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Detection and pharmacological modulation of nicotinamide mononucleotide (NMN) *in vitro* and *in vivo*

Laura Formentini, Flavio Moroni, Alberto Chiarugi *

Department of Preclinical and Clinical Pharmacology, University of Florence, Italy

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ABSTRACT

The emerging key role of NAD-consuming enzymes in cell biology has renewed the interest in NAD resynthesis through the rescue pathways. The first step of the nicotinamide-dependent NAD-rescue pathway is operated by nicotinamide phosphoribosyl transferase (NaPRT) forming nicotinamide mononucleotide (NMN). Because of the difficulties in measuring NMN, numerous open questions exist about the pathophysiological relevance of NaPRT and NMN itself. Here, we describe a new method of fluorimetric NMN detection upon derivatization of its alkylpyridinium group with acetophenone. By adopting this method, we analyzed the kinetics of nicotinamide-dependent NAD recycling in HeLa and U937 cells. Measurement of NMN contents in subcellular fractions revealed that the nucleotide is highly enriched in mitochondria, suggesting intramitochondrial NAD synthesis. NMN increases in cells undergoing hyperactivation of the NAD-consuming enzyme poly(ADP-ribose) polymerase (PARP)-1, or exposed to gallotannin, a putative inhibitor of NMN-adenylyl transferases. Evidence that the inhibitor of NAD resynthesis FK866 selectively inhibits NaPRT having no effect on NMNAT activity is also provided. Importantly, NMN reduces NAD and ATP depletion in cells undergoing PARP-1 hyperactivation, significantly delaying cell death. Finally, we show that a single injection of FK866 in the mouse induces long-lasting (up to 16 h) but mild (~20%) reduction of NMN contents in different organs, suggesting slow rate of basal NAD consumption *in vivo*. Data provide new information on the biochemistry and pharmacology of NAD biosynthesis, allowing a better understanding of pyridine nucleotide metabolism.
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1. Introduction

Pyridine nucleotides have long been considered hydride ion carriers exclusively involved in oxidoreduction reactions. However, the recent identification of different enzyme families having nicotinamide adenine dinucleotide (NAD) as substrate has significantly renewed the interest in the biochemistry and pharmacology of pyridine nucleotides [1–3]. In particular, being these enzyme families responsible for the irreversible transformation of NAD into different products, attention has been focused on the mechanisms of NAD resynthesis into eukaryotic cells. Classically, two metabolic pathways regulate the formation of NAD in mammalian cells; i.e. the *de novo* (also called the "kynurenine") pathway, leading to NAD from tryptophan, and the rescue pathway using the nicotinamide moiety produced as a by-product by the NAD-consuming enzymes. The Preiss–Handler route also contributes to pyridine nucleotide formation, transforming nicotinic acid absorbed from the gut into NAD [1,4,5]. Notably, a recently identified metabolic route leading to NAD formation from nicotinamide riboside also exists [6,7]. Conversely, vertebrates are unable to convert nicotinamide into nicotinic acid [5].

A central metabolite in NAD resynthesis is nicotinamide mononucleotide (NMN, Fig. 1). It is produced by nicotinamide phosphoribosyl transferase (NaPRT) and by nicotinamide riboside kinase (NRK). NMN is then converted into NAD by three isoforms of NMN-adenylyl transferase (NMNAT). Recently, NaPRT received much attention by the scientific community because of its pleiotypic functions. Specifically, besides being an intracellular enzyme involved in NAD resynthesis, the protein has been also identified as a released factor behaving as an adipokine and as an inflammatory cytokine [8]. Adipokines are fat tissue-derived hormones with central roles in metabolism and disease pathogenesis. A protein with the same amino acid structure of NaPRT has been identified as a 52-kDa release product of visceral fat and therefore called visfatin [9,10]. Controversies exist as for the endocrine properties of visfatin/NaPRT. Although originally identified as an insulin receptor-interacting protein able to improve glucose-stimulated insulin secretion, the protein is now thought to be devoid of insulin receptor-interacting properties but

* Corresponding author at: Dept. Pharmacology, University of Florence, Viale Pieraccini 6, 50139 Firenze, Italy. Tel.: +39 055 4271230.
E-mail address: alberto.chiarugi@unifi.it (A. Chiarugi).

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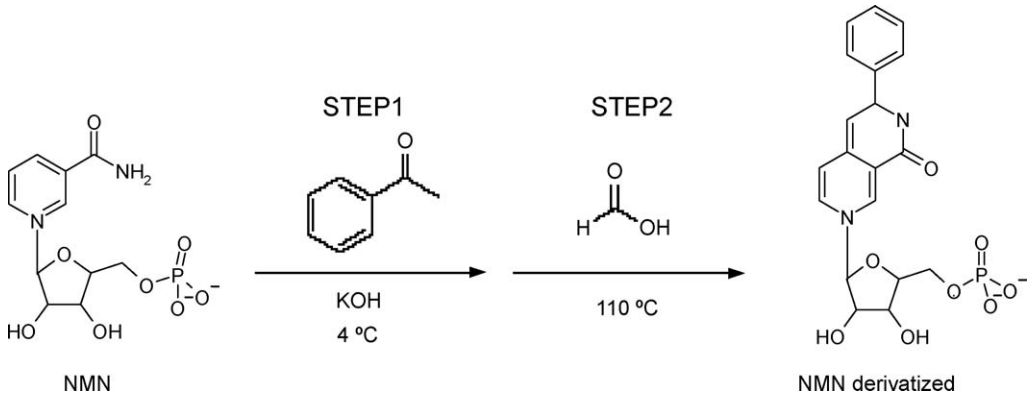

Fig. 1. Derivatization of NMN. NMN is converted into a fluorescent compound upon reaction with acetophenone and formic acid.

still able to regulate glucose homeostasis by promoting pancreatic islet insulin secretion. Of note, these properties are entirely dependent on NMN production by secreted visfatin/NaPRT [11]. The secreted protein can also behave as a cytokine called Pre-B cell colony-enhancing factor (PBEF). NaPRT/visfatin/PBEF is released by various immune cells, promotes TNFα, IL1β and IL-6 production, and is increased in the sera of patients affected by sepsis, acute lung injury, myocardial infarction, rheumatoid arthritis and inflammatory bowel disease. The protein is also able to inhibit neutrophil apoptosis, thereby promoting the immune response [12]. Whether NMN production contributes to the immunoregulatory functions of NaPRT/visfatin/PBEF is still unknown but a specific receptor has not been identified. Also, the permeability of NMN through the plasma membrane is not clear and, again, a possible NMN-interacting receptor waits to be identified.

Given the potential relevance of NaPRT/visfatin/PBEF to pathophysiology, several ELISA kits able to detect the protein have been recently made commercially available. Yet, these kits have different sensitivities and are heterogeneous in nature. These inconsistencies are probably responsible for the apparent contrasting properties of secreted NaPRT/visfatin/PBEF present in the literature. Also, since the functional properties of NaPRT/visfatin/PBEF may in part depend on its enzymatic activity [11,13], evaluation of intra as well as extracellular NMN production can certainly help understanding the pathophysiological role of the protein. Unfortunately, analytical determination of NMN is difficult because of its physicochemical features. Indeed, very few studies have measured NaPRT activity and/or NMN concentrations in biological fluids or tissue extracts using radioactive or complex HPLC/MS techniques [11,13,14]. In the present study we provide new information on NMN metabolism and pharmacological modulation by using an original method of NMN measurement as well as the newly identified inhibitor of NaPRT (E)-N-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3-yl) acrilamide (FK866) [15,16].

2. Materials and methods

2.1. Cell culture and treatment

Unless otherwise stated, all chemicals and cell culture products were from Sigma (Milan, Italy). HeLa or U937 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum and antibiotics. Cultures were brought to 50–70% confluence and exposed to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 100 μM), nicotinamide 10–1000 μM, NMN 10–1000 μM, NAD 0.1–1 mM. (E)-N-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3-yl) acrilamide (FK866, 1-100 μM) was obtained from NIH, Bethesda, USA. Cell viability was evaluated by measuring lactate dehydrogenase (LDH) release in the incubating media or reduction of methylthiazolyl tetrazolium (MTT) and described [17]. An inverted Nikon TE-2000U microscope equipped with a CDD camera was used for cell visualization.

2.2. Cell fractionation

As previously described [17], cells were disrupted using a glass/glass homogenizer in 500 μl of buffer A (Tris HCl 50 mM, pH 7.4, mannitol 225 mM, saccarose 75 mM, 1 mM PMSF, 10 μl of protease inhibitor cocktail), and centrifuged at 600 × *g* to obtain the nuclear pellet. Supernatants were centrifuged at 12,000 × *g* to obtain the mitochondrial pellet and the cytosolic fraction. Cell fractions were then processed for NMN determination as described above.

2.3. NMN, NAD and ATP measurement

Ultra pure NMN standards (Sigma, Milan, Italy) were dissolved in water and solutions analyzed by HPLC with UV or fluorimetric detection. For UV detection, cells grown in a 48-well plate were scraped with 100 μl of HCl 0.6. The cell extract was centrifuged at 14,000 × *g*/5 min and 25 μl of the supernatant injected in an HPLC system consisting in a mobile phase of 0.1 M buffer phosphate pH 6.5, 1% acetonitrile, 10 mM tetra butyl-ammonium bromide (TBAB), a Supelco 25 cm column (5 μm) and an UV detector (PerkinElmer) set at 260 nm. For fluorimetric detection, we modified a method previously described [18]. Briefly, cells grown in a 48-well plate were lysed with 100 μl of $HClO_4$ 1N, whereas mouse organs were sonicated in $HCLO_4$ 1N (1:4, w/v). Then, 100 μl of the extract were neutralized with KOH 1N and, after 5 min, additional 100 μl of 0.1 M bicine pH 7.4 were added. The cell extract was centrifuged at 14,000 × *g*/5 min and 10 μl of the supernatant were mixed with 100 μl of KOH 1N and 50 μl of acetophenone. The solution was incubated for 15 min at 4 °C, then 100 μl formic acid were added and the solution incubated 5 min at 100 °C. By means of this derivatization procedure, NMN is converted into a highly fluorescent compound as shown in Fig. 1. Excitation and emission spectra of the fluorescent compound were determined by means of a spectro fluorophotometer RF5000 (Shimadzu, Milan, Italy). Samples were injected into the HPLC system consisting in a mobile phase of 0.1 M buffer phosphate pH 6.5, 10% acetonitrile, a Supelco 25 cm column (5 μm) and a fluorimetric detector (PerkinElmer) with excitation and emission wavelength of 332 and 454 nm, respectively.

NAD contents were quantified by means of an enzymatic cycling procedure as described [19]. ATP was measured by the ATPlight kit (PerkinElmer, Milan).

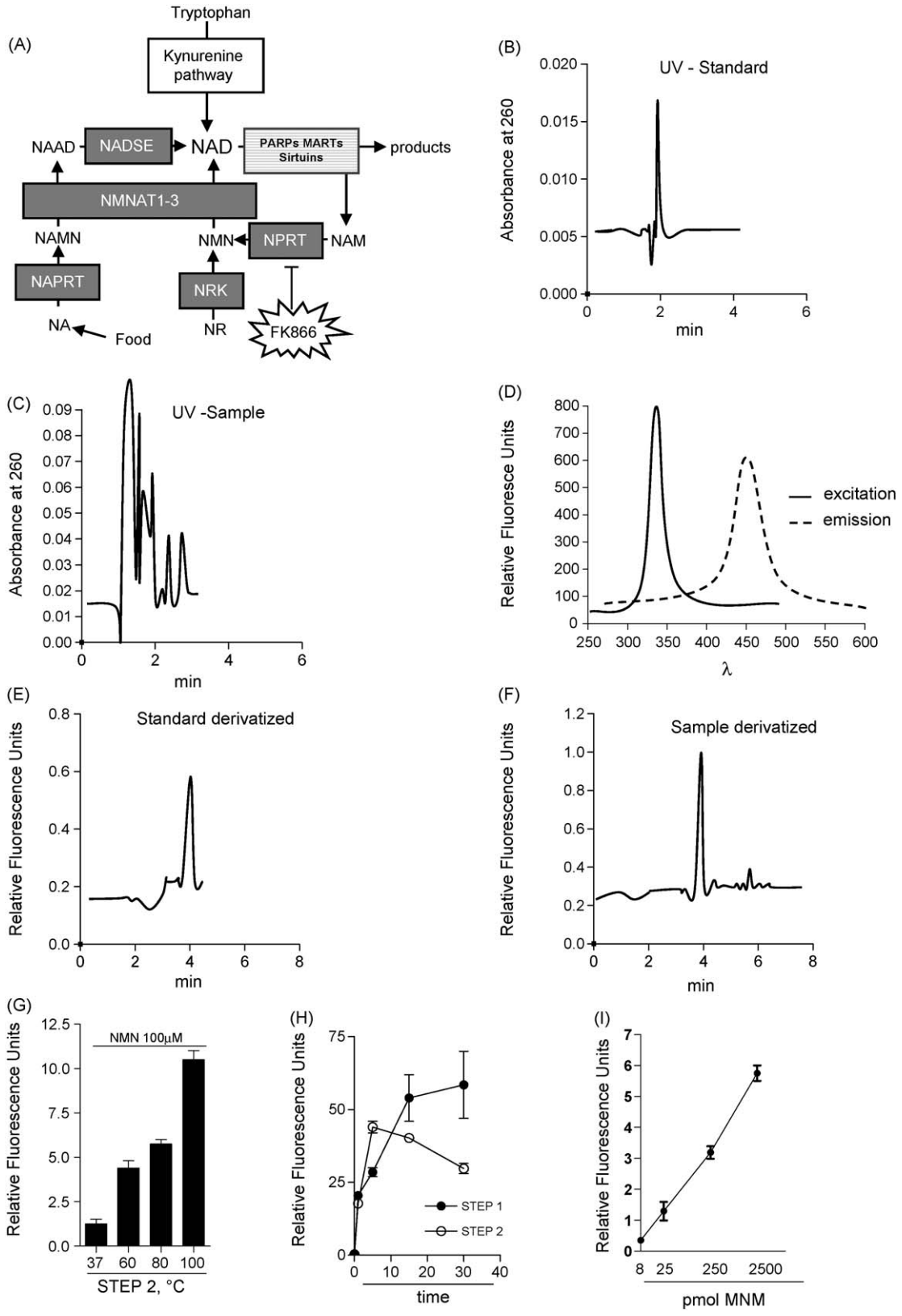

Fig. 2. Optimization on HPLC analysis of NMN. (A) NAD biosynthetic pathways in mammals. NAD is synthesized *de novo* through the kynurenine pathway originating from tryptophan. Intracellular NAD degrading enzymes such as PARPs, mono(ADP-ribosyl)transferases (MARTs) and sirtuins hydrolyze NAD forming different products and nicotinamide (NAM). The later is utilized to re-synthesize NAD through the NAD-rescue pathway including NaPRT and NMNAT1-3. Nicotinic acid (NA) originating from food is transformed into NAD through the Preiss–Handler pathway composed by NA phosphoribosyl transferase (NAPRT), NMNAT1-3 and NAD deamidase (NADASE). Nicotinamide riboside kinase (NRK) converts nicotinamide riboside (NR) into NMN. The site of action of the NaPRT inhibitor FK866 is shown. (B), Chromatogram of NMN (250 pmol) obtained with an HPLC apparatus coupled to an UV detector (see Section 2). (C), Chromatogram of a whole cell extract (U937 cells) injected into the same apparatus described in (B). (D) Absorption and emission spectra of NMN derivatized as described in Section 2. (E), Chromatogram of derivatized NMN (250 pmol) obtained with an HPLC apparatus coupled to a fluorimetric detector (see Section 2). (F) Chromatogram of a whole cell extract (U937 cells) derivatized as described in Section 2 and injected into the same

2.4. In vivo experiments

CD1 male albino mice (20–25 g) (n = 4 per group) were injected i.p. with FK866 (100 mg/kg) and sacrificed 4, 8 and 16 h later. Organs were rapidly collected and processed as described above for NMN content determination. Procedures involving animals and their care were conducted in compliance with the Italian guidelines for animal care (DL 116/92) in application of the European Communities Council Directive (86/609/EEC) and was formally approved by the Animal Care Committee of the Department of Pharmacology of the University of Florence.

3. Results

3.1. Spectrofluorometric HPLC analysis of NMN

Isocratic HPLC analysis of NMN with UV absorbance detection is virtually impossible because of its extremely short elution time. Indeed, under our experimental conditions (see Section 2), standard NMN had a retention time of 1 min 54 s (Fig. 2B). Addition of tetra butyl-ammonium bromide (up to 10 mM) to the mobile phase to increase NMN retention only minimally influenced this parameter (not shown). We were unable to identify an unequivocal peak with a retention time corresponding to NMN in a whole cellular extract from different cell lines (Fig. 2C and not shown).

It is well known that N-alkylpyridinium compounds are transformed into fluorescent compounds through reactions with ketone moieties followed by heating in acidic environment. Accordingly, methyl-nicotinamide and NAD have been quantified spectrofluorometrically for different purposes [18,20]. We therefore attempted to adapt this method to NMN measurement (see Section 2). Upon derivatization, NMN became fluorescent with excitation and emission maxima at 332 and 454 nm, respectively (Fig. 2D). Of note, elution time of derivatized NMN standard was increased up to 4 min 18 s (Fig. 2E), and a single peak with the identical elution time was present in a whole cellular extract derivatized as described above (Fig. 2F). The peak area linearly increased by spiking the extract with different amounts on NMN (not shown). Optimization of the derivatization procedure showed that NMN fluorescence increased linearly by augmenting the temperature of step 2 up to 100 °C (Fig. 2G). At this temperature, incubations longer than 5 min reduced fluorescence (Fig. 2H). Maximal efficiency of step 1 was reached with derivatization times of 15 min or longer. NMN fluorescence increased linearly over the range of 8–2500 pmol with a threshold sensitivity of 750 fmol (Fig. 2I).

3.2. Modulation of NMN content in HeLa and U937 cells

Basal contents of NMN in U937 cells were 3.7 ± 0.1 nmol/mg prot, and about 10-fold lower in HeLa cells (0.48 ± 0.02 nmol/mg prot). These findings suggested low basal activity of NaPRT in HeLa cells. We therefore attempted to evaluate in intact cells the contribution of NaPRT to NMN contents by adding nicotinamide to the cell medium. As shown in Fig. 3A, nicotinamide increased NMN contents in a concentration-dependent manner in U937 but not in HeLa cells, again suggesting low expression levels/activity of NaPRT in this cell type. In contrast with this interpretation, however, addition of nicotinamide to the culture medium similarly increased NAD contents in both cell types. Taken together, these findings suggested that NaPRT is active in both U937 and HeLa cells, and that NMN does not accumulate in HeLa because of its rapid transformation into NAD by NMