+ loss and resulting tissue damage after ischemia and stroke [106-108]. Stroke injury is caused by an acute blockage of arterial blood flow to the brain, causing oxygen starvation of the affected tissues. When the deprived tissue becomes reperfused with oxygen again, a cascade of serious negative consequences begins. The production of reactive oxygen species provokes oxidative stress, thus inducing DNA and extensive tissue damage caused by PARP-1 over activation and concomitant loss of NAD^+.

Furthermore, preservation of NAD^+ levels was effective in neurodegenerative disorders [109, 110]. The activation of SIRT-1 in models for Parkinson's or Alzheimer's disease significantly improved the pathology. It is supposed that the effects are mediated by PGC-1, an important regulator of oxidative stress defence proteins [111].

Nicotinic acid, combined with other drugs, is clinically used as cholesterol lowering compound due to its artheroprotective effects [112].

As outlined in the above sections, the intracellular NAD^+ metabolism has a significant function in the overall cellular metabolism, as cofactor for individual enzymes and as sole substrate for NAD^+ consuming proteins, including PARPs and sirtuins. Thus it displays an attractive point of intervention for the treatment of various pathologies as highlighted.

Strategies to modulate PARP-1 activity and poly(ADP-ribosyl)ation

The diverse implication of PARPs in a variety of biological processes, not least due to their major impact on global NAD^+ metabolism, makes the modulation of their activity a clinical

target. There are different approaches that are experimentally or clinically used to modulate PARP activity.

Modulation of PARP activity & poly(ADP-ribosyl)ation

Experimental approaches to suppress cellular PAR formation

Classically the ablation of the PARP gene or protein using knock-out systems [61, 113, 114] or by knock-down approaches [115] was used to identify the cellular impact of poly(ADP-ribosyl)ation. Additionally, the trans-dominant inhibition offers a molecular genetic system to inhibit PARP in living cells, which employs the selective overexpression of the DNA-binding domain of PARP as a dominant negative mutant [116].

In order to diminish the massive PARP induction, PAR formation and NAD^+ depletion, a number of chemical PARP inhibitors were developed for clinical use [117]. Most inhibitors are structurally based on the substrate NAD^+ and act on a competitive level. They are potential candidates to preserve tissue integrity after oxidative stress occurring e.g. during ischemia injury or for the therapy of neurodegeneration and neuroinflammation [118]. These therapeutic applications address the same purpose as the earlier mentioned NAD^+ therapies.

More importantly, PARP inhibitors seem to evolve as promising drugs in cancer treatment [119]. PARP inhibitors have been shown to (i) sensitize tumour cells to DNA-damaging agents in chemo- or radiotherapy [120, 121] and (ii) selectively kill cancers that are defective in distinct DNA repair pathways, e.g. BRCA mutated cells which harbour defects in HR repair [122]. There are currently diverse clinical trials employing PARP inhibitors either in combination with chemotherapeutic agents exploiting the described synthetic lethality or as stand-alone treatment of HR-deficient tumours.

Strategies to intensify PAR formation

Apart from the beneficial effects of the inhibition, there is also a scientific interest to increase PARP activity and thereby support DNA repair and maintenance of genomic stability. Strategies to intensify the poly(ADP-ribosyl)ation reaction range from genetic approaches as the overexpression of the PARP protein [63, 123] or knock-ins [124] to the application of

substances that are able to potentiate the poly(ADP-ribosyl)ation capacity [125, 126]. One indirect alternative is the modulation of the substrate levels of NAD^+, which was used in our study (Chapter I-II) to investigate the effects of altered PAR levels on diverse biological aspects.

Strategy to modulate intracellular NAD^+ levels

As already mentioned, the cellular NAD^+ levels are maintained by the conversion of NA, NAM, NR or tryptophan. Theoretically, all four precursors can increase NAD^+ when applied additionally in an experimental approach. However, not all cells respond equally well to a supplementation. Dependent on the expression profile of intra- and extracellular transporters or proteins of the NAD^+ biosynthesis pathways (e.g. NMNAT enzymes), distinct precursors are predominantly used for NAD^+ synthesis [127]. Animal feeding studies showed that exogenously added NA is able to increase NAD^+ levels in blood or organs like kidney and liver with higher efficiency compared to NAM [128].

Beside its role as NAD^+ precursor, NAM directly inhibits NAD^+ consuming enzymes by binding to a conserved pocket, which is crucial for substrate binding and catalysis [129], making NAM not the ideal system to intensify or enhance PARP activity and associated cellular processes. In contrast to NA, which causes flushing at higher concentrations and NAM, which has inhibitory effects, NR would be a better potent precursor.

Interestingly, some cells are able to use extracellular added NAD^+ for biosynthesis as reported for astrocytes [130] and neuronal cells [131].

Apart from the addition of extracellular precursors, the overexpression of distinct enzymes of the NAD^+ biosynthesis pathway was shown to increase NAD^+ levels and SIRT activity, respectively. Increased expression of NAMPT, which is responsible for recycling nicotinamide to NAD^+, resulted in elevated intracellular NAD^+ levels [92]. Another option to maintain cellular NAD^+ is the addition of an activator of AMP-activated protein kinase (AMPK), which was reported to increase NAD^+ concentrations and SIRT-1 activity [132].

OBJECTIVE

To date the main focus on PARP research relies on the attempt to inhibit its activity to either specifically kill tumour cells or prevent from pathophysiological outcomes, which is tightly associated with the intolerable decline of NAD^+. However, permanent inhibition of PARP may also be linked with decreased genomic stability due to impairment of DNA repair functions. For that reason alternative attempts modulating PARP-1 activity and its related functions are of scientific interest, in order to maintain cellular integrity.

Objective of this study was to promote the PARP-mediated cellular poly(ADP-ribosyl)ation by increasing the basal NAD^+ level and to investigate the stress-related biological responses and effects in a human system.

According to the magnitude of damage, PARP-1 can critically affect NAD^+ levels thus influencing deleterious or health-enhancing processes. Under mild genotoxic stress, poly(ADP-ribose) formation facilitates DNA repair by recruitment of DNA repair factors and modification of the chromatin structure, whereas severe genotoxic stress and the ensuing overactivation of PARP-1 induces cellular NAD^+ depletion, energy failure and ultimately cell death. To cover both situations, biological relevant but also severe DNA damaging conditions, inducing massive PAR formation, were investigated for their respective consequences.

Firstly, the effect of the *ex vivo* added NAD^+ precursor nicotinic acid on primary human, mononuclear blood cells was evaluated, considering the optimal concentrations to get an effective augmentation.

In a second step the direct influence on PAR formation was monitored. To gain insights, if the NA supplementation and the associated stronger poly(ADP-ribose) formation is able to promote cellular processes, three specific pathways were analyzed.

This includes the impact on cell death rates, with the distinction between apoptosis and necrosis. As PARP-1 activation is implicated in both pathways, it is of interest whether cells undergo apoptotic or necrotic forms of cell death when NAD^+ pools are maintained even in acute genotoxic situations. This can play an important role in the development of tissue pathologies.

Depending on the level of DNA damage and intracellular NAD^+ status, PARP-1 and its product PAR mediate the recruitment of DNA repair factors to sites of lesions, facilitate DNA repair and thus maintain genomic integrity. Therefore the impact on DNA damage and repair was determined in regard of time- and dose dependent aspects. To access modulations at the chromosomal level, the impact on genomic stability was analysed by scoring micronuclei as marker.

To account for the fact that not only PARPs, but also the NAD^+-dependent sirtuins can be influenced by improving the intracellular NAD^+ levels, their activity was determined in addition.

In summary, these scientific findings should help to understand, if there is a benefit for cellular key process by supporting PARP via NAD^+ modulation under stress conditions. The intracellular levels of PAR and NAD^+ are important parameters for biological responses to genotoxic stress and influence diverse cellular functions including DNA repair or maintenance of genomic stability. Notably, loss of genomic stability is a hallmark of both carcinogenesis and the ageing process. Together, the data could allow for the design of more effective pharmacological interventions in that context.

CHAPTER I

Ex-vivo supplementation with nicotinic acid enhances cellular poly(ADP-ribosyl)ation and improves cell viability in human peripheral blood mononuclear cells

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Abstract

Poly(ADP-ribosyl)ation is a posttranslational modification of proteins, which is mainly catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) by using NAD^+ as substrate and is directly triggered by DNA strand breaks. Under mild genotoxic stress poly(ADP-ribose) (PAR) formation plays an important role in DNA repair whereas severe genotoxic stress and the ensuing overactivation of PARP-1 induce cellular NAD^+ depletion, energy failure and ultimately cell death. We are interested in studying the consequences of moderately enhanced enzymatic activity under conditions of DNA damage. Here we chose supplementation of cells with the NAD^+ precursor nicotinic acid (NA) as a strategy. In order to reliably assess PAR accumulation in living cells we first developed a novel, sensitive flow-cytometric method for the rapid analysis of poly(ADP-ribose) accumulation (RAPARA). Our data showed that *ex vivo* supplementation of human peripheral blood mononuclear cells (PBMC) with low concentrations of NA significantly raised cellular NAD^+ levels by 2.1-fold. Upon X-irradiation or exposure to hydrogen peroxide or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, PAR accumulation was significantly increased and sustained in NA-supplemented cells. Furthermore, NA-supplemented PBMC displayed significantly higher cell viability due to a lower rate of necrotic cell death. In summary, *ex-vivo* supplementation of human PBMC with NA increases cellular NAD^+ levels, boosts the cellular poly(ADP-ribosyl)ation response to genotoxic treatment, and protects from DNA-damage-induced cell death.

Introduction

Poly(ADP-ribosyl)ation is a reversible posttranslational modification of cellular proteins, which is greatly induced after DNA damage [133]. The reaction is catalyzed by poly(ADP-ribose) polymerases (PARPs), a family of enzymes encoded by 17 different genes in the human genome [134, 135]. PARP-1 is the best studied family member and is responsible for about 90% of cellular poly(ADP-ribose) (PAR) formation after genotoxic stress [18]. PAR formation has been implicated in several cellular processes including DNA repair [44], transcription, chromatin remodelling, and maintenance of genomic stability [136]. After infliction of DNA damage, PARP-1 binds via its zinc finger motifs to DNA single and double strand breaks [21]

and synthesizes long polymers of up to 200 units of ADP-ribose on different target proteins, mainly PARP-1 itself, using NAD^+ as substrate [4]. The negatively charged polymers have been shown to recruit different DNA repair proteins of the base excision repair (BER) pathway like XRCC I, DNA polymerase β and DNA ligase III [39, 41]. Probably due to charge repulsion, automodified PARP-1 is released from DNA breaks, allowing repair to proceed [137]. PARP activity is counteracted by the enzyme poly(ADP-ribose) glycohydrolase (PARG), of which several splice variants are expressed. PARG possesses exonucleolytic as well as endonucleolytic degrading activity against PAR [13, 138]. Thus, the polymer level detectable in live cells represents a steady state of anabolic and catabolic activity. However, excessive activation of PARP-1 under circumstances of massive DNA damage induces (i) the depletion of the cellular NAD^+ pool, resulting in energy failure concomitant with necrotic cell death [65], or (ii) PAR-dependent release of apoptosis inducing factor (AIF) from mitochondria, thus triggering a caspase-independent form of apoptosis[68]. In mammalian cells, NAD^+ can be synthesized *de novo* from tryptophan and in the so-called salvage pathway either from nicotinic acid (NA) or nicotinamide, collectively termed niacin or vitamin B3 [139], or from nicotinamide riboside [89]. Cellular supplementation with NA increases the NAD^+ pool, thus preventing the extensive loss of NAD^+ [128]. Cells growing in medium without nicotinamide have much lower NAD^+ content and as a consequence, DNA repair is negatively affected when such cells are exposed to genotoxic agents [140]. *In vivo* studies on the influence of niacin deficiency [141-143] revealed a decrease in NAD^+ levels in cells of different tissues accompanied with an impaired PAR content under these conditions. On the other hand, additional intake of NA *in vivo* increases bone marrow cell NAD^+ levels as well as poly(ADP-ribosyl)ation [143]. However, those studies measured PAR content several hours after the treatment with alkylating agents, when cellular response to genotoxic agent could already have influenced other processes. Therefore, it was not defined when and to what extent PARP-1 has been activated initially, as the DNA damage was caused over a longer period of time. But it is important to know if NA supplementation, either in the short term or in the long term, has an impact on the immediate response to DNA damage concerning the duration or intensity of PAR production, as PARP-1 (over)activation is relevant in the pathophysiology of inflammatory or neurodegenerative diseases. The modulation of NAD^+ /PAR metabolism by pharmacological NA supplementation could be an alternative application for pharmacological intervention by altering the activity of PARP-1 and preserving cellular NAD^+ levels to reduce

inflammatory responses. To gain a more detailed insight into the sequence of events leading to DNA damage-driven poly(ADP-ribosyl)ation, a higher time resolution and improved detection method is needed. To our knowledge, no study has been published that shows direct consequences of NA supplementation on the duration and intensity of the poly(ADP-ribosyl)ation response to genotoxic treatment and to survival in normal human cells.

Therefore, the goal of our study was to determine the short-term effect of NA supplementation in freshly isolated human peripheral blood mononuclear cells (PBMC) exposed to DNA damaging treatment *ex vivo*. We addressed the question if increased availability of NAD^+ can lead to an enhanced cellular poly(ADP-ribosyl)ation response to DNA damage and, as a consequence, to improved cell viability as a long-term effect. To investigate PAR formation in intact cells, a new flow cytometric assay for the detection of cellular PAR in human PBMC had to be established. Using this method termed RAPARA (rapid analysis of poly[ADP-ribose] accumulation), we could show that supplementation with NA indeed leads to a increased and sustained poly(ADP-ribosyl)ation after X-irradiation, hydrogen peroxide or MNNG treatment in PBMC, further demonstrating that the cellular NAD^+ content is a critical determinant for the level of PAR formation. In addition, the supplemented cells showed a higher viability after DNA damage.

Materials and Methods

Cells

Blood samples (collected in citrate S-Monovettes from Sarstedt, Nümbrecht, Germany) were obtained from healthy male or female donors aged 20 - 45. The research was conducted in accordance with the Declaration of Helsinki, and ethical clearance was obtained from the University of Konstanz Ethics Committee. For all experiments freshly isolated PBMC were used. These were cultured between 5 h and 1 day in RPMI containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2 in a humidified atmosphere. Medium and supplements were purchased from Invitrogen (Darmstadt, Germany), FCS from Biochrom (Berlin, Germany).

Isolation of PBMC

Ten ml freshly obtained peripheral venous blood was diluted with 20 ml phosphate buffered saline (PBS; 137 mM NaCl [Carl Roth, Karlsruhe, Germany], 3 mM KH_2PO_4, 10 mM Na_2HPO_4 pH 7.4 [Sigma-Aldrich, Munich, Germany]), placed on top of 15 ml Biocoll (Biochrom) in a 50-ml conical tube and centrifuged without brake at 800 *g* for 10 min at room temperature. The PBMC recovered were washed twice with PBS and used for the experiments or kept in culture [144].

Supplementation of PBMC with nicotinic acid

For each experiment, one half of the freshly isolated PBMC were supplemented with 15 µM NA (Merck, Darmstadt, Germany) for at least 5 h. The other half was incubated in the culture medium without NA supplementation as a control.

PARP inhibition

Cells were incubated with PARP inhibitor PJ-34 (Enzo Life Sciences, Lörrach, Germany) for at least 5 h at final concentrations of 0.03 µM, 0.3 µM or 3 µM before and during genotoxic exposure.

Determination of PARP-1 activity in intact PBMC by flow cytometry

Aliquots of 5 x 10^5 – 1 x 10^6 cells resuspended in 80 µl PBS were placed into each well of a 96-well v-bottom plate (Sarstedt). Cells were X-irradiated (RT 100, Müller, Hamburg, Germany) on ice at doses between 0 and 25 Gy, incubated at 37°C for the time periods indicated and subsequently fixed and permeabilized with 180 µl methanol (Sigma-Aldrich) /acetic acid (3+1) (Carl Roth) for 5 min at room temperature. Alternatively, cells were treated with hydrogen peroxide (Merck) at final concentrations as indicated for 3 minutes at 37°C or with MNNG (Sigma-Aldrich) for 10 minutes at room temperature to induce PAR synthesis. Both chemicals were removed by centrifugation, 200 *g* for 3 minutes, and a further washing step with PBS. Cell pellets were resuspended with 80 µl PBS and subsequently fixed as described.

Cells were centrifuged at 1,180 *g*, washed twice with FACS buffer (PBS; 0.5% FCS; 2 mM NaN_3 [Merck]) and incubated with 100 µl primary antibody 10H (5 µg/ml) recognizing PAR [145] for 1 h at room temperature. Cells were centrifuged at 1,180 *g* and washed twice with 200 µl FACS buffer before incubated with the Alexa488-labeled goat-anti-mouse secondary antibody

(Invitrogen) for 1 h at room temperature. Finally, cells were centrifuged at 1,180 *g*, washed twice with FACS buffer and kept on ice until flow cytometric analysis was performed (FACS Calibur II or LSR II, Becton Dickinson, Heidelberg, Germany). A total of 10,000 events for each sample were acquired and analyzed using FlowJo V7 software (Tree Star Inc., Ashland OR, USA).

Assessment of cell viability by flow cytometry

Cell viability was determined using Annexin V staining (Enzo Life Sciences) according to the manufacturer's protocol. Cells were irradiated with 0 or 5 or 25 Gy. After 24 h, cells were washed with PBS and 10^6 cells were resuspended 1 ml binding buffer (140 mM NaCl [Carl Roth], 10 mM HEPES and 2.5 mM $CaCl_2$ [both from Sigma-Aldrich], pH 7.4). For each treatment 100 µl of the cell suspension was mixed with 5 µl annexin V and propidium iodide (final concentration 1 µg/ml; Sigma-Aldrich) and incubated in the dark at room temperature. After 15 min, 400 µl of binding buffer was added to each sample and flow cytometric analysis was performed (FACS Calibur II or LSR II, BD). A total of 10,000 events for each sample were acquired and analyzed using FlowJo V7 software (Tree Star).

Quantification of cellular NAD^+ content

The cellular NAD^+ content was determined using a modified NAD^+ cycling assay [146]. For each treatment 3-5 x 10^6 cells were resuspended in 500 µl PBS, X-irradiated as indicated (0-25 Gy) and incubated at 37°C for different time periods (0-15 min). If treated with H_2O_2 or MNNG, the cells were subsequently centrifuged, washed and resuspended in 500 µl PBS. Thereafter, cells were precipitated with 0.5 M $HClO_4$ on ice. After 15 min samples were centrifuged at 1,500 *g* for 10 min and the supernatant was combined with 350 µl of 1 M KOH, 0.33 M K_2HPO_4, 0.33 M KH_2PO_4 followed by incubation on ice for 10 min. All chemicals were from Sigma-Aldrich. Cells were centrifuged at 1,500 *g* for 10 min and the supernatant was frozen at -20°C before NAD^+ determination by an enzymatic cycling assay [146].

Statistical analysis

All statistical analyses were done with Prism or InStat (GraphPad Inc., La Jolla CA, USA). If two groups were compared, a two-tailed paired t-test was performed. For the comparison of

more than two groups, analysis of variance (ANOVA) was used. A p-value < 0.05 was considered significant.

Results

Establishing a new method to detect PARP activity and determination of cellular PAR contents in intact cells

To determine cellular PAR accumulation in intact cells, a new flow-cytometry based assay termed RAPARA (rapid analysis of poly[ADP-ribose] accumulation) was established.

Using this new assay, it is possible to closely monitor the time-dependent (Figure 1A) as well as dose-dependent (Figure 1B) cellular polymer formation after irradiation with high resolution. Upon irradiation with 25 Gy we detected significant induction of PAR formation already after 1 min of incubation at 37°C ($p<0.05$) (Figure 1A).

At the time of highest PAR accumulation (*i.e.*, between 3-5 min after irradiation), we were able to measure induction of PARP activity already after a dose of 0.5 Gy. PARP activity was significant different from control when irradiated with 2 Gy ($p<0.05$) or higher doses (Figure 1B).

The results confirm that poly(ADP-ribosyl)ation is a very fast response to DNA damage in PBMC, as there is a detectable increase even after one minute of incubation, and maximum PAR accumulation can be observed already after 3-5 min in this cell type. For comparison, Supplementary Figure S1 shows a western blot analysis of whole cell lysates of cells treated with X-irradiation. With this standard technique PAR formation is clearly detectable after high dose irradiation, but compared to the RAPARA assay lacks sensitivity in the low dose range.

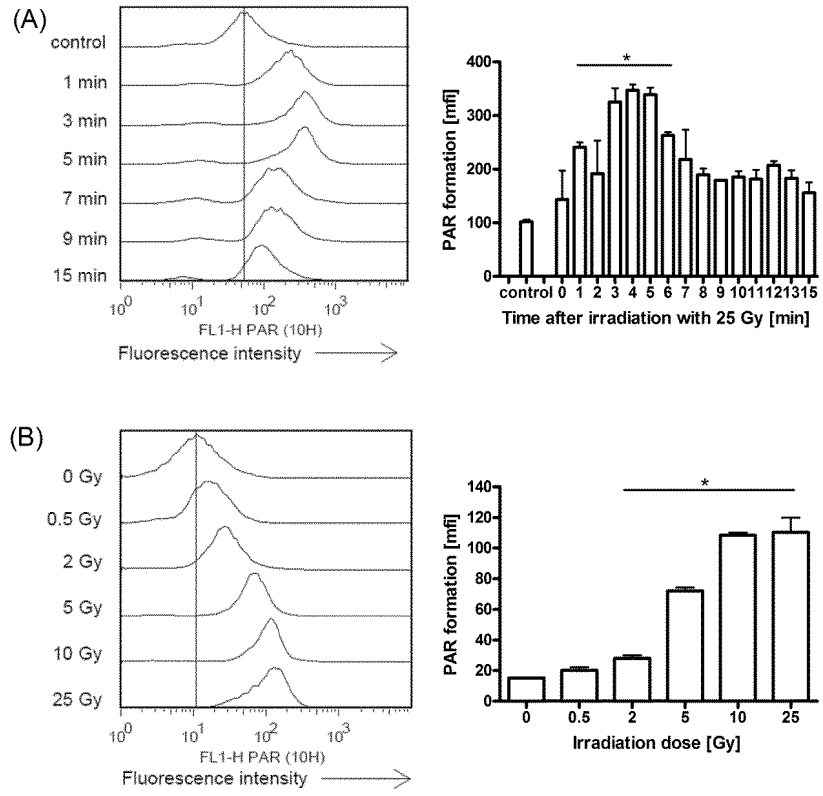


Figure 1: Evaluation of RAPARA for the detection of poly(ADP-ribosyl)ation in intact PBMC in response to X-irradiation.

Single-parameter histograms of poly(ADP-ribose)-associated mean fluorescence intensities and quantitative evaluation are displayed.

A: Cells were treated with 25 Gy and incubated at 37°C for different time periods. PAR level is increased up to 4 min after damage; thereafter the PAR-degrading activity of PARG exceeds PAR synthesis. A significant increase is detectable from 1 minute on (*p<0.05, repeated measures ANOVA). Control represents non-irradiated cells. B: Cells were treated with different doses of X-irradiation and incubated at 37°C for 4 min. There is already a noticeable increase in polymer formation after an irradiation dose of 0.5 Gy and a significant increase after a dose of 2 Gy (*p<0.05 repeated measures ANOVA), thus highlighting the high sensitivity of the new assay. Histograms are representative of one out of 3 measurements of one donor. Each bar represents triplicate analysis of PBMC from one donor and is expressed as mean ± SD.

Effect of nicotinic acid supplementation on cellular NAD^+ level

NA is a precursor of the PARP substrate NAD^+. Supplementation with NA was done immediately, if not indicated otherwise, after PBMC isolation in culture medium for 5 h, in order to ensure efficient uptake and metabolism of NA. To determine which concentrations of supplemental NA can induce increased biosynthesis of NAD^+ and therefore boost the NAD^+ pool, a range of concentrations were tested. Addition of increasing concentrations of NA to routine culture medium, which usually contains about 8.2 µM NA, elevated total cellular NAD^+

levels in PBMCs in a concentration-dependent manner (Supplementary Figure S2). Significant increase was observed above 5 µM. Robust and effective augmentation was achieved with a concentration of 15 µM NA (Table 1), which was therefore used for all subsequent experiments. An increase in NAD^+ level was recorded in the cells from 13/13 donors, regardless if freshly isolated or one day cultured cells (labeled with asterisk) were supplemented. Amongst the donors, there was no significant gender effect detectable with regards to basal NAD^+ levels or the effect of supplementation. Furthermore, supplementation had no effect on cell volume (200 μm^3 on average).

Table 1: Inter-individual variation in cellular NAD^+ concentration in human PBMC supplemented or not with NA.

m, male; f, female; asterisks indicate that cells were cultured for 1 day before NA supplementation. Two-tailed paired t-test was used for statistical evaluation.

Donor	Gender	Intracellular NAD^+ concentration [µM] without NA supplementation	Intracellular NAD^+ concentration [µM] with NA supplementation	Fold increase
01	m	194.6	295.5	1.5
02	m	170.0	307.3	1.8
03	m	174.9	382.5	2.1
04	f	181.8	380.4	2.1
05	f	190.0	489.0	2.6
06*	f	215.1	490.7	2.2
07*	f	239.5	579.1	2.4
08*	f	134.9	329.1	2.4
09*	f	230.9	560.3	2.4
10	f	202.4	390.7	1.9
11*	m	249.3	383.1	1.5
12	m	173.1	370.3	2.1
13	f	202.6	487.6	2.4
		Mean ±S.D.		2.1 ± 0.3

Effect of nicotinic acid supplementation on PAR accumulation

To induce cellular poly(ADP-ribosyl)ation, PBMC were X-irradiated with doses from 1 to 25 Gy and further incubated for different time periods. PAR accumulation was determined by RAPARA. For the dose-response experiments (Figure 2A and B), cells were incubated for 3 min after irradiation to measure the peak of cellular poly(ADP-ribosyl)ation, as was determined in Figure 1A. Our data revealed that the NA- dependent increase of NAD^+ levels also increased cellular PAR formation in PBMC, which was significant when treated with doses of 5 Gy and above (Figure 2A). The dose-dependent increase in poly(ADP-ribosyl)ation was enhanced when cells were supplemented with NA, with the strongest effect being apparent at the highest dose tested (25 Gy). In contrast, there was no change in the basal activity of PARP-1

by NA supplementation (Figure 2A). Concomitant with the increased poly(ADP-ribosyl)ation observed at higher irradiation doses, there was a decrease of cellular NAD^+ level, which was mitigated in cells supplemented with NA (Figure 2B). The results from the time-course experiments are perfectly in line with this observation (Figure 2C and D).

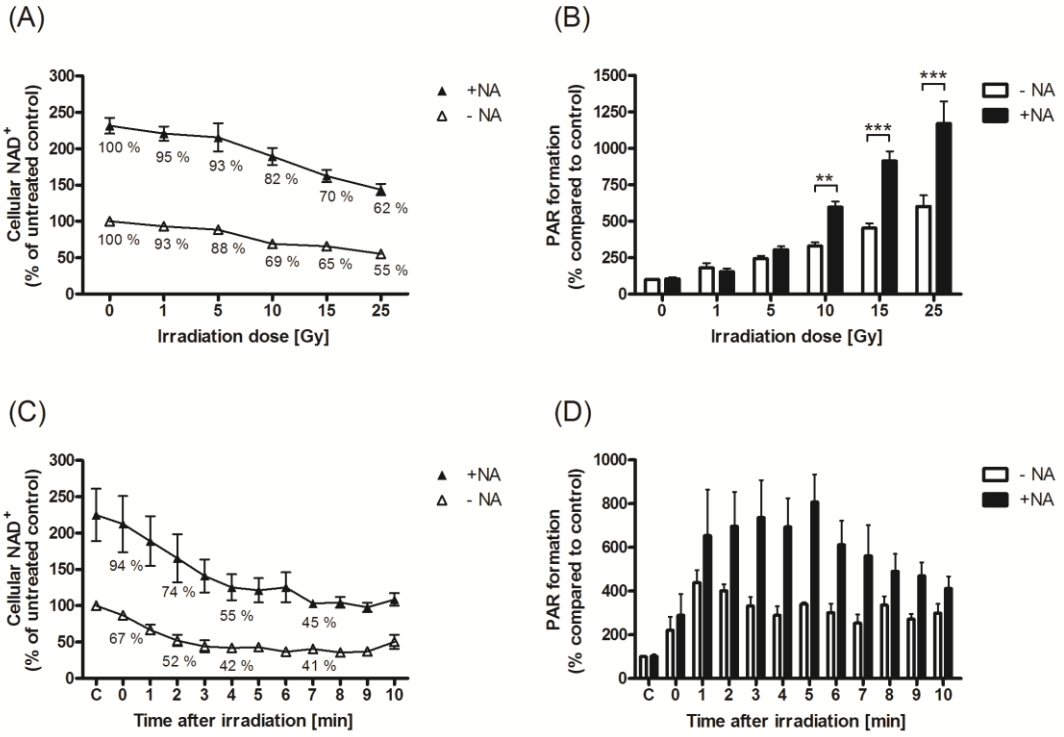


Figure 2: Effect of NA supplementation on PAR accumulation and the cellular NAD^+ level.

Poly(ADP-ribosyl)ation and NAD^+ was measured upon X-irradiation of PBCM. The cells were irradiated with doses as indicated and incubated for 3 min at 37°C (A and B) or PBMC were irradiated with 25 Gy and incubated at 37°C for different time points as indicated (C and D). Each bar represents the mean of triplicate measurements from three donors, respectively, and is expressed in % (± S.E.M.)

A: Dose dependent response of PARP-1 activity in NA-supplemented compared with non-supplemented controls. A significant difference was detected at 10 Gy or above (**p<0.01; ***p<0.001, repeated measures ANOVA). B: Effect of NA supplementation on the cellular NAD^+ concentration compared with non-supplemented controls after different irradiation doses. C: Time-dependent response of PARP-1 activity in non-supplemented and NA supplemented cells. D: Time-dependent effect of NA supplementation on the cellular NAD^+ concentration compared with non-supplemented controls.

The response of PARP-1 to DNA damage is time-dependent, showing a very fast maximum within the first minutes, before PAR becomes degraded (Figure 2C). In PBMC the highest level of PAR can always be detected at the same time point, *i.e.* 1 minute after irradiation, independent of the available NAD^+. Cellular NAD^+ levels dropped simultaneously within the first minutes after irradiation (Figure 2D). The total amount of both, PAR and NAD^+ was much

higher at all time points with NA supplementation than without. It is also apparent that the increased PAR level persisted longer in NA-supplemented cells.

Additionally we monitored PAR formation and cellular NAD^+ depletion in response to oxidant or alkylation-induced DNA damage. The DNA-damaging compounds used, H_2O_2 and MNNG, led to an increase of polymer formation (Figure 3A and C) with significantly higher levels in NA-supplemented cells. The effect on cellular NAD^+ consumption (Figure 3B and D) was comparable with that in Figure 2B, where DNA damage was induced by irradiation, showing that NAD^+ pools decline gradually with increasing PAR formation and independent of the DNA-damaging agent applied.

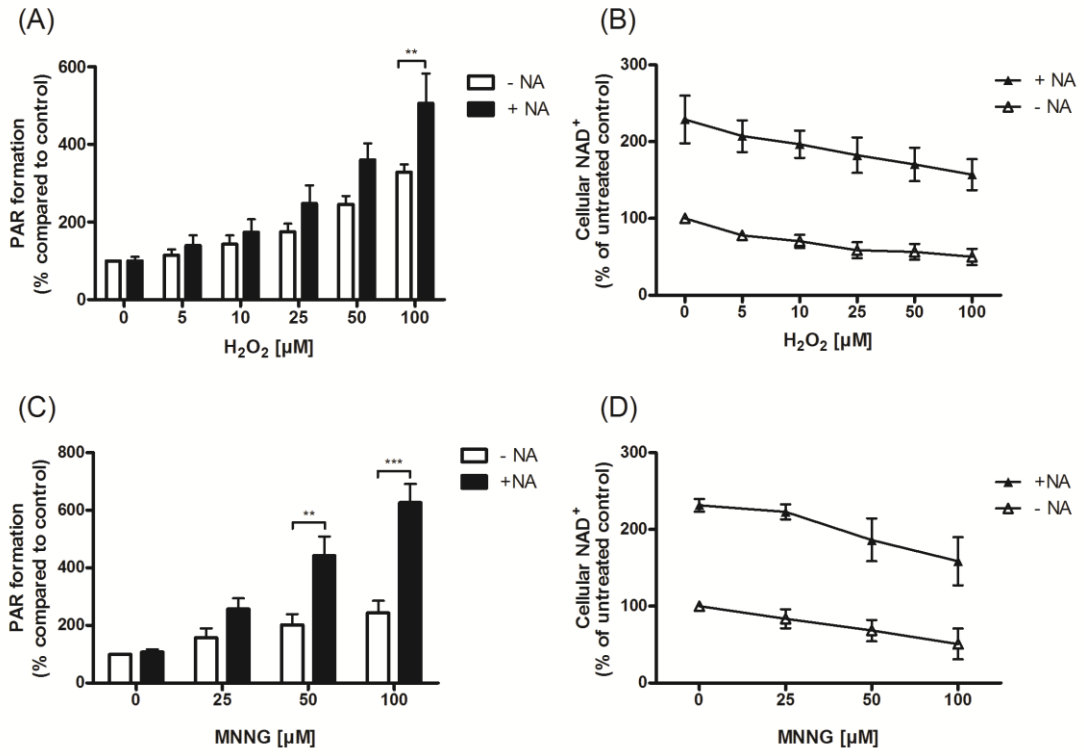


Figure 3: Effect of NA supplementation on PAR accumulation and the cellular NAD^+ level in response to oxidative stress (A and B) or alkylating agents (C and D).

Each bar represents the mean of triplicate measurements from four donors, respectively, and is expressed as % (± SEM). A, C: PAR levels induced by different concentration of H2O2 (A) or MNNG (C) were significantly higher for NA-supplemented cells as indicated (**p<0.01; ***p<0.001, repeated measures ANOVA).B, D: Cellular NAD^+ levels decreased in a concentration-dependent manner after treatment with hydrogen peroxide (B) or MNNG (D).

We used PARP inhibitor PJ-34 in a range of concentrations in order to monitor the impact on PAR formation and NAD^+ depletion (Figure 4). In response to 25 Gy X-irradiation the cells show concentration-dependent inhibition of PAR formation (Figure 4A). At the lowest concentration of PJ-34 the PAR level of NA-supplemented cells was still higher compared to control, but further increasing PJ-34 concentration led to a complete block of PAR formation. The drop of NAD^+ level after irradiation with 25 Gy was reduced as a function of PJ-34 concentrations (Figure 4B). Low concentrations of PJ-34 attenuated the consumption of NAD^+ partially, whereas PARP inhibition with 3 µM PJ-34 totally prevented NAD^+ loss, due to the inability of cells to produce PAR.

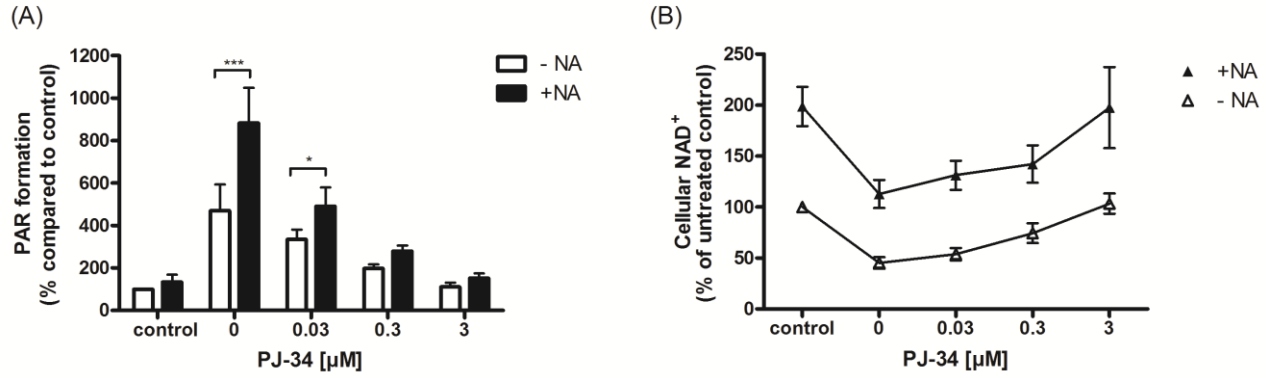


Figure 6: Effect of PJ-34 on PAR accumulation and NAD^+ levels after X-irradiation.

PBMCs were treated with PJ-34 as indicated and assayed for PAR formation and cellular NAD^+ concentration 3 min after irradiation with 25 Gy. Each bar represents the mean of triplicate measurements from four donors, respectively, and is expressed as % (± SEM).
A: Impact of increasing concentrations of PJ-34 on PAR levels in non-supplemented and NA-supplemented cells.
B: Impact of increasing concentrations of PJ-34 on cellular NAD^+ levels in NA-supplemented cells compared to non-supplemented controls.

Effect of NA on cell viability

As the strand break-stimulated PARP-1 activity can consume large amounts of NAD^+, it was important to investigate if the increased NAD^+ concentration and enhanced poly(ADP-ribosyl)ation after NA supplementation is linked with an increase in cell viability after DNA damage. To analyze the impact of NA supplementation on apoptosis and necrosis, supplemented and non-supplemented cells were irradiated with 0 or 5 or 25 Gy and incubated in culture medium (-/+ NA) for 24 h at 37°C, followed by annexin V and propidium

iodide staining. Ionizing radiation induced a dose-dependent increase in cell death (Figure 5A).

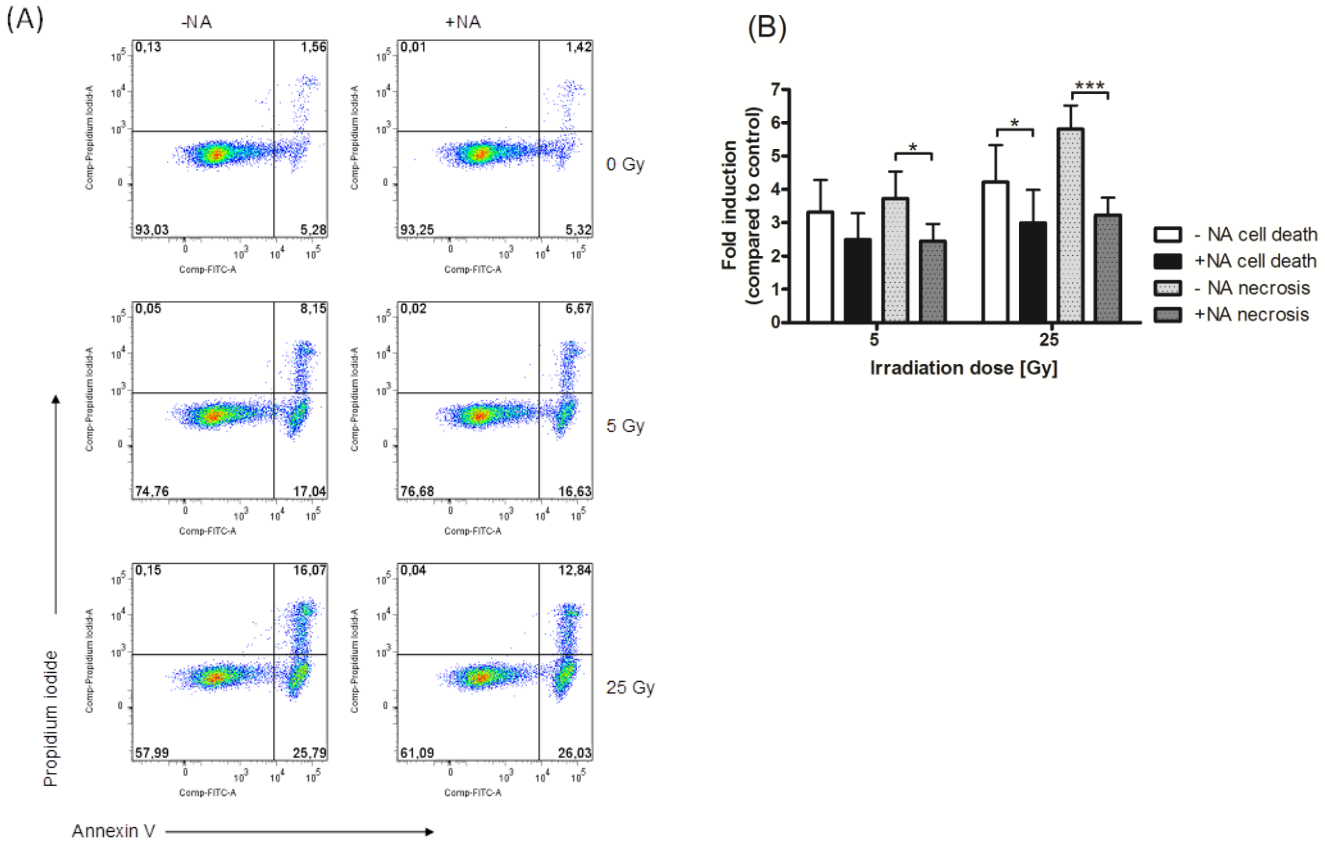


Figure 5: Effect on cell viability

NA-supplemented and non-supplemented cells were irradiated with 5 Gy or 25 Gy, and after 24 h cell viability, apoptosis and necrosis was determined by flow cytometry using annexin V and propidium iodide staining.
A: Flow cytometric analysis. Viable cells (annexin V-negative/ PI-negative), apoptotic cells (annexin V-positive) and cells in the necrotic stage (annexin V-positive/PI-positive) were expressed as percent. Data are representative of one out of tree measurements of one donor. B: Comparison of the fold induction of total cell death (apoptotic and necrotic cells) between non-supplemented and supplemented cells. A significant difference was observed in irradiated cells between non-supplemented and supplemented PBMC (*p<0.05; **p<0.01, repeated measures ANOVA). Each bar represents the mean value ±SEM of duplicate measurements of three different donors, respectively.

In undamaged cells, there was no difference in cell viability between NA supplemented and non-supplemented cells. We calculated the ratio of total and necrotic cell death of non-supplemented or NA-supplemented PBMCs to unirradiated controls (Figure 5B). Upon irradiation, there was a decrease in cell death rates in NA supplemented cells by 30% (4.2-fold

induction without NA vs. 3-fold induction with NA). This was mainly the result of a reduced rate of necrosis, which dropped by 45% after NA supplementation compared to non-supplemented cells. Thus, under conditions of higher PARP-1 activity and an increased NAD^+ concentration, cells are more resistant to DNA damage.

To test if the observed effect on cell death is a direct consequence of the increased PARP-1 activity or only correlated to it, we inhibited PARP with PJ-34 (Figure 6). Total cell death after 5 or 25 Gy irradiation was diminished in NA-supplemented cells in contrast to non-supplemented (Figure 6A and B). This was independent of the addition of PJ-34. The frequency of necrotic cell death significantly decreased at concentrations of 0.3 µM and 3 µM PJ-34 in NA or non-supplemented PBMC (Figure 6C and D). By contrast, increasing concentrations of the PARP inhibitor led to an increase in the apoptotic fraction after irradiation with 5 Gy or 25 Gy (Figure 6E and F).

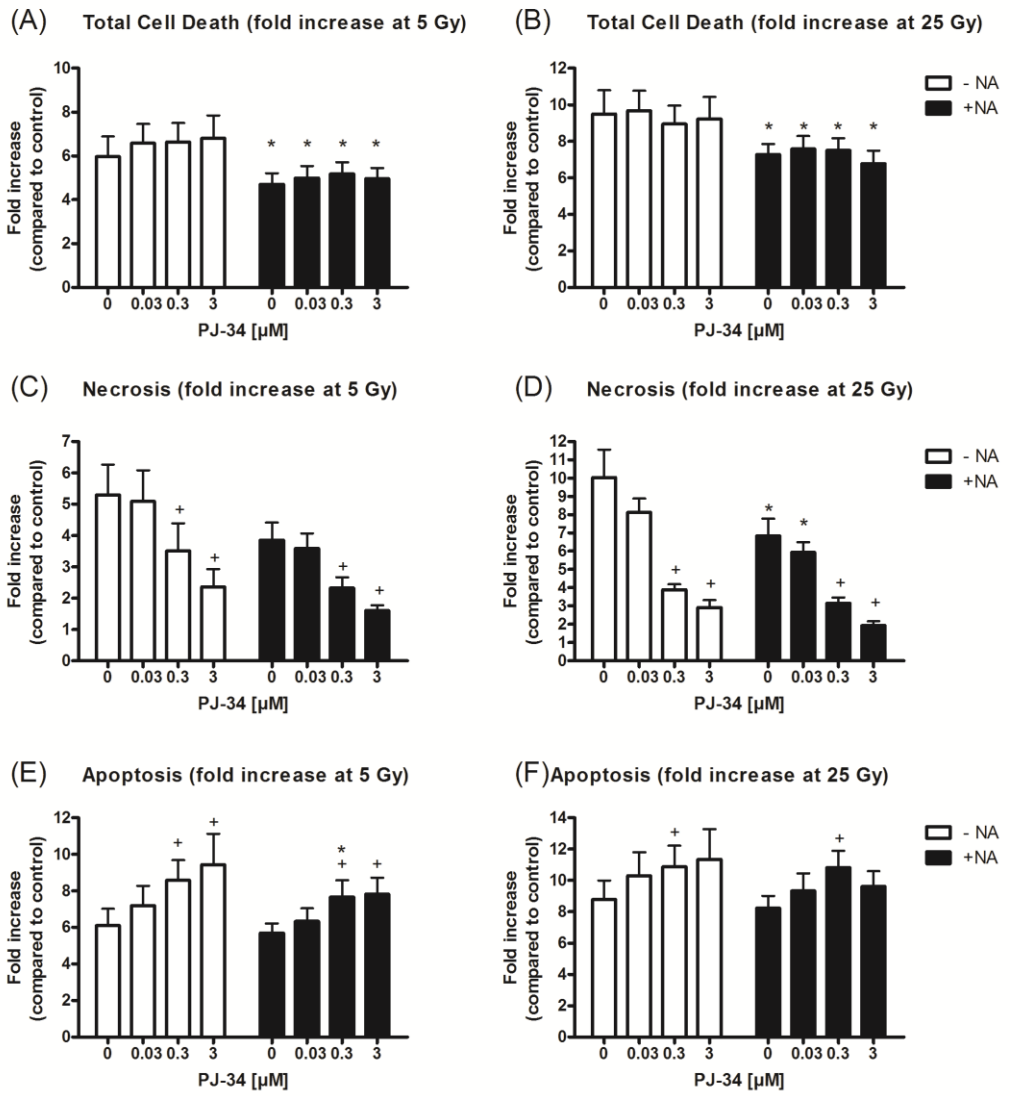


Figure 7: Effect of PARP inhibition on cell death.

PBMCs were treated with different concentrations of PJ-34 as indicated and analyzed for total cell death (A and B), necrosis (C and D) and apoptosis (E and F) after irradiation with 5 Gy (A,C,E) or 25 Gy (B,D,F). A, B: A significant difference in fold-induction of total cell death was observed in irradiated cells between non-supplemented and supplemented PBMC (*P<0.05, Wilcoxon matched pairs test). C, D, E, F: A significant decrease of the necrotic fraction with increasing concentrations of PJ-34 was detected within non-supplemented or within NA-supplemented, respectively (*p<0.05, repeated measures ANOVA), or between non-supplemented compared to NA-supplemented (*p<0.05, repeated measures ANOVA). Each bar represents the mean value ±SEM of triplicate measurements of six different donors.

Discussion

In this study we focused on the consequences of elevated NAD^+ levels regarding PARP-1 activation after DNA damage induction by ionizing irradiation, H_2O_2 or MNNG treatment. Intracellular NAD^+ levels of human PBMC were raised by *ex vivo* supplementation of culture medium with low concentrations (15 µM) of NAD^+ precursor NA. We found a strong and robust increase in cellular NAD^+ levels, with the basal NAD^+ status being increased 2.1-fold on average. This concentration of the precursor is much higher than fasting levels of NA in human blood plasma, which is approximately 0.08 µM [147] or barely detectable, because NA is largely converted to NAD^+ and therefore does not persist in circulating blood.

As non-supplemented cells were kept in culture medium, whose components are designed to maximize cell growth and survival rather than to perfectly model in vivo conditions, controls were also exposed to NAD^+ precursors due to the routine media supplements [148]. However, this led only to a slight increase of basal NAD^+ pools after incubation of 5 h (medium control, Supplementary Figure S2) or longer (24 h, marked with asterisk, Table 1). Nevertheless, we decided to use the shorter (5 h) incubation period in order to avoid non-physiological conditions.

We then monitored the immediate impact on PAR formation in response to DNA damage using the newly developed RAPARA. We could track the time and dose-dependent PAR formation after PARP activation with high resolution. Next, we analyzed cell viability, since PARP-1 is implicated in cell death processes by excessive consumption of NAD^+. We found that cell death in NA-supplemented cells was reduced after irradiation due to effective protection from necrotic cell death.

RAPARA enabled us to assess PAR levels in intact living primary cells under physiological conditions. Using this new approach we can detect PAR levels and the direct impact on PAR formation after addition of nutritional factors like NA, the effect of DNA-damaging agents (Figure 2 and 3) or PARP inhibitors (Figure 4). It should be noted that there are several types of PARP activity assays available, but most of them can only be used for measurements in permeabilized cells [149, 150] or in cell lysates [151]. For example, another flow cytometric PAR assay we have published previously comprises permeabilization of cells and postincubation of the cell “ghosts” with saturating concentrations of exogenous NAD^+ and an

PARP activator oligonucleotide in order to assess total cellular poly(ADP-ribosyl)ation capacity [150]. On the other hand the determination of PARP-1 activity in intact cells, *i.e.* the PAR detected being produced by the living cell, as a function of its PARP activation status, endogenous NAD^+ pools and perhaps other factors, was so far restricted to either biochemical extraction of PAR followed by HPLC-based quantitative detection of enzymatic digestion products [126, 152], which is sensitive and accurate but requires large cell numbers, or immunofluorescence microscopy [116], which is neither quantitative nor useful for the comparison of large sets of samples.

In contrast, RAPARA is very sensitive as we are able to detect routinely PAR formation even after low-level genotoxic stimuli application, which escape detection by the current gold standard, *i.e.* western blotting (Figure S1). A slight increase, though not statistically significant, in PAR levels can be detected by RAPARA after X-irradiation with as little as 0.5 Gy or H_2O_2 addition of 5 µM compared to controls. Significantly increased levels were observed after 2 Gy irradiation or 25 µM H_2O_2, which was not achieved using the western blot technique (Figure S1). Furthermore with RAPARA it is possible to determine PARP activity conveniently and with high temporal resolution (Figure 1A). The apparent difference in the maximal PAR accumulation between Figure 1A and Figure 2D can be explained by the inter-individual variability of PAR formation in response to DNA damage [150]. As RAPARA is able to yield results quickly and with high sensitivity, it holds great opportunity for measuring PARP-1 activity in intact PBMC. This is an enormous advantage in studies where PARP-1 activity is, for example, used as a biomarker, as it has already been done in permeabilized PBMC [125].

In the present work we used RAPARA to assess the effect of elevated intracellular NAD^+ levels on PAR formation after DNA damage induced by genotoxic agents, and we could confirm that the higher availability of NAD^+ positively affects cellular poly(ADP-ribosyl)ation, which is in agreement with other studies [128, 153]. Moreover we showed that the same amount of DNA damage leads to a higher level of poly(ADP-ribosyl)ation in NA-supplemented cells, even after low-dose irradiation when the availability of NAD^+ is not limited. We also used hydrogen peroxide and MNNG, two DNA-damaging substances triggering PARP activity, to show that the observed effects concerning NAD^+ and PAR formation after NA-supplementation are independent of the type of DNA damage. In NA-supplemented cells, PAR persists for extended time periods (Figure 2D). It has been shown *in vitro* that NAD^+ concentration can

affect length and frequency of branching sites of PAR chains [10]. Thus, the higher NAD^+ concentration may possibly lead to higher amount of branched polymer, which was reported to be degraded more slowly by PARG [154]. Also, the increased availability of NAD^+ could extend the time span of high PARP-1 activity so that equilibrium with the catabolic activity of PARG be maintained for a longer period of time, *i.e.* between 1 and 7 min. Diminished PARP-1 activity can be brought about either by resealing DNA strand breaks or, if increased damage persists, by depletion of the NAD^+ pool. Thereafter, degradation of the polymer can be observed. In the non-supplemented cells it is likely that the restricted NAD^+ content is not able to maintain PARP-1 in a fully active state. Thus, the activity of PARG becomes dominant, resulting in rapid degradation of PAR. If the polymer is degraded before the repair process can start properly, the DNA repair process will fail, leading to mutations or apoptosis.

There are few studies in which the effect of NA on poly(ADP-ribosyl)ation and the cellular consequences were investigated; most of them, however, refer to correction of established NAD^+ deficiency [141, 142]. Nevertheless, those studies showed that PARP activity is negatively influenced by decreased cellular NAD^+ concentration. In an *in-vivo* study in rats, it was shown that supplementation of NA increases the NAD^+ content as well as poly(ADP-ribosyl)ation in bone marrow cells when treated with the alkylating agent ethylnitrosurea (ENU) [143]. The authors investigated the effect of NA on PAR levels 3 h post-treatment, when ENU starts to induce DNA damage, measured by western blot technique. However in the present study we investigated the immediate response of PARP-1 to DNA damage with the addition of cell viability analysis as readout. As overactivation of PARP-1 caused by severe DNA damage leads to depletion of NAD^+, and consequently of ATP, which results in cellular dysfunction, necrosis and inflammatory response of tissue, we investigated if PBMC can be rescued from energy-loss dependent cell death by supplementation with NA. Indeed we observed increased cell viability in NA-supplemented cells after exposure to ionizing radiation (Figure 5), which was caused by a 50% reduction in the fraction of necrotic cells. It may also be that the higher NAD^+ content influences other pathways such as sirtuin function, leading to higher cell viability [109, 155]. In order to test if this effect was a direct consequence of the increased PARP-1 or only correlated to it, we inhibited PARP activity with the potent competitive inhibitor PJ-34. The rates of necrotic cell death of NA-supplemented and non-supplemented PBMCs were comparable at low levels when PARP-1 activity was fully inhibited by high inhibitor concentrations, but significantly different in the absence or at low

concentrations of PJ-34 (Figure 6D). This was also reflected in the levels of PAR formation detected in response to DNA damage (Figure 4B). We propose that preserved NAD^+ levels even under circumstances of high PARP-1 activity after DNA damage is responsible for prevention of necrotic cell death. The increased NAD^+ content allows for higher PARP-1 activity, which possibly supports DNA repair by avoiding the depletion of the NAD^+ pool and the subsequent cellular energy failure. Our data are in line with other studies, showing that early inhibition of PARP activity can induce a switch in the mechanism of cell death in lymphocytes [156]. Moreover, we could show that combined incubation of NAD^+ precursor NA and PARP inhibitor PJ-34 leads to an additive effect, pushing cells from pro-inflammatory necrosis to less inflammatory apoptosis, which may be therapeutically useful.

In conclusion, our data show that keeping the NAD^+ pool high by supplementation of NA leads to significantly increased poly(ADP-ribosyl)ation in response to DNA damage, even at doses where NAD^+ is not a limiting factor for the reaction. This results in preservation of the NAD^+ pool and decreases potentially harmful necrosis, comparable with the effect of PARP inhibitors. Interestingly, PAR-mediated release of apoptosis-inducing factor (AIF) from mitochondria, which leads to caspase-independent cell death [68], is not a dominant mechanism in this system, as the excessive PAR formation by nicotinic acid supplementation is *not* associated with increased cell death, but, on the contrary, with increased cell survival. Therapies that augment NAD^+ pools may be alternative pharmacological approaches to treat diseases, where PARP is pathophysiologically implicated. The consequences related with PARP-mediated reactions analyzed in this study concentrate on cell death, but the effect on DNA repair is barely understood. Further studies will address the underlying mechanisms.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft through the International Research Training Group 1331 (fellowships awarded to K.W. and A.K.). The authors thank Professors M. Miwa and T. Sugimura (Tokyo, Japan) for the kind gift of 10H hybridoma cells. We thank Professors A. Wendel and C.R. Hauck (University of Konstanz) for kindly giving us access to their flow-cytometers.

Supplementary Data

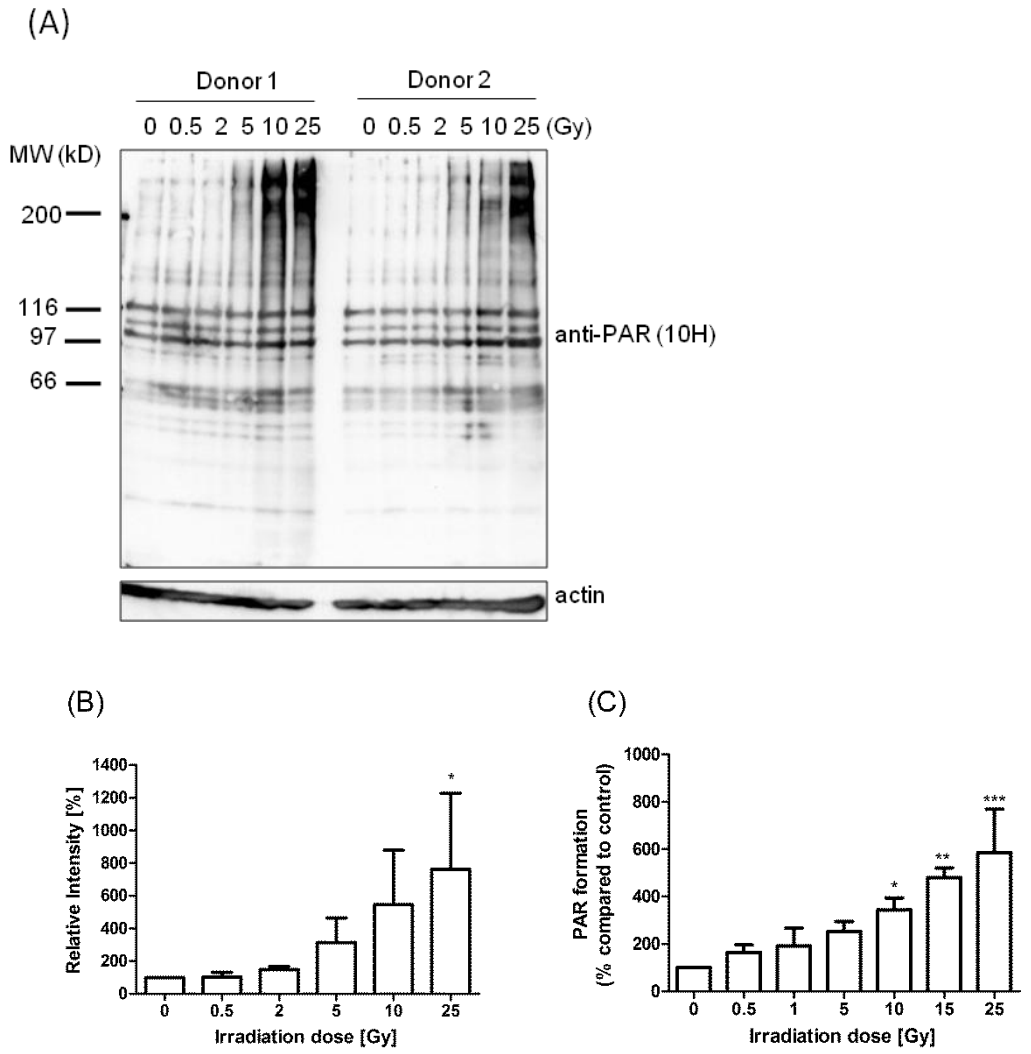


Figure S1: Comparison of RAPARA assay and western blot technique.

A: Detection of PAR formation by western blotting. Total proteins from 1.5×10^6 PBMC were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with the anti-PAR antibody 10H. B: Quantitative evaluation of dose-dependent PAR formation by western blot analysis. The signal intensity was determined using image analysis system and expressed as relative intensity compared to unirradiated controls. (*P<0.05, repeated measures ANOVA). Data represent the means ±SD of independent experiments on PBMC from 3 donors. C: Detection of dose-dependent PAR formation by RAPARA assay. Data represent the means ±SD of independent experiments of 3 donors (**P<0.01, ***P<0.001, repeated measures ANOVA).

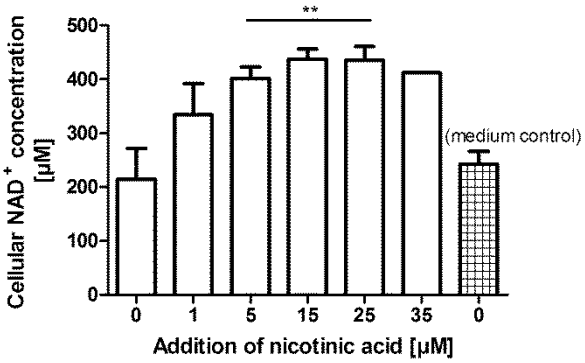
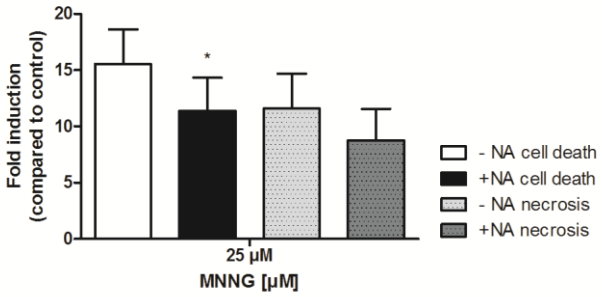


Figure S2: PBMC were incubated in RPMI medium with various NA concentrations as indicated for 5 h, respectively.

Control cells were analyzed immediately after isolation without incubation (0 h). Medium control cells were kept in culture medium without additional NA (0 µM) supplementation for 5 h. Total cellular NAD^+ levels were measured with an enzymatic cycling assay. (**P<0.01, repeated measures ANOVA). Each bar represents the mean value ±SD of triplicate measurements of PBMC from three different donors.

Additional Data



Additional Figure A1: Effect of NA on cell viability 24 hours after MNNG treatment.

Comparison of the fold induction of total cell death or necrosis between non- and NA-supplemented cells. Each bar represents the mean value ±SD of three different donors. (*p<0.05, repeated measures ANOVA).

CHAPTER II

The NAD^+ precursor nicotinic acid improves genomic integrity in human peripheral blood mononuclear cells after X-irradiation

Kathrin Weidele, Sascha Beneke, Alexander Bürkle

submitted to Biochemical Pharmacology

Abstract

The DNA damage-activated poly(ADP-ribose) polymerase 1 (PARP-1) acts as a nick sensor and modifies target proteins by poly(ADP-ribosyl)ation, with NAD^+ serving as substrate. The intracellular levels of poly(ADP-ribose) (PAR) and NAD^+ are important factors for biological responses to genotoxic stress and influence diverse functions, such as DNA repair or maintenance of genomic stability. Abrogation of cellular PAR formation by *Parp1* gene deletion or gene silencing, low-molecular weight PARP inhibitors, or lowering cellular NAD^+ levels through niacin starvation sensitises cells to an array of genotoxic agents and increases genomic instability. Here we investigated the impact of raising the NAD^+ and PAR levels in resting or mitogen-activated human blood peripheral mononuclear cells (PBMC), through *ex vivo* supplementation with the NAD^+ precursor nicotinic acid (NA), on DNA damage and DNA repair as well as on genomic stability under genotoxic stress. We observed that upon NA supplementation, resting PBMC displayed slightly lower levels of DNA damage after ionising radiation and showed more efficient repair of DNA strand breaks within 40 min repair time compared to non-supplemented controls. We also detected less micronucleus formation after irradiation at 5 Gy. Likewise, in proliferating PBMC, DNA repair was significantly more efficient and the frequency of micronuclei was significantly decreased after irradiation at 3 Gy or more. Our data reveal that NAD^+ and PAR levels may be limiting under conditions of genotoxic stress in primary normal human cells and that boosting the NAD^+ / PAR system with nicotinic acid supplementation can improve genomic maintenance beyond normal.

Introduction

Immediately after genotoxic insults cells activate various responses that contribute to cell survival or death. One of the first reactions is poly(ADP-ribosyl)ation (PARylation) of proteins, mediated by nuclear poly(ADP-ribose) polymerases (PARPs) [20, 134]. The most active enzyme in this regard is PARP-1 (and to a lesser extent PARP-2 [3]), which covalently attaches ADP-ribose units in a stepwise fashion to target proteins including itself, thus synthesizing a negatively charged polymer by using nicotinamide adenine dinucleotide (NAD^+) as substrate. Depending on the level of DNA damage and intracellular NAD^+ status, PARP-1 and its product PAR mediate the recruitment of DNA repair factors to sites of lesions, facilitate DNA repair and help maintain genomic integrity under conditions of moderate stress [19, 28]. In this

scenario a tolerable amount of total cellular NAD^+ is used for polymer synthesis. In contrast, drastic and irreversible NAD^+ depletion [64] as a result of hyperactivation of PARP-1 under severe stress conditions can lead to cell death [157]. This paradigm is also apparent in inflammatory diseases and neurodegenerative disorders [66, 67]. One important parameter determining the cellular response to stresses is the level of available NAD^+, which is crucial for adequate PAR synthesis [87]. Several studies have reported that administration of NAD^+ precursors such as nicotinamide (NAM), nicotinic acid (NA) or nicotinamide riboside can increase the intracellular NAD^+ concentration in diverse tissues or cellular compartments *in vitro* and *in vivo* [127, 128, 158]. In a recent study we could show in human peripheral blood mononuclear cells (PBMC) that *ex vivo* supplementation with a low dose of nicotinic acid (15µM) raised cellular NAD^+ levels and enhanced PAR formation after genotoxic treatments. Strikingly, the cells were protected from DNA damage induced necrotic cell death [159]. Conversely, the importance of NAD^+ was highlighted by other studies reporting that niacin (NA or NAM) deficiency results in increased chromosomal instability [160, 161], increased cancer incidence [162] or impaired PARP-1 functions in rats [141, 160, 163]. Likewise, abrogation of cellular PAR formation by *Parp1* gene deletion or silencing; or low-molecular weight PARP inhibitors is known to sensitise cells to many genotoxic agents and to increase genomic instability [19, 28]. In the present work, we wanted to gain new insights into how up-modulation of NAD^+ status can influence cellular responses after genotoxic treatment in non-niacin-deficient healthy subjects. We focused on the biological consequences of elevated NAD^+ levels in respect to PARP-1 mediated reactions in human PBMC in the context of genomic integrity. To assess how modulated NAD^+/PAR levels contribute to physiological/pathophysiological outcomes we set out to investigate various end points including (I) DNA damage and repair and (II) the influence on genomic stability after treatment with ionising radiation (IR) using varying doses.

Human PBMC are primary cells proficient in DNA damage response cascades and repair pathways and therefore ideal for DNA repair studies and most relevant to understand biochemical and molecular mechanisms in human physiology. Furthermore, they are widely used in epidemiological studies to investigate the correlation of various parameters including DNA repair capacity and cancer risk [164]. As DNA repair might vary throughout the cells cycle, we used unstimulated, non-cycling cells, which are mostly in G_0 phase and compared them with proliferating cells, stimulated with phytohaemagglutinin (PHA-L). Based on an

automated version [165] of the fluorimetric alkaline DNA unwinding (FADU) technique [166, 167] we monitored DNA strand break formation in PBMC after IR and the early phase of DNA repair after damage. While FADU assay cannot discriminate between single and double strand breaks, the former are known to be 5-10-fold more frequent than the latter after X-irradiation and so the FADU largely represents single strand breaks. Independent of proliferation state, we observed significantly modified DNA repair kinetics in NA-supplemented cells at doses > 5 Gy. Since the FADU technique can yield quantitative information on DNA repair but cannot measure the quality of repair, we additionally determined the influence on genomic stability as a further parameter. Our results from the evaluation of micronuclei (MN), a well-established marker of chromosome breakage or loss [168], reveal an improvement of genomic stability after NA supplementation in unstimulated PBMC and even more so in mitogen-stimulated PBMC.

Materials and methods

Chemicals and reagents

Biocoll and FCS were purchased from Biochrom (Berlin, Germany). Antibiotics and RPMI were from Invitrogen (Darmstadt, Germany). Standard chemicals were purchased from Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany) if not stated otherwise.

Isolation of peripheral blood mononuclear cells [159]

The blood sampling was carried out, in accordance with the Declaration of Helsinki and with approval of the University of Konstanz Ethics Committee, from healthy donors aged 24-45 years. Venous blood was obtained using S-Citrate-Monovette blood collection system from Sarstedt (Nümbrecht, Germany). Cells were separated via Biocoll gradient centrifugation. Briefly, the freshly drawn blood was mixed with an equal volume of PBS (137 mM sodium chloride; 10 mM disodium hydrogen phosphate; 3 mM potassium dihydrogen phosphate; pH 7.4) and layered on 15 ml of Biocoll separating solution, followed by centrifugation at 800 x *g* for 15 min at room temperature. The PBMC layer was collected and washed twice with PBS. Isolated cells were incubated in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere.

Nicotinic acid was added to the culture medium at a final concentration of 15 µM and cells were incubated 5 h before DNA damage induction.

Mitogen stimulation of cells

In order to investigate NAD^+ levels and DNA strand break repair at different stages of the cell cycle we challenged cells with X-irradiation, either in G_0 phase or 44 h after mitogenic stimulation with Leucoagglutinin (PHA-L) [169]. Figure 1 presents an overview of the treatment and time schedule used for the experiments conducted in this study. PHA-L was used for the stimulation of cell proliferation. 5 µg/ml PHA-L was added to cell cultures in DNA repair studies, NAD^+ measurements and cell cycle analysis, or 20 µg/ml in micronucleus induction tests according to Fenech et al. [170].

Flow cytometric analysis of cell cycle

Methanol fixed cells (10^6/ml) were washed in PBS and incubated with 100 µg/ml RNase A for 1 h at room temperature. Then 10 µg/ml propidium iodide was added to determine DNA content. Samples were analysed with a LSR II from Beckton Dickinson (Heidelberg, Germany) and cell cycle distribution was quantified using FlowJo Software (Tree Star, Ashland, USA).

Detection of intracellular NAD^+ levels

Cellular NAD^+ concentration of proliferating cells was determined by an enzymatic cycling assay adapted from Jacobson et al. [146]. $5x10^5$ - $1x10^6$ cells per sample were irradiated in 500 µl PBS, incubated for 10 min at 37°C to allow PARP-1 activity, and precipitated with ice-cold perchloric acid (0.5 M). After centrifugation at 1500 x *g* for 10 min, the supernatant, comprising the cellular NAD^+, was removed and processed for the cycling assay as described [146]. Intracellular NAD^+ concentrations of controls and irradiated samples were determined on the basis of defined NAD^+ standards.

Automated fluorimetric alkaline DNA unwinding (FADU) assay

For assessment of DNA strand breakage and repair, cell number was adjusted to $4x10^6$ cells/ml suspended in culture medium. Aliquots of 100 µl were irradiated on ice (8 Gy/min) using an X-ray source (RT 100; Müller, Hamburg, Germany). Subsequently, samples were

incubated for DNA repair at 37°C for the time periods indicated and then kept on ice until analysis by FADU. A modified and automated version of the FADU method was used for the assessment of induced DNA damage and repair as previously described [165]. Briefly, 900 µl of suspension buffer (0.25 M *meso*-inositol; 10 mM sodium phosphate, pH 7.4; 1 mM magnesium chloride) was mixed with each cell sample and 70 µl per well was transferred in triplicates onto a 96-well plate. The subsequent automated steps included cell lysis, alkaline DNA unwinding and neutralisation of the samples, followed by addition of fluorescent dye SybrGreen (Invitrogen) to monitor the amount of DNA remaining double stranded. T values are controls representing the total amount of double-stranded DNA. P_0 values reflect the SybrGreen fluorescence after alkaline unwinding in untreated controls, thus representing the level of basal DNA lesion sites in cellular DNA. P_x values represent SybrGreen fluorescence after alkaline unwinding measured immediately after irradiation of cells with a dose of x Gy. P_r values represent SybrGreen fluorescence after alkaline unwinding measured after incubation of cells after irradiation in order to allow DNA repair. Data are plotted on a logarithmic scale. [165].

***In vitro* cytokinesis-blocked micronucleus test**

The induction of micronuclei was performed according to a protocol from Fenech with slight modifications [170]. Aliquots of $7.5x10^5$ cells in 750 µl were irradiated in G_0 phase and then stimulated with 20 µg/ml PHA-L. X-ray exposure in proliferating cells was at 44 h after PHA-L stimulation. Cytochalasin B was added at a final concentration of 6 mg/ml at 44 h post-irradiation (Figure 1A) or 48 h (1B). Seventy-two hours later, cells were transferred to glass slides by cytospin at 500 x *g* for 3 min. After methanol fixation (10 min) cell staining was done with Giemsa (Merck) diluted in Sörensen buffer (pH 6.8) (3:1) for 5 min. After drying, the cells were embedded with Mowiol. Micronuclei were scored in 500-1000 binucleated cells (BN) per sample. Micronucleus frequencies were calculated by dividing the total number of MN by the total number of BN cells counted.

Statistical analysis

To test for deviations from Gaussian distribution we used GraphPad Prism (La Jolla, USA) using the Kolmogorov-Smirnov test. For statistical analysis, means of data were compared by

two-way ANOVA and Bonferroni posttest using Graph Pad Prism. p-values < 0.05 were considered significant and are labelled with one asterisk (*), ** p < 0.01, *** p < 0.001.

Results

We have recently shown that NA supplementation reduces cell death after genotoxic stress in freshly isolated human PBMC and shifts the residual fraction from necrosis to apoptosis, which is less harmful in regard to tissue integrity [159]. In order to get deeper insight into the protective effect of NA supplementation, we analysed its impact on DNA repair and genomic stability, *i.e.* micronucleus formation, after treatment with ionising radiation, and we determined the influence of the cell cycle phase, during which damage is applied to the cells, on the respective parameters.

For clarity, the general treatment schedule including the time points of NA supplementation, PHA-L stimulation and irradiation at different cell cycle stages is summarised in Figure 1.

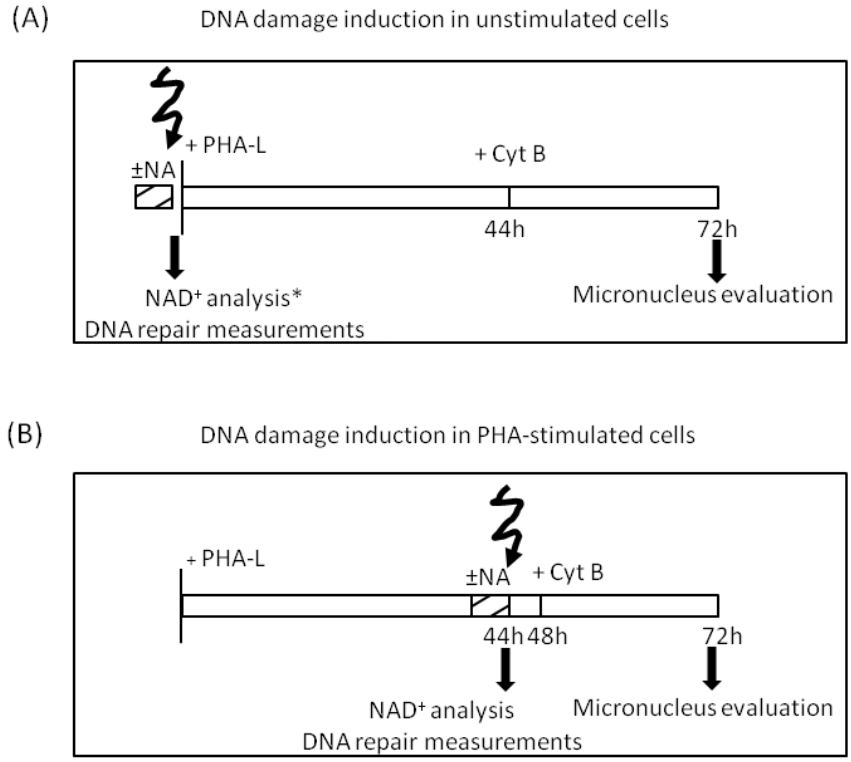


Figure 1: Experimental treatment schedule.

Human PBMC were either exposed to ionising radiation (A) as unstimulated cells or (B) 44h post PHA-L-stimulation and prepared or cultured for the assays indicated, respectively. If required, cells were supplemented 5 hours before irradiation with 15 µM nicotinic acid (NA) as indicated. For further details see Materials and Methods.* Note that NAD^+ measurements for unstimulated PBMC have previously been published in Ref. [159].

Distribution of cell cycle phases in unstimulated and PHA-L stimulated PBMC and intracellular NAD^+ levels

We used flow cytometry to monitor cell cycle distribution of PBMC. As depicted in Figure 2, freshly isolated PBMC show a distinct peak representing the DNA content of cells in G_0 phase, as expected. By contrast, after 44 h of stimulation cells are found in all cell cycle phases, with 65-75% in G_0/G_1 and 20-30% in $S/G_2/M$. NA supplementation did not influence cell cycle profile at the time points examined.

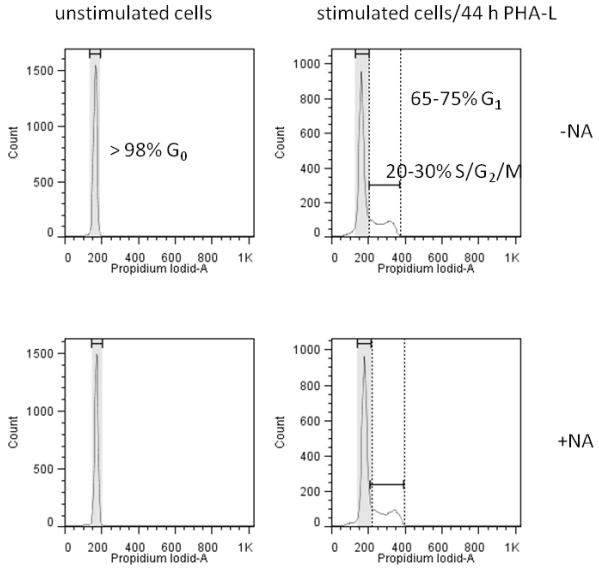


Figure 2: Distribution of cell cycle phases in non-damaged resting or proliferating PBMC.

Flow cytometric analysis of cell cycle distribution without and after PHA-L stimulation. The DNA content is plotted against the cell number; histograms are representative of one out of three measurements. Mean distribution of cell cycle phases of three measurements is indicated as percentage.

In our previous work we found mean NAD^+ concentrations in unstimulated cells of about 200 µM [159]. In analogy to those data, we tested PHA-L-stimulated samples for their physiological and stress-related NAD^+ concentrations. The mean NAD^+ level in undamaged proliferating cells was approximately 217 µM, which could be raised by NA supplementation up to 315 µM, *i.e.* the mean increase achieved by 15 µM NA application within 5 hours incubation was up to 1.5-fold (Table 1). In contrast, the basal cellular NAD^+ status in unstimulated PBMC was increased 2.1-fold on average as reported previously [159].

Table 1: NAD^+ levels of PHA-L stimulated PBMC detected via enzymatic cycling assay.

	Intracellular NAD^+ concentration without NA supplementation (mean ±SEM)	Intracellular NAD^+ concentration with NA supplementation (mean ±SEM)	Fold increase
PHA-L stimulated (n=4)	217±51 µM	315±83 µM	1.5

The cellular consumption of NAD^+ as a result of DNA damaged induced PARP activation was dose-dependent and was most pronounced after 25 Gy irradiation. Under those conditions about 50% of total NAD^+ was consumed. Independent of the irradiation dose applied, the supplemented cells displayed significantly higher NAD^+ levels compared to controls (Figure 3).

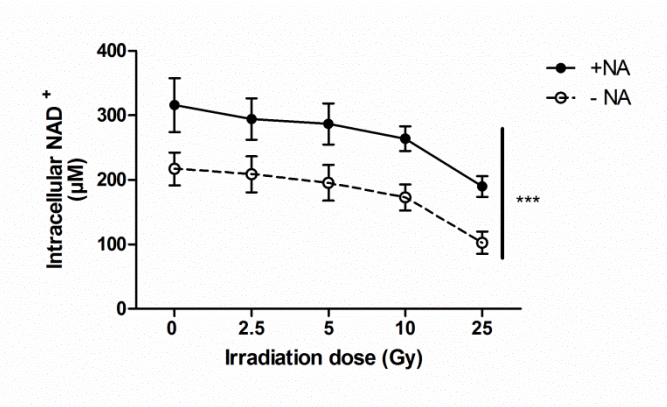


Figure 3: Intracellular NAD^+ levels, effect of nicotinic acid supplementation and DNA damage.

Cells were irradiated with doses as indicated and then incubated for 10 min at 37°C to allow poly(ADP-ribosyl)ation to occur. The NAD^+ concentrations shown of PHA-L-stimulated PBMC pre-treated or not with NA represent the mean ± SEM of four different donors ($^{***}p<0.001$, repeated measures ANOVA).

Baseline damage in unstimulated versus PHA-L-stimulated PBMC and the effect of NA supplementation

To estimate if stimulation with PHA-L or nicotinic acid supplementation *per se* influences baseline damage of the genome we compared the P_0 values from PBMC, representing the basal level of DNA breakage, of 6 different donors (Figure 4). The T value of a given sample represents the total amount of double stranded DNA and was set to 100%. In unstimulated cells (G_0 phase) the P_0 levels were approximately 85% of the total DNA amount. Proliferating

cells exhibited higher amounts of physiological strand breaks, with the mean P_0 values reaching only 67% of the respective T-values. Neither in unstimulated nor in stimulated PBMC did NA supplementation change baseline P_0 values.

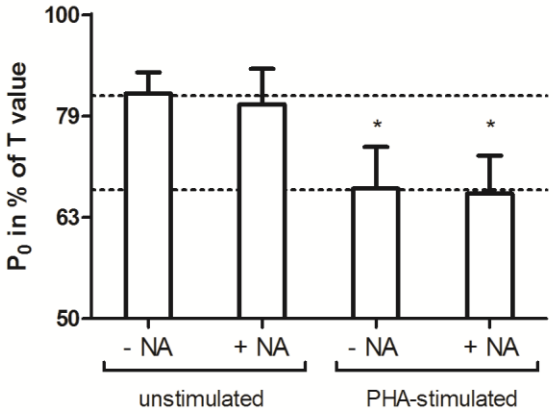


Figure 4: Baseline levels of DNA damage in unstimulated and proliferating PBMC.

Endogenous levels of DNA strand breaks in undamaged cells are expressed as percentage of fluorescent signal in control cells (P_0) in relation to the total amount of DNA (T values). Each bar represents the mean value ± SEM of six independent experiments using PBMC ex vivo supplemented (+NA) or not (-NA) with nicotinic acid. P_0 values of PHA-stimulated cells were significantly lower compared to unstimulated P_0 values ($^{*}p<0.05$, ANOVA).

Effect of NA supplementation on DNA damage induction and repair

To test if elevated NAD^+ levels can contribute to altered DNA repair in human PBMC we damaged cells immediately after 5 hours of NA incubation and monitored DNA damage and DNA repair characteristics by using the FADU assay. As expected, IR caused a dose-dependent induction of DNA strand breakage (Supplementary Figure 1), with a linear decrease of the SybrGreen fluorescence signal recorded at low doses. As NAD^+ levels are strongly affected by intense DNA damage and subsequent PARP hyperactivation, we monitored strand break formation also for higher doses. As expected, massive strand break induction in unstimulated cells was achieved with high-dose irradiation (25 Gy; Figure 5D) whereas 2.5 Gy led to moderate damage (Figure 5A). Within 40 minutes repair time, rejoining of DNA breaks was achieved, as is evident from the recovery of fluorescent signals.

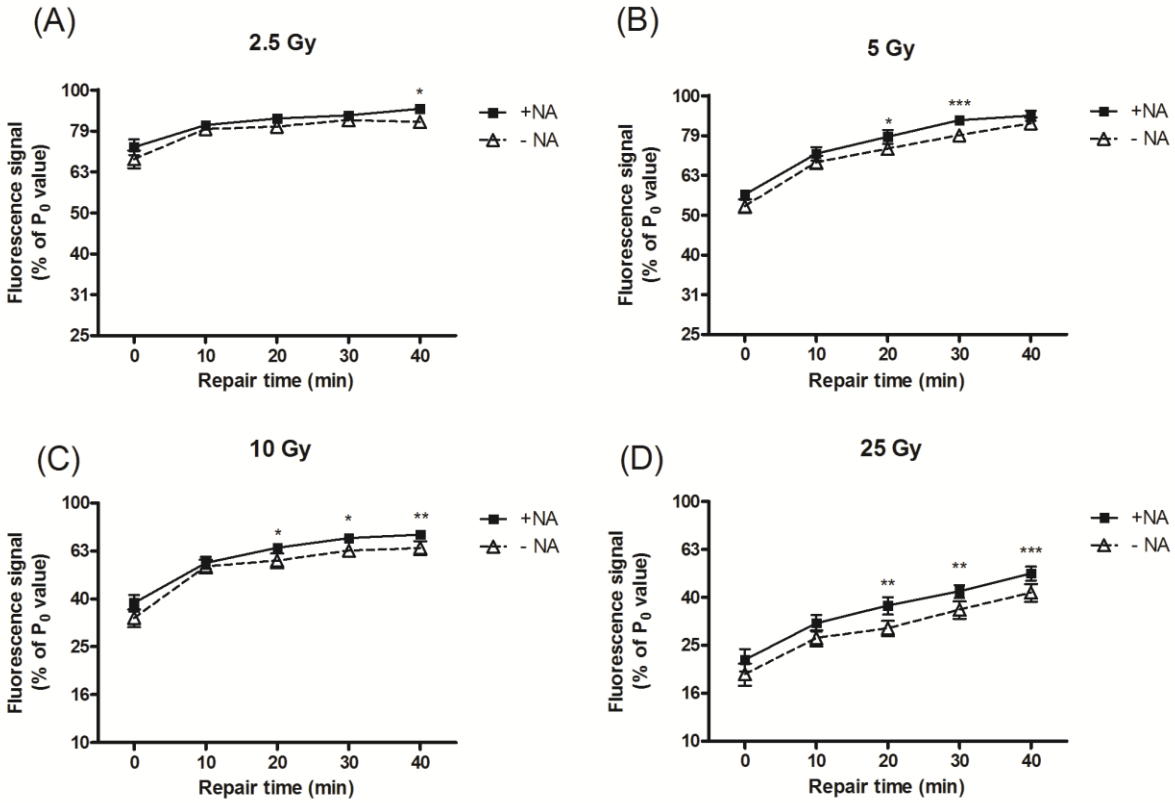


Figure 5: DNA damage and repair in unstimulated PBMC.

Cells were irradiated in G_0. Strand break formation and subsequent DNA repair within the first 40 minutes after DNA damage was measured using the automated FADU assay. Values represent the mean fluorescence (± SEM) of double stranded DNA in relation to non-irradiated controls (P_0) as obtained in independent experiments covering (A) n=12 donors (B) n= 13 donors (C) n= 11 donors, and (D) n=10 donors. Significant differences between –NA compared to +NA were indicated ($^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ repeated measures ANOVA).

Strand break formation in supplemented PBMC was slightly, but non-significantly decreased compared to non-supplemented cells (Figure 5A-D, 0 min). We observed, however, that repair of strand breaks was significantly more efficient in supplemented cells at later time points (20 - 40 min repair), especially when doses of 5 Gy and higher were applied.

As the FADU assay largely detects single strand breaks, we monitored specifically the formation and repair of double strand breaks via the detection of γH2AX signals (Supplementary Figure S2). Using a flow cytometric assay a dose-dependent increase of γH2AX positive cells was observed, but there was no significant difference between non-supplemented and NA-supplemented cells (A). Likewise the repair process after DSB induction with 5 Gy was not significantly influenced in NA supplemented PBMC (B).

In order to study the role of cell cycle progression in DNA damage formation and repair, we stimulated cells 44 h with PHA-L and induced DNA damage in proliferating PBMC cultures (Figure 6). As mentioned above, there was an increase in baseline endogenous DNA breakage in PHA-L-stimulated cells as detected by FADU assay (Figure 4), yet the strand break induction by IR was comparable to unstimulated cells (Supplementary Figure S1 and Figure 6). NA supplementation in proliferating PBMC significantly improved DNA repair at doses above 5 Gy, which is consistent with data obtained for quiescent cells.

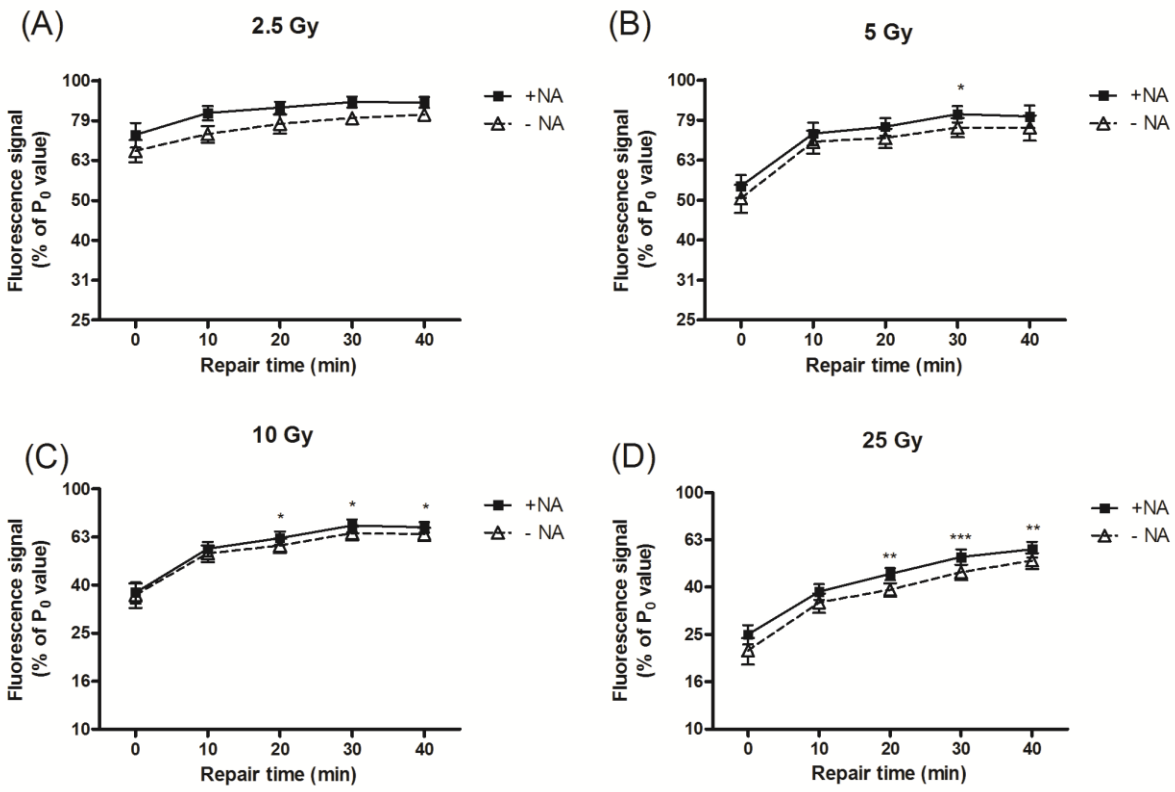


Figure 6: DNA damage and repair in proliferating PBMC.

Cells were irradiated after PHA-L stimulation. Repair of strand breaks after genotoxic treatment was measured within the first 40 min by using the automated FADU assay. Values represent the mean fluorescence (± SEM) of double stranded DNA in relation to non-irradiated controls (P_0) as obtained in independent experiments covering (A) n=11 donors (B) n= 13 donors (C) n= 10 donors, and (D) n=10 donors. Significant differences between –NA and +NA were indicated ($^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, repeated measures ANOVA).

Effect of NA supplementation on genomic stability

Studies have demonstrated a positive role of poly(ADP-ribosyl)ation in the maintenance of genomic integrity [63] and a negative influence by niacin deficiency [161], which compromises cellular PAR formation. Thus we investigated the impact of NA supplementation under genotoxic stress conditions on the formation of micronuclei in the low dose range of ionising radiation (1-5 Gy). The basal micronucleus frequency in undamaged cells was independent of cell phase, but clearly increased in response to DNA damage (Figure 7). In cells exposed in G_0, a reduced MN frequency was detectable at a dose of 5 Gy (Figure 7A) after supplementation. A substantial impact of NA was detected in proliferating cells where supplemented samples showed significantly decreased levels of MN frequency after doses of 3 Gy and 5 Gy (Figure 7B). Cells treated in G_0 phase in general had higher MN frequencies (about 40% at 3 Gy and 60-70% at 5 Gy) compared to cells damaged during proliferation (30% at 3 Gy and 40% at 5Gy).

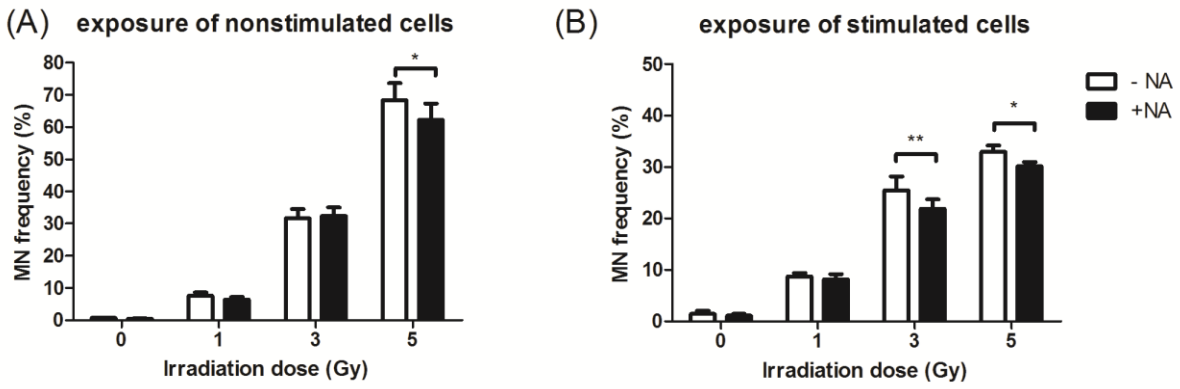


Figure 8: Genomic stability in human PBMC exposed to DNA damage, as a function of nicotinic acid supplementation.

(A) Unstimulated or (B) PHA-L stimulated cells were challenged with low doses of ionising radiation. Percentage micronucleus frequency ± SEM in binucleated cells was determined for n=6 donors. NA-supplemented cells showed significant lower MN frequencies compared to non-supplemented cells ($^{*}p<0.05$, $^{**}p < 0.01$, repeated measures ANOVA).

Discussion

Apart from the important function of intracellular NAD^+ as a coenzyme in vast range of oxido-reduction reactions, NAD^+ also participates in DNA repair and maintenance of genomic stability, by providing the substrate for the DNA strand break-activated enzymes PARP-1 and PARP-2 in response to genotoxic stress. Dependent on the level of DNA damage and the available physiological NAD^+ concentration, cellular functions related with PARPs are affected and therefore an optimal NAD^+ level is essential.

In the present study we investigated if an above-normal supply of NAD^+ can enhance DNA repair and genomic stability in human PBMC that display physiological NAD^+ levels before supplementation, *i.e.* in the absence of NAD^+ deficiency. We supplemented PBMC *ex vivo* with the NAD^+ precursor NA, which was shown to lead to increased and sustained poly(ADP-ribose) formation after IR [159], and monitored strand break formation and subsequent repair after irradiation at moderate or high doses, which induces PARP-1 (hyper)activation. Using the automated FADU assay [165], we were able to detect IR-induced strand break formation in unstimulated or mitogen-stimulated PBMC and the progression of DNA repair over 40 min. NA supplementation improved DNA repair in a significant way. The highest impact was visible at 20-40 minutes repair time, especially when doses of 10 or 25 Gy were used. At such doses NAD^+ is substantially consumed by PARPs [159] and cellular NAD^+ levels are dramatically reduced [87] accompanied by ATP loss. However, NA supplementation also enhanced repair after 5 Gy irradiation where NAD^+ is not a limiting factor (Figure 3). The rate of irradiation-induced DNA damage and repair capacity of quiescent and proliferating PBMC was comparable, but the endogenous level of DNA breakage (P_0 value) was significantly higher in mitogen-stimulated cells, due to their higher replicative activities, which was also reported by Mayer and colleagues using analysis by comet assay [171]. Interestingly, the yield of strand breaks induced by IR was marginally and non-significantly reduced in NA-supplemented cells, suggesting some protective effect.

J.B. Kirkland's group demonstrated the relevance of niacin status on genomic integrity, DNA repair and protection from carcinogenesis using animal models focusing on niacin deficiency [142, 160, 172]. A study by Sims and colleagues reported the ability of nicotinamide to increase the level of unscheduled DNA synthesis, used as a parameter for DNA repair, in unstimulated human lymphocytes, but this was accompanied by *diminished* poly(ADP-ribose)

synthesis after treatment with the alkylating genotoxin MNNG, as nicotinamide at the concentration used actually inhibits PARP-1 activity by product inhibition [173]. Likewise, high concentrations of nicotinamide were reported to improve repair capacity after γ-irradiation above 40 Gy in mouse melanoma cells [174]. In contrast, in the present study we used medically relevant doses of X-irradiation and a low concentration of nicotinic acid, a NAD^+ precursor not interfering with the basal PARP activity [159], to clarify if elevated NAD^+ levels, which contribute to higher PAR formation after genotoxic stress, enable improved DNA repair. Our observations that NA supplementation reduced initial strand break formation in PBMC are in line with other studies demonstrating that the administration of niacin reversed the increase of single-strand breaks in lymphocytes exposed to genotoxic treatment [158, 175], although there cells were pre-incubated shortly before treatment, not assuring the conversion into NAD^+. We speculate that in our system, higher intracellular NAD^+ levels achieved by NA supplementation potentially influence chromatin compaction in the context of epigenetic changes, thus making these cells less susceptible to ionising radiation.

As a further endpoint we investigated the effects on genomic stability and monitored if increased NAD^+ levels are accompanied by changes in micronucleus frequency. In response to doses of 3 - 5 Gy IR we found decreased MN frequency in proliferating cells pre-incubated with NA. Furthermore, there was a tendency towards lower MN frequency in unstimulated lymphocytes after NA addition, with a significant difference apparent at 5 Gy. Fewer MN appeared in PBMC damaged during proliferation resulting in lower MN frequencies compared to cells irradiated in G_0. This was consistent with a report revealing that cells irradiated in G_2/M display fewer MN than in G_1 or S phase [176].

As double strand breaks are one of the precursors of micronuclei, we specifically determined γH2AX levels in damaged cells. We monitored the dose- and time dependent response in unstimulated PBMC and found no significant effects regarding NA supplementation in the tested three individuals (Supplementary Figure S2). Although the number of individuals tested and thus statistical power is low, we assume that DSB induction and repair is not primarily affected by modulated NAD^+ levels, which is also apparent from data for stimulated PBMC (unpublished data).

Here we show that NAD^+ levels could be raised successfully not only in unstimulated, but also proliferating PBMC. Interestingly, NAD^+ levels fluctuate during cell cycle, with highest levels in G_1 and lowest in S/G_2 phase [177]. As proliferating cells represent a population containing

about 20-30% S/G_2 cells, we assume that specifically these cells are more susceptible for NA supplementation due to their lower rate of NAD^+, therefore resulting in decreased MN frequencies after NA addition.

Supplementation with NA elicits a 1.5-fold increase of NAD^+ in proliferating cells (Figure 3 and Table 1), which is lower than the effect detected in quiescent cells we have previously reported [159]. This may be due to the longer time of maintaining the cells in standard culture medium, which comprises niacin. In order to clarify if specifically the cells in S/G_2/M exhibit lower basal NAD^+ levels and decreased MN frequencies after NA supplementation, G_1 and S/G_2/M cells should be analysed separately. Data from E.L. Jacobson, based on PARP inhibitors in combination with DNA-damaging agents, have revealed that the cytotoxicity is potentiated in dividing cells and mutation frequencies decreased, whereas the inhibitor has little or no impact on cell survival in quiescent cells [178]. Our hypothesis that especially cells in S/G_2/M phase are susceptible to modified NAD^+/PAR levels is in perfect agreement with those findings.

In the present study we have investigated the impact of elevated NAD^+ levels in non-niacin-deficient PBMC from healthy young donors, with the highest effects appearing under conditions of extensive DNA damage. We propose that nutritional intervention with NA targeting human subjects with decreased PARP activity or low basal NAD^+ levels, which occurs during the ageing process [179], in the pathogenesis of diseases [104] or cancer therapy [180, 181], may provide a new strategy in stabilizing genomic integrity under stress conditions, thus antagonising the development of cancer and delaying the ageing process. It was shown in mice that inactivation of PARP-1 leads to acceleration of aging, shortened life span and increased spontaneous carcinogenesis [182], suggesting that increased PAR formation by NA supplementation is a potential tool to counteract these effects.

In the past, nicotinic acid has been in clinical use, at millimolar concentrations in the tissue, as a cholesterol lowering drug due to its artheroprotective effects [112, 183]. In our previous study we could show that NA influences NAD^+ levels and PAR formation in human PBMC *ex vivo* significantly already at low micromolar concentrations; thus NA could be a potent substance for pharmacological intervention to correct suboptimal NAD^+ levels and prevent any possible negative consequences.

As PARPs are the main, but not the only NAD^+ consumers in a cellular system playing part in the above mentioned processes, further factors should be included in future investigations. Other NAD^+-dependent enzymes, especially from the sirtuin family such as SIRT-1 and SIRT-6, should be taken into account as both are involved in DNA repair and maintenance of genomic instability [83, 184]. Taken together, our present data show a significant beneficial effect on DNA strand break repair and maintenance of genomic stability as a result of NA supplementation of normal human primary cells obtained from young healthy subjects. Surprisingly, the cellular maintenance systems can further be optimised with this simple pharmacological intervention, showing effects even in cells without niacin deficiency. NA medically applied could be an additional tool to enhance the protection against genomic instability *e.g.* during cancer therapy or the ageing process.

Acknowledgements

This work was supported by the *Deutsche Forschungsgemeinschaft* through Research Training Group 1331 (fellowships awarded to K.W.). We thank Prof. Christof R. Hauck, University of Konstanz for kindly giving us access to the flow cytometer.

Supplementary Data

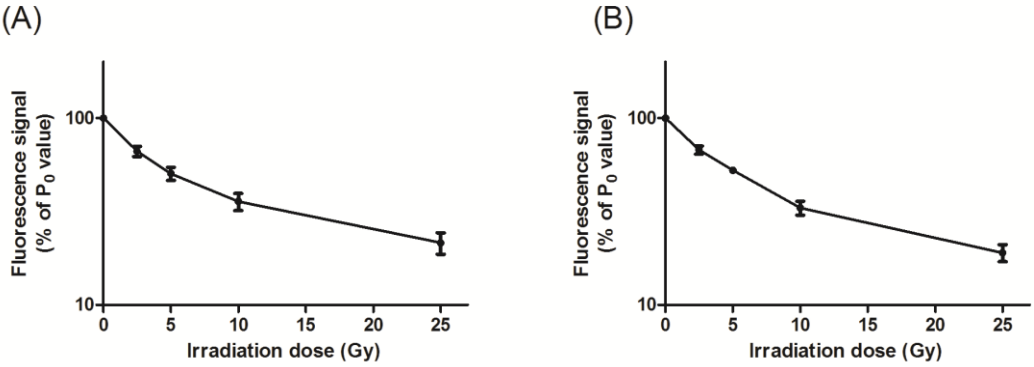


Figure S1:

DNA strand break induction (P_x values) of (A) unstimulated and (B) PHA-L stimulated PBMC as function of radiation dose. The data correspond to those shown in Figures 5 and 6 and represent the DNA strand breakage induced by ionising radiation as analysed by the automated FADU assay.

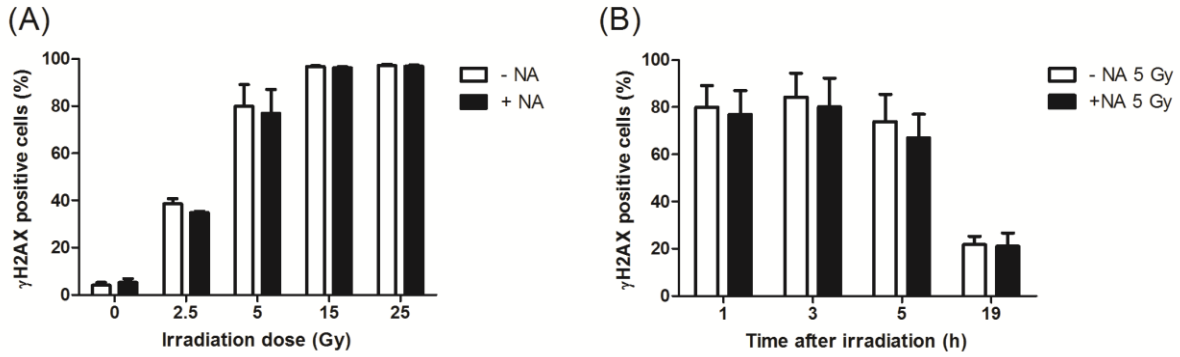
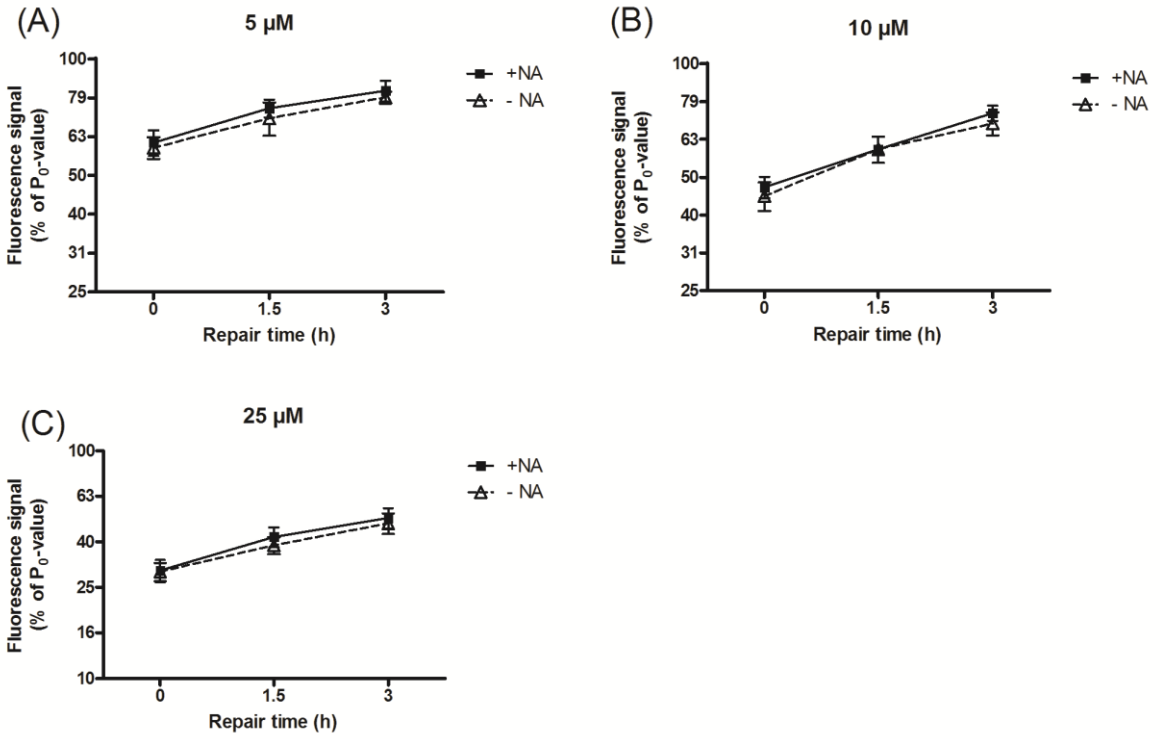


Figure S2: **Determination of γH2AX formation after irradiation.**

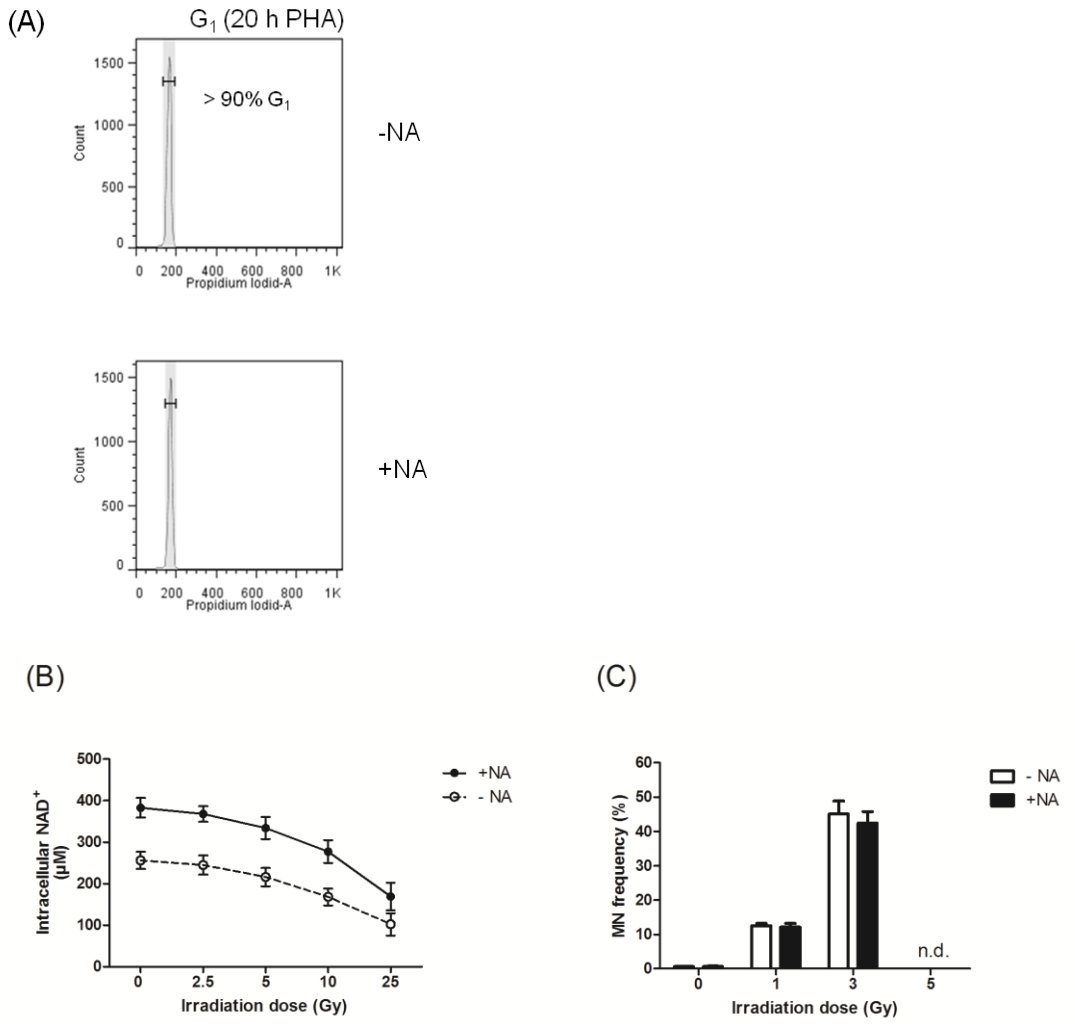
(A) Unstimulated PBMC were irradiated with indicated doses and fixed with methanol/acetic acid (3+1) 1 h after damage induction. Samples were stained with anti-phospho-histone H2A.X (Ser139), clone JBW301 antibody (Upstate, Millipore, Schwalbach, Germany). Fluorescent signal of secondary antibody Alexa 488 (Invitrogen) was analysed by flow cytometry. Using FlowJo software non-irradiated control cells were used to set the threshold gating to determine the percentage of γH2AX positive cells [185]. (B) γH2AX formation and time-dependent decrease after irradiation with 5 Gy. Data represent γH2AX percentages ± SEM of 3 donors.

Additional Data



Additional Figure A1: DNA damage and repair in unstimulated PBMC after exposure to alkylating agent MNNG.

Cells were treated for 10 minutes with (A) 5 µM (B) 10 µM or (C) 25 µM MNNG at room temperature and washed in PBS two times to remove the alkylating substance. Samples were incubated in a reaction tube with perforated lids in medium for indicated time points and subjected to FADU procedure afterwards. Values represent the mean fluorescence (± SEM) of double stranded DNA in relation to non-irradiated controls (P_0) as obtained in independent experiments of 4 donors. No significant impact was observed in NA vs. non-supplemented cells.



Additional Figure A2: Cell cycle distribution, NAD^+ levels and micronucleus formation in PHA-stimulated cells damaged in G_1 phase

(A) Cell cycle profile of PBMC stimulated for 20 h with PHA-L. Cells are in G_O/G_1 phase and have not entered S/G_2[186]. (B) NAD^+ levels in response to various doses of irradiation. Mean ±SEM of 4 donors. (C) Frequency of micronucleus appearance. Mean ±SEM of 6donors is shown. MN formation for 5 Gy was not detectable (n.d.).

CHAPTER III

Evaluating the impact of nicotinic acid supplementation on sirtuin deacetylase activity

Kathrin Weidele, Sascha Beneke, Alexander Bürkle

Abstract

Apart from the crucial role as coenzyme in the cellular redox state, NAD^+ is used as substrate by the NAD^+-dependent enzymes poly(ADP-ribose)polymerases (PARPs) and sirtuins (SIRTs), members of the broad families of ADP-ribosyltransferases and protein deacetylases that regulate fundamental cellular processes. As both are implicated in stress responses and dependent on NAD^+, a modulation of the substrate levels will impact their respective activities under DNA damaging conditions. Recently we showed that the supplementation of nicotinic acid, a precursor able to increase intracellular NAD^+, results in enhanced poly(ADP-ribosyl)ation levels and affects cell death, DNA repair and genomic stability in a positive manner. To elucidate if the activity of sirtuins is also affected, we used (i) a commercial available SIRT fluorimetric kit and (ii) monitored the acetylation status of SIRT-1 target protein p53 in human peripheral blood mononuclear cells. Although endogenous SIRT activity measured with the fluorimetric kit was diminished when salermide, a SIRT inhibitor was applied, no NAD^+ dependent impact was detectable in terms of basal SIRT-1 activity. However, the immunoassays revealed that the DNA damaged induced p53 accumulation and acetylation was clearly reduced in nicotinic acid supplemented cells, showing a dependency of the available substrate.

Introduction

Nicotinamide adenine dinucleotide (NAD+) is a cellular molecule involved as coenzyme in cellular oxidation/reduction reactions. Beside its key role in energy metabolism as electron carrier, it is constantly consumed by NAD^+ degrading enzymes. This includes (i) poly(ADP-ribose)polymerases (PARP) (ii) sirtuins (SIRT) and (iii) cADP-ribose synthases. Activated PARPs transfer the ADP-ribose unit from NAD^+ on an amino acid receptor and catalyze the formation of polymers, releasing nicotinamide (NAM) [1]. Among the 17 homologous genes containing a conserved PARP motif, PARP-1 and 2 are stimulated immediately after DNA damage and consume massive amounts of NAD^+ for the poly(ADP-ribosyl)ation (PAR) reaction. The most active in this context is PARP-1 which accounts for over 90 % of total PARP activity [18]. PARPs and the long branched homopolymer PAR affect chromosomal architecture, DNA repair

processes and are also implicated in the regulation of cell death [20]. Sirtuins belong to a family of NAD^+ dependent protein deacetylases [187] which are related to silent information regulator 2 (Sir2) in yeast [188]. In principle, they remove acetyl groups on lysines under consumption of NAD^+ and the release of NAM and O-acetyl-ribose [189]. In mammalians seven SIRT homologs are localized in diverse cellular compartments [190], whereby SIRT-1, SIRT-6 and SIRT-7 are nuclear proteins like PARP-1. Sirtuins function in the regulation of gene expression and chromatin structure and are implicated in energy metabolism [73]. Interestingly, some sirtuins have a similar mode of action as PARPs, they possess ADP-ribose transferase activities (e.g. SIRT-6) [82]. The third NAD^+ consuming protein class are cADP-ribose synthases, which use NAD^+ as substrate to generate the second messenger cADP-ribose, for the mobilization of calcium [191].

The contributions of NAD^+ metabolism, sirtuins and PARPs in mechanisms that influence cell survival under conditions of stress give evidence of a close connection [192]. As both, SIRTs and PARPs, consume the same substrate, their activities may be regulated by each other [94]. In a recent study we analysed the effects of an increased NAD^+ level on PARP mediated reaction in response to stress. PAR formation was significantly increased and cellular integrity was positively modified, as genomic instability was reduced and necrotic cell death was decreased [159]. However, beneficial effects may also be related to strengthened SIRT activity as consequence of boosted NAD^+ levels. It was reported that NAMPT [92] and the overexpression of NAD^+ biosynthetic enzyme NMNAT-1 concomitantly increased SIRT-1 activity [109] in mammalian cells [91]. There is substantial evidence that increasing NAD^+ levels by supplementation with one of the biosynthetic precursors may also influence the activity of SIRTs. To test this, we focused on the modification of p53, a classical deacetylation target of SIRT-1 and marker for sirtuin activity. The tumor suppressor p53 is an essential transcription factor in the regulation of cellular processes after DNA damage. Under normal physiological conditions p53 is inhibited by its negative regulator Mdm2, maintaining cellular p53 levels low. However in stress situations p53 is stabilized, leading to the transcription of p53-responsive genes implicated in cell cycle arrest and apoptosis [55]. As p53, PARPs and SIRT are all part of DNA damage response cascade, it is not surprising that interconnections have been described. As mentioned before, p53 is a target of nuclear SIRT-1 [75]. The deacetylation of p53 downregulates its activity and consequently affects lifespan and cell survival [76]. On the other hand p53 can also be modified by PARP-1 [54, 193].

Based on publications of Aksoy [194] and Escande [195], we choose two methods to determine the endogenous SIRT activities and deacetylation capacity upon genotoxic stress. Using (i) a commercial available deacetylase detection kit and (ii) western blot technique, we evaluated the impact of raised intracellular NAD^+ levels on endogenous deacetylase activity, with the focus on SIRT-1. Furthermore we determined if the endogenous expression and acetylation level of p53 in human blood mononuclear cells (PBMC) is affected by the supplementation with NAD^+ precursor nicotinic acid (NA) in response to x-irradiation.

Materials and methods

Chemicals and reagents

Standard chemicals were purchased from Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany) and Merck (Darmstadt, Germany) if not stated otherwise.

Isolation of peripheral blood mononuclear cells

In accordance with the Declaration of Helsinki and permission of the ethical committee of the University of Constance blood sampling was carried out from healthy donors aged 24-45 years. After blood collection using S-Citrat-Monovettes (Sarstedt, Nümbrecht, Germany) cells were separated via Biocoll (Biochrom, Berlin, Germany) gradient centrifugation as described in detail earlier [159]. Isolated cells were incubated in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Darmstadt, Germany) at 37°C with 5% CO_2 in a humidified atmosphere. Five hours before DNA damage induction nicotinic acid was added to the culture medium at a final concentration of 15 µM.

Deacetylase activity assay

To determine the effect of increased NAD^+ levels on deacetylase activity, we used Fluor de Lys system from Biomol. The Fluor de Lys® Substrate comprises an acetylated lysine side chain and is incubated with a sample containing deacetylase activity. The addition of a developer generates a fluorophor as a result of the deacetylation, which allows the detection of

deacetylase activity. In combination with trichostatin A, which inhibits class I/II HDACs, but not sirtuins (class III HDACs), the specific SIRT related deacetylase activity can be monitored.
To examine the in vitro deacetylase activity in PBMC, the SIRT Fluorescent Activity Assay kit (Biomol, Hamburg, Germany; catalogue no. AK-555) was used with slight modifications as published earlier [195]. Proteins lysates containing cellular deacetylase activity of $5x10^5$ cells per sample were prepared with the lysis buffer of the kit plus protease inhibitor cocktail and DTT. The lysates were incubated with the assay buffer (25 mM Tris-HCl, pH 8.0, 2.7 mM KCl, 137 mM NaCl, 1 mM $MgCl_2$, and 1 mg/ml bovine serum albumin) containing the Fluor de Lys-p53 peptide (amino acid residues 379–382 of human p53, Arg-His-Lys-Lys [Ac]) in the presence of 200 µM or 400 µM NAD^+ for 1 h at 37°C. To inhibit other HDACs than SIRTs 1 µM TSA was added additionally. The deacetylase activity was measured in a microplate-reading fluorimeter after addition of the developer and 1 hour incubation.

Western blot analysis of p53 and ac-p53 status

Cells were supplemented with NA for 5 hours and 1 µM TSA for 1 hour before irradiation. At distinct time points after DNA damage, cells were collected, washed in PBS and cell number was adjusted to $5x10^5$/20 µl per sample. For cell lysis appropriate volume of hot SDS sample buffer (5x; 100 mM Tris-HCl pH 8.0, 25 % ß-mercaptoethanol, 5 % glycerol, 12.5 % SDS, 0.01 % bromphenol blue) was added to the cell suspension and incubated for 5 min at 95°C. Afterwards extracts were sonicated with 3-4 bursts of 15 seconds each. Proteins were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with the anti-p53 antibody (Calbiochem, Merck, Darmstadt, Germany) or ac-p53 (Epitomics, Biomol, Hamburg, Germany). Same membranes were re-probed with anti-actin antibody (Upstate, Millipore, Schwalbach, Germany) to ensure equal loading. ImageQuant LAS 4000 system was used for quantitative imaging of blots by Amersham ECL chemiluminescence (both from GE Healthcare, Munich, Germany).

Statistical analysis

For statistical analysis means of data were compared with two-way ANOVA and Bonferroni posttest using Graph Pad Prism (La Jolla, USA). P-values < 0.05 were considered significant and indicated with an asterisk (*), ** $P < 0.01$, *** $P < 0.001$.

Results

Deacetylase activity in human PBMC

To determine the intracellular deacetylase activity - or to be precise the SIRT activity in human PBMC - we tested a commercial available kit from Biomol for its specificity, practicability and usability in that context. Figure 1A shows the fluorescence related deacetylase activity of cell lysates pre-incubated without HDAC class I/II inhibitor TSA to monitor the total deacetylase activity and with TSA to test for the respective sirtuin activity. High fluorescence values as determined for non-TSA treated samples indicate strong deacetylase activity. To determine the SIRT related proportion, the respective deacetylase activity of SIRTs was measured including TSA. The detected signal was dramatically decreased when samples were treated with TSA, showing the “pure” SIRT activity.

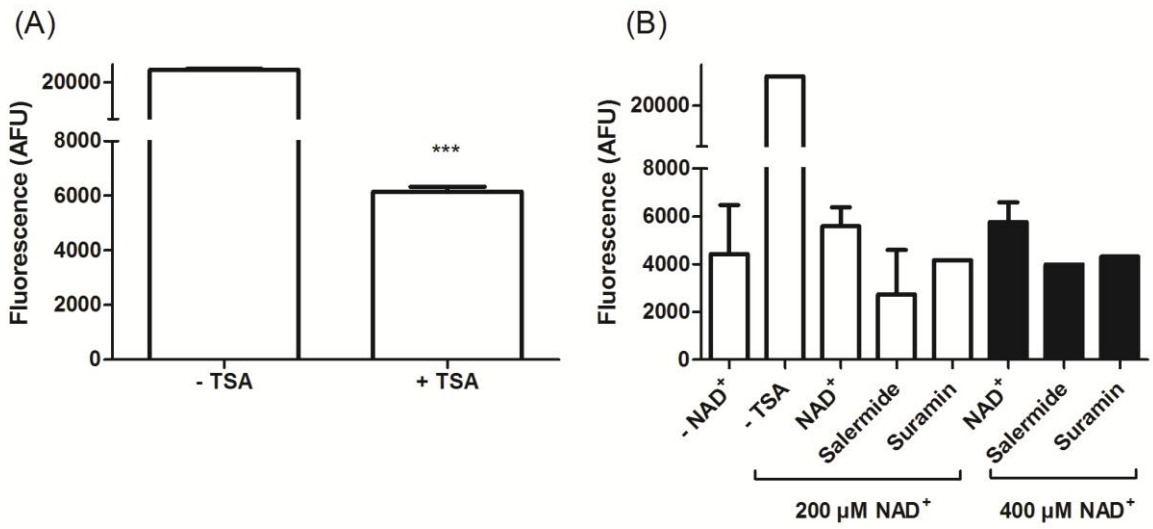


Figure 1: Endogenous activity of HDACs and SIRTs.

(A) Cellular PBMC lysates (5x105 cells per sample) were incubated with a substrate mix as described in Materials and methods with or without TSA to monitor total deacetylase activity and the SIRT related part, respectively(*p<0.05, Student`s t-test) (B) Samples were treated as described in (A) but incubated with various NAD^{+} concentrations and SIRT inhibitors. We used 200 µM or 400 µM NAD^{+}, which corresponds to the concentration in non- and NA-supplemented cells, respectively. Graphs represent the mean fluorescence signal ± SEM of 3 independent experiments of various donors.

In order to test the influence of modified NAD^+ levels, different concentrations of endogenous added NAD^+ 200 µM or 400 µM were used, which reflects the physiological and NA supplemented intracellular NAD^+ levels [159]. Control samples without exogenous added NAD^+ were also included. No differences were observed independent of the added NAD^+ concentration (Figure 1B). However, in combination with sirtuin inhibitors salermide or suramin, fluorescence signals were additionally reduced compared to controls.

Expression level of p53 and ac-p53

As alternative method to monitor SIRT activity cellular extracts were loaded on a SDS PAGE and proteins were blotted on a membrane. With the application of specific antibodies recognizing either total p53 protein or only p53 acetylated at K382, the SIRT-1 deacetylase activity was determined.

Cells were irradiated with 10 Gy and incubated afterwards as indicated to induce SIRT under stress conditions. As shown in Figure 2, the detected levels of total p53 were low in non-irradiated samples, but dramatically increased after DNA damage induction. Signal intensities were increased in a time-dependent manner after irradiation. The analysis of ac-p53 of the same samples showed that non-irradiated cells have no signals or barely detectable levels of acetylated p53 on lysine 382 (upper band). However there was a time dependent increase of acetylated p53 when the samples were irradiated.

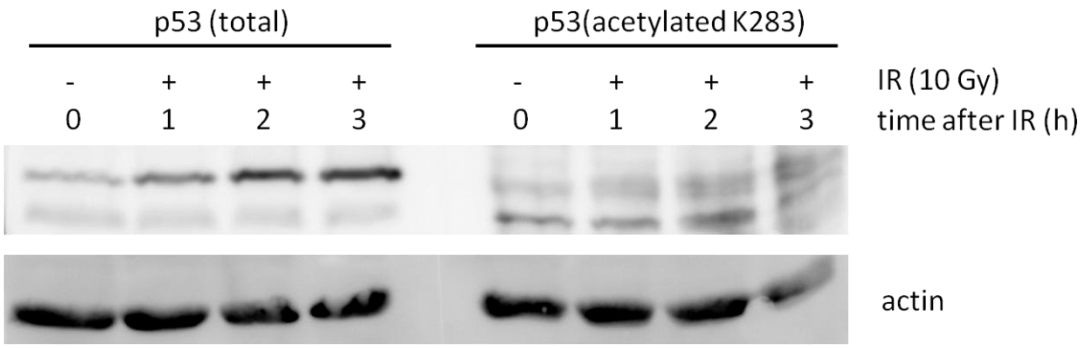
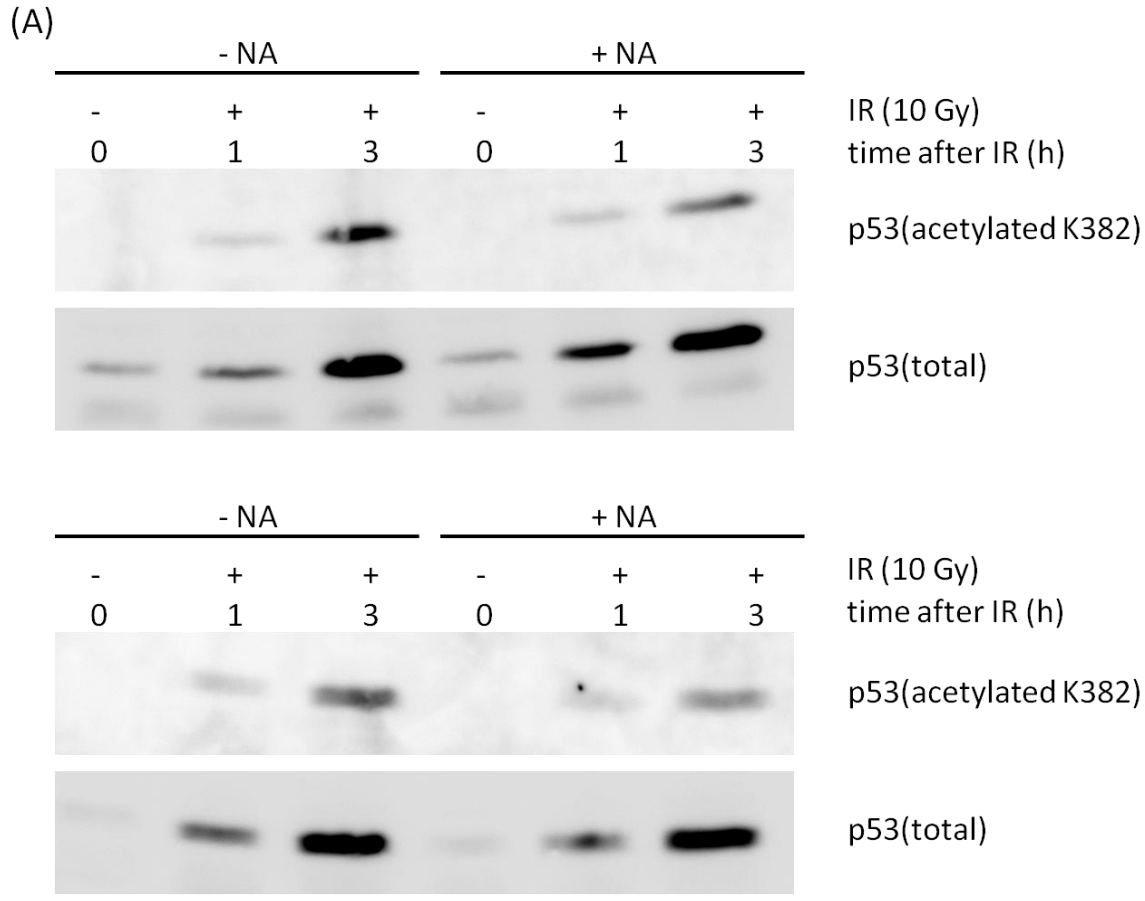


Figure 2: Time-dependent accumulation of total p53 and p53 acetylation on lysine 382 in response to irradiation.

Total proteins from 5×10^6 PBMC per lane were loaded onto SDS PAGE and analysed by western blot. Blots were incubated with indicated antibodies.

In order to detect NAD^+ dependent differences in the acetylation state, intracellular NAD^+ levels were modified via 15 µM NA supplementation prior DNA damage induction. In Figure 3 representative blots of two donors are shown (3A) and the quantitative analyses of 5 independent experiments (3B-D). The total intracellular p53 was stabilized as consequence of DNA damage in non- and supplemented samples to a similar extent. The levels of p53 protein at 3 hours post IR were slightly lower in NA-supplemented cells. Significant differences were observed for ac-p53 levels in NA-supplemented cells also at 3 h post IR, where intensities were decreased in comparison to non-supplemented controls.



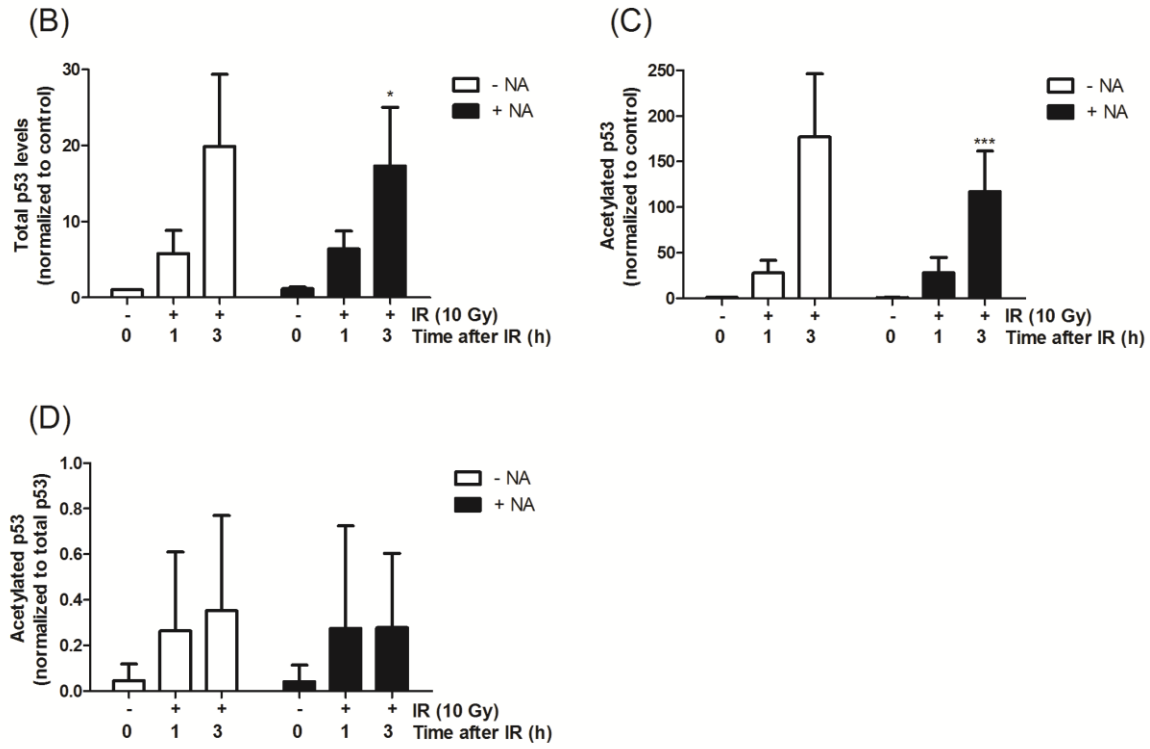


Figure 3: Total p53 accumulation and p53 acetylation on lysine 382 in response to DNA damage.

PBMC were pre-treated -/+ NA, TSA and irradiated with 10 Gy. Levels of p53 and ac-p53 were measured in whole cell lysates via immunoblot analysis at indicated time points after irradiation. (A) Representative blots of 2 donors are shown. (B-D) Quantitative analysis of band intensities for total p53 (B), acetylated p53 (C) and total p53 to ac-p53 ratio (D) are shown for 5 independent experiments ±SEM. A significant difference in NA-supplemented cells compared to non-supplemented is indicated with $^{*}p<0.05$, $^{***}p<0.001$, repeated measures ANOVA.

Discussion

Genotoxic events can moderately or seriously decline intracellular NAD^+ levels as stress-related NAD^+ consuming enzymes become active. Aside from PARPs, which are the main consumers, sirtuins are affected by altered NAD^+ levels or differences in the NAD/NADH ratio. Recent reports showed that upregulated NAD^+ biosynthesis, which increases the available NAD^+ pool, had protective cellular effects under genotoxic stress [91, 109]. Furthermore Pillai and co-workers underlined that modulation of SIRT activity either by repletion of cellular NAD^+ levels by directly adding NAD^+ to the culture medium or by upregulating NAD^+ biosynthetic enzymes is implicated in enhancement of cell protection against genotoxicity, which is directly linked to PARP activity [97]. This led us to the question, if extracellular added nicotinic acid, which was proven to increase intracellular NAD^+ levels and DNA-damage activated PARP activity in human PBMC [159], also controls sirtuin functions, thereby regulating diverse targets and transcriptional programs.

Independent of NA supplementation we analyzed the impact of exogenous added NAD^+ on endogenous deacetylase activity in PBMC lysates using SIRT fluorimetric detection system. Although we identified differences in the fluorescence related deacetylation of the substrate when HDAC class 1 and 2 were inhibited with TSA (more than 70 % reduction), the SIRT related signal was independent of added NAD^+. In contrast to the publication of Escande, where deacetylase activity in rat nuclei liver extracts was strongly dependent on exogenous added NAD^+ [195] no impact was visible in our samples. We expected that the addition of exogenous NAD^+ will lead to an increase in deacetylation of the added substrate comparable to *ex-vivo* NA-supplementation; however the fluorescence signals for controls without exogenous added NAD^+ were equal to the samples when 200 µM or 400 µM were used for incubation. This could mean that endogenous NAD^+ in our samples is not limiting and sirtuins stable active. Using salermide and suramin, inhibitors of sirtuins [196], we confirmed that the remaining fluorescence after TSA addition is specifically SIRT activity and is decreased after their application. As the used system only detects basal, not stress related SIRT activity, additional samples, e.g. irradiated with x-rays could be included for clarity. However, this approach was not suitable to determine the dependency of sirtuin deacetylase activity on NAD^+ using this *in vitro* system.

Apart from the *in vitro* approach we chose a classical deacetylation target of SIRT-1, p53, to investigate the cellular deacetylse activity [75]. PBMC lysates pre-treated with NA were analyzed via western blot technique for their (i) p53 levels and (ii) (de-) acetylation status of p53 as consequence of DNA damage. As expected, the total p53 levels are low under basal conditions, as it is constantly degraded. However, after DNA damage induction with 10 Gy a time-dependent increase was detectable in non- and NA-supplemented cells to a similar extent. From the evaluation of band intensities for modified p53 we observed slightly lower acetylation levels for NA supplemented PBMC 3 h post IR, which could be evidence for stronger deacetylation potentially caused by higher SIRT-1 activities. It is thought that activation of PARP-1 limits NAD^+ for subsequent SIRT functions, which was supported by data from Bai and coworkers, where the attenuation of PARP-1 leads to increased NAD^+ levels and enhanced SIRT activity [100]. As earlier published by our group, the *ex-vivo* supplementation of NA raises intracellular NAD^+ levels and increases poly(ADP-ribosyl)ation levels under genotoxic circumstances, but doesn`t limit the substrate availability to the same extent compared to non-supplemented cells. The remaining adequate NAD^+ level could favour SIRT functions and supporting cellular survival, respectively. An animal study showed that an increase in NAD^+ concentration is linked to increased SIRT-1 catalytic activity in fasting mice [80]. However, to our knowledge, there is so far no study with human primary cells addressing the impact on sustained NAD^+ levels on SIRT-1 deacetylase activity.

Here we show evidence that NA supplementation obviously affects p53 acetylation status, which indicates for stronger deacetylation by SIRT-1 within the first hours after irradiation. As strongest acetylation occurred after the latest measured time point at 3 h, a prolonged time course would give information about the progression of deacetylation rate. Additionally, we plan to include SIRT inhibitor experiments, as already done for the *in vitro* approach, to further evaluate our results.

Herein we focused specifically on deacetylase activity of SIRT-1, as its directly linked due to its cellular localization, the dependence of NAD^+ as well its function in regulating cellular processes, including gene expression, DNA repair, cell-cycle progression, chromatin remodelling and genome stability [197]. However, other nuclear sirtuins like SIRT-6, a deacetylase and mono ADP-ribosyl transferase [82], which seems to be implicated in DNA repair and genomic stability [83, 84], could be affected as well by altered NAD^+ levels. Upcoming work will bring some light in the understanding of the impact of NAD^+ modulation

not only on PARP proteins and functions, but also other NAD^+ dependent proteins and their potential therapeutic versatility.

Acknowledgements

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GENERAL DISCUSSION

One of the most crucial processes in regard to the stabilization of genetic information is the cellular DNA damage response system. Among various specialized proteins and signalling pathways, the proteins PARP-1/2 and the associated poly(ADP-ribosyl)ation reaction is activated, thereby affecting physiological and pathophysiological aspects, including DNA repair, maintenance of genomic stability and cell death. For this reason the modulation of the enzymatic activity represents an interesting target for pharmacological intervention. Although there is a great potential of PARP inhibitors in antitumor therapy and therapy of ischemia-reperfusion damage, it should be taken into account that PARP-1 has a key role in genomic maintenance and its ablation impacts fundamental cellular processes. Thus, alternative strategies not interfering, but rather supporting PARP-1 activity, could be a new promising application.

One possible approach is the modulation of the substrate NAD^+ concentration, a parameter that dictates PARP-1 activity, the formation of PAR, ultimately affecting other NAD^+ dependent processes and thereby strongly influences the balance between prevention and aggravation of disease processes.

Former studies by Kirkland and colleagues demonstrated the importance of an adequate NAD^+ status in regard to tissue function and health, showing dramatic effects of niacin deficiency on ADP-ribose metabolism [142] and genomic instability in bone marrow cells in an *in vivo* rat model [160]. Conversely, the external supply of niacin is capable of elevating the cellular NAD^+ [143] thereby influencing NAD^+ dependent processes, most notably ADP-ribosylation reactions mediated by PARP-1 as a major NAD^+ consumer [173].

Considering these background information, we aimed to investigate the biological consequences of modulated, specifically increased NAD^+ levels on intracellular PARP activity and poly(ADP-ribosyl)ation under DNA damaging conditions via the administration of extracellular added NAD^+ precursor nicotinic acid, an effective NAD^+ booster (Chapter I and II). As activated PARP-1 reduces the NAD^+ bioavailability for NAD^+ dependent deacetylases as sirtuins, thereby limiting their activity, maintained NAD^+ levels by NA supplementation will consequently benefit the activity of both enzymes, PARP-1 and SIRT-1, which is summarized in Chapter III.

Human blood cells as cellular system and NAD^+ levels

In contrast to the niacin deficient models investigated by Kirkland, we screened primary cells from human subjects with niacin proficient background for protective effects attributed to increased NAD^+ levels, either by strengthened PARP-1 or SIRT-1 activity. Although there are some human studies describing the aspects of niacin intake and contribution to increased resistance to DNA damage, reliable data concerning DNA repair, PARP activity and chromosomal integrity in a human system are limited, not consistent, or even absent for SIRT activity. For example, Weitberg reported that lymphocytes from two *in vivo* supplemented volunteers exhibited less strand breaks in relation to their intracellular NAD^+ concentrations, when treated *ex vivo* with oxygen radicals [175], whereas Hagemann found no evidence that cigarette smoke-related cytogenetic damage can be decreased in PBMC of smokers via an increase of the niacin status by supplementation with NA [198].

In addition, numerous *ex vivo* experiments were conducted using high doses of nicotinamide [173], a nutritional NAD^+ precursor with inhibitory effects on both PARPs [199] and SIRTs [200]. *In vitro* studies showed that high levels of NAM negatively interfere with PARP, thereby influencing genomic stability by increasing SCEs [201, 202] and delaying the rejoining of DNA strand breaks [174, 203]. Thus, NAM as supplement would be counterproductive, when the basic idea is to systematically stimulate the activity of PARP. In order to robustly increase the intracellular NAD^+ levels, but not interfering with basal PARP functions, nicotinic acid was exclusively used to for this work.

As described in Chapter I, the *ex vivo* application of low concentrations of nicotinic acid significantly increased intracellular NAD^+ levels in unstimulated human PBMC up to 2 fold on average; the application of the same concentration to mitogen-stimulated cells (Chapter II Figure 3) resulted in slightly lower augmentation, approximately 1.5 fold. We choose the minimal effective concentration of 15 µM NA, which improved the NAD^+ levels in PBMC of healthy human donors within 5 hours incubation time. Interestingly, our data are consistent with a study on human culture cells where NA in the micromolar range significantly increased NAD^+ levels within few hours, whereas NAM did not at the same concentrations [204]. In contrast to neuronal cell types, which are able to incorporate NAD^+ via specific transporters [130], addition of exogenous NAD^+ to PBMC did not increase cellular nucleotide pools (Schmitz M. Diploma thesis 2007).

NAD^+ and poly(ADP-ribosyl)ation

We monitored as first parameter sensitive to altered NAD^+ levels cellular poly(ADP-ribosyl)ation using flow cytometric based RAPARA (Chapter I). RAPARA enabled us to assess PAR levels in intact living primary cells under physiological conditions. Using this new approach we can detect PAR levels and the direct impact on PAR formation after addition of nutritional factors like NA, the effect of DNA-damaging agents or PARP inhibitors in direct comparison to control cells within one experimental setup.

We quantified how an excess of the available NAD^+ pool impacts the immediate response to DNA damage concerning the duration or intensity of PAR production. Although basal NAD^+ levels were increased after NA supplementation, basal PAR levels were not affected, suggesting no short-term impact of NA and maintained signalling under normal conditions when applied for 5 hours. However, the DNA damage induced PAR formation was significantly enhanced in supplemented cells. The application of ionising radiation, hydrogen peroxide or MNNG – substances able to induce double and/or single strand breaks - all significantly increased PAR levels in control cells and strongly intensified PAR formation in NA-supplemented cells. Time-course experiments also demonstrated that the polymers persist longer, suggesting that intracellular NAD^+ levels influencing PAR metabolism. Indeed, the size and complexity of the polymer seems to be dependent on substrate availability as ADP-ribose chains increase with elevated NAD^+ concentrations [205].

PARP-1 activation concomitantly decreased NAD^+ levels by polymer synthesis within the first minutes, which is in agreement with other publications [87]. Here we showed the direct correlation of polymer formation and NAD^+ consumption in dose- and time dependent manner in an intact cell system (Chapter I Figure 2 and 3). High DNA damaging doses (10 - 25 Gy) or concentrations (> 50 µM H_2O_2; >50 µM MNNG) lead to the hyper-accumulation of PAR, which results in detrimental NAD^+ reduction to approximately 40-60 % of the initial values, showing PARP`s major impact on NAD^+ metabolism. Inhibitor studies with PJ-34 attenuated NAD^+ depletion, clearly identifying PARP-1 as immediate NAD^+ consumer after DNA damage (Chapter I, Figure 4).

Under severe DNA damaging conditions, but also at lower genotoxic insults, NA supplementation causes (i) higher poly(ADP-ribosyl)ation but (ii) preserves an adequate NAD^+ pool, which both can be advantageous for further cellular reactions.

(i) The complex PAR product attracts proteins with high affinity binding sites including DNA repair proteins and signalling factors. Boosted PAR levels as measured in NA supplemented cells could lead to a more effective recruitment of DNA repair factors to the lesion site with a consequent higher survival rate or less mutations.

(ii) Strong NAD^+ depletion as part of PARP hyper-activation lowers ATP pools and triggers necrotic cell death pathway. The prevention of the excessive NAD^+ depletion could favour concomitant ATP depletion and subsequent cellular energy failure, minimizing cell death.

(iii) PARP as major NAD^+ consumer limits NAD^+ in relation to cell damage, thereby repressing SIRT activity by competing for the same substrate. The prevention of the excessive NAD^+ depletion could favour SIRT activity and thus cell viability, respectively.

To uncover if elevated NAD^+/PAR levels have beneficial effects on above mentioned reactions, appropriate assays for determining cell death, DNA repair, maintenance of genomic stability and SIRT-1 deacetylase activity were evaluated.

Effects on cell death

As mentioned above, there are two possible determinants playing a role in protecting cells under stress conditions. The activation of PARP-1, the concomitant poly(ADP-ribosyl)ation and also the functions of sirtuins are both implicated. As NAD^+ depletion is a key step in PARP-1 mediated cytotoxicity [130, 206], we initially investigated if sustained NAD^+ and increased PAR levels impacts on cell viability, as reported for NA supplemented damaged PBMC (Chapter I, [159].

In Chapter I we showed that NA supplementation reduces cell death after genotoxic stress (when damaged with IR or MNNG) and shifts the residual fraction from necrosis to apoptosis. Furthermore the addition of PARP inhibitor PJ-34 revealed strong implication of PARP-1, as

the beneficial effects on necrosis in NA supplemented cells were significantly dependent on PARP activation, especially at 25 Gy. Thus we propose that preserved NAD^+ levels even under circumstances of high PARP-1 activity after DNA damage is responsible for prevention of necrotic cell death. This is in line with recent publications showing that NAD^+ depletion is the cause of PARP-1-mediated cell death in neuronal excitotoxic injury and that the addition of exogenous NAD^+ effectively inhibits cell death [206, 207] and restore glycolytic functions [208].

The second sequential step contributing to cell death is the depletion of cellular NAD^+ stores and subsequent attenuation of SIRT-1 deacetylase activity. It has been proposed that the decline of NAD^+ and the rise of nicotinamide induced by PARP-1 activation may downregulate the activity of SIRT-1 [94]. As consequence of increased PARP activity and NAD^+ consumption, diminished deacetylase activity can lead to hyper-acetylation of pro-apoptotic factors as p53, which impacts negatively on cell survivability [209]. As indirect marker for SIRT-1 deacetylase activity we therefore analysed p53 acetylation levels in irradiation stressed cells and the impact of NA supplementation, respectively (Chapter III). Irradiation with 10 Gy induced acetylation of p53 in a time-dependent manner, with strongest signals at latest measured time points. We observed slightly lower acetylation levels for NA supplemented PBMC 3 h post IR, which could be evidence for stronger deacetylation potentially caused by higher SIRT-1 activity, which could be a additional factor shifting the balance towards cell survival.

There are publications indicating that sufficient NAD^+ and proper SIRT deacetylase activity is essential for protective effects in cardiac myocyte [97] or axonal cell death [109]. For example, Pillai showed that PARP mediated myocyte cell death could be prevented by repletion of cellular NAD^+ by adding NAD^+ to the culture medium or by overexpressing NAD^+ biosynthetic enzymes [97].

In summary, our data support that depletion of cellular NAD^+ levels forms a link between PARP activation and reduced SIRT-1 deacetylase activity and that preventive intervention via enhancement of basal NAD^+ pools effectively diminishes cell death.

Effects on DNA damage and repair

Aside from the cytoprotective effects caused by preservation of cellular NAD^+ levels and concomitant avoidance of energy failure, efficient DNA repair mediated by the formation of PAR is the other determinant positively influencing cell survival [210]. The polymer formation facilitates the assembly of repair proteins proximal to DNA strand breaks and enables the opening of the condensed chromatin structure. Thus we hypothesized that increased PAR synthesis improves the response to DNA damage and favours DNA repair (Chapter II).

On average, we observed significantly modified strand break repair in NA supplemented cells after genotoxic stress, which was most pronounced at higher doses, when NAD^+ is limited due to strong PARP-1 activation. However, beneficial effects were also detectable under lower damaging conditions. In addition to the effects on irradiation induced damaged and repair as included in the manuscript in Chapter II, we used the alkylating agent MNNG (Additional Figure A1). The repair rate was slightly but not significantly affected. In order to clarify exactly whether the low donor number or differences in the repair pathway is responsible for these results as detected for MNNG, more experiments should be conducted.

Interestingly, although the impact on DNA repair was only marginal in most cases of the tested donors, there was no impairment of strand break rejoining caused via the NAD^+/PAR modulation. Briefly, the supplementation of NA to PBMC before damage infliction can be supportive or ineffective in context to DNA repair, but obviously not negatively interfering with ongoing repair processes. Conversely, niacin deficiency, as evaluated by the Kirkland group in mice, delays excision repair and causes double strand break accumulation, which in turn favours chromosome breakages and translocations [161]. It is considered, that catalytically inactive PARP, lacking the ability for automodification, negatively affects the repair process.

In our model it is conceivable, that the induced amount of PAR as a result of DNA damage is sufficient for recruitment of repair factors under normal conditions, but especially in the case of severe damage crucial for the faster or increased attraction, which could be managed by sustained PAR formation in NA supplemented cells. The complexity of poly(ADP-ribose) structures, i.e. the length and branching of produced polymer could play a role in determining specific functional outcomes as observed for non- and NA-supplemented cells.

Regardless of a positive contribution of an increased PAR level in the repair process, there is mounting evidence for an involvement of SIRT proteins in damage detection and repair [83, 211]. Specific inhibitors or ablation systems for PARPs and SIRTs in that context could figure out the cellular interplay and the impact of varying NAD^+ levels. Indeed, SIRT-1 mutant mice show impaired DNA damage response and reduced ability to repair DNA damage [184].

Effects on genomic stability

As described in the previous parts, diverse NAD^+ dependent reactions are implicated in maintaining genomic stability, particularly the nuclear enzymes PARP-1 and SIRT-1 [136, 212]. They both play strong integrative roles, which are supported by diverse studies. The inhibition/ablation of PARP-1 increases the formation of spontaneous SCE and enhances SCE and MN formation following exposure to genotoxic agents [213-215]. Moreover it was demonstrated, first in yeast and then mammalians, that sirtuins have an important task in genomic integrity by maintaining proper chromatin structure [84, 184, 212].

To address if altered NAD^+ levels influence the genomic integrity of damaged cells, we analysed micronucleus formation as classical marker for genomic instability as further endpoint. We observed that increased NAD^+ levels are accompanied by changes in micronucleus frequency. In response to doses of 3 - 5 Gy we found decreased MN frequency in proliferating cells pre-incubated with NA. Furthermore, there was a tendency towards lower MN frequency in unstimulated lymphocytes after NA addition, and a significant difference apparent at 5 Gy. Of note, the cells damaged during proliferation seem to be more susceptible for NA supplementation than unstimulated cell. Possible cell-cycle dependent differences could account for that. It was reported that NAD^+ levels fluctuate during cell cycle, with highest levels in G_1 and lowest in S/G_2 phase [177], which was consistent with variations in PARP activities during different stages [216]. NAD^+ measurements with PBMCs stimulated for 20 h with PHA, which are solely in G_1, had indeed higher basal NAD^+ levels than proliferating cells, when about 20-30 % are in S/G_2 stage (Chapter II Additional Figure A2). However, the supplementation was effective in both cases. Cells damaged in G_1 were highly sensitive to X-irradiation, but MN formation seems not to be significantly affected when

supplemented with NA. In order to clarify in which cell cycle phase the responses are divergent, G_1 and $S/G_2/M$ cells should be analysed separately.

Furthermore one has to consider, that PHA is a selective human T cell activator, which induces resting T lymphocytes to undergo cell division [217]. The slightly diverse composition of cell types or subpopulations in unstimulated compared to PHA-stimulated PBMC could as well account for differences in the sensitivity, as reported, as well [218].

In contrast to our data, pharmacological intakes of niacin do not appear to be further protective against the genotoxic properties of etoposide (ETO) compared to adequate intakes in mouse models [160]. However, the ETO induced formation of MN in that study was relatively low compared to controls. Our results also indicate no or less protective effects when the damage is minor (e.g. 1 Gy), but significant effects starting from 3 Gy or higher, which correlates with all investigated endpoints. Of note, the effects may depend on the type of carcinogen, target organ and organism.

Interestingly, a human study with 190 volunteers identified micronutrients appearing to be protective against genome instability, including preformed nicotinic acid, which was related with reduced baseline micronucleus levels [219]. Notably, the intake of NA originated from dietary habit and subject groups with higher intakes had significantly lower frequencies of MN. As additionally applied NA, as performed in this study, also give evidence to maintain genome health under damaging conditions, a maintained high level of intracellular NAD^+, e.g. via supplementation, seem to be valuable.

Concluding remarks & perspectives

The obtained results in that study showed that sufficient available NAD^+ is necessary for appropriate cellular functions in terms of DNA damage activated PARP-1 and presumably SIRT-1 activity, which positively impacts cell death, DNA repair and genomic stability.

Supplying exogenous precursor nicotinic acid seems to be a valuable approach to augment NAD^+ levels especially in the case of acute damage or to correct suboptimal NAD^+ levels to prevent from possible negative consequences. Although not included in that study, in theory the precursor nicotinamide riboside, which was shown to increase yeast Sir2 activity and

replicative life span [220], could alternatively be used in humans and tested for the explored effects.

While nicotinic acid supplementation in part has been studied *in vivo* in humans for its effects on NAD^+ levels [175], profound data concerning PARP activity are still insufficient [198] or lacking for sirtuin activity. Using this *ex vivo* approach we were able to monitor effects on PARP-1 activity, PARP-1 mediated reactions in response to DNA damage and for the first time consequences on human SIRT-1 deacetylase activity. The next consequent step to get a further understanding is the evaluation on *in vivo* effects. As nicotinic acid is medically applied as extended-release niacin due to its effects on lipids, atherosclerotic plaque, and cardiovascular outcomes, the respective drugs could potentially used as supplement for human trials.

RECORD OF CONTRIBUTIONS

Chapter I: *Ex-vivo* supplementation with nicotinic acid enhances cellular poly(ADP-ribosyl)ation and improves cell viability in human peripheral blood mononuclear cells

The included data are part of my diploma thesis prepared in 2008 under the supervision of A. Kunzmann. A. Kunzmann authored the first version of the manuscript which was included in her dissertation in 2009. Together with S. Beneke and A. Bürkle additional experiments were designed and conducted by myself as part of my PhD. I rewrote the manuscript with valuable comments from S. Beneke and A. Bürkle, which was finally published in Biochemical Pharmacology in 2010.
Further involved was M. Schmitz, she developed the protocol for the RAPARA in her diploma thesis in 2007.

Chapter II: The NAD^+ precursor nicotinic acid improves genomic integrity in human blood mononuclear cells after X-irradiation

As part of my PhD I investigated the effects of NA supplementation in human PBMC on cellular parameters. I designed and performed the experiments under supervision of A. Bürkle. S. Beneke gave me useful tips concerning the experimental design. I wrote the manuscript with valuable comments from S. Beneke and A. Bürkle. The manuscript was submitted to Biochemical Pharmacology in January 2012.

Chapter III: Evaluating the impact of nicotinic acid supplementation on sirtuins deacetylase activity

I planned and conducted the experiments under supervision of A. Bürkle. S. Beneke gave me helpful instructions for the design of the SIRT activity assay. I wrote the manuscript with very valuable comments of S. Beneke.

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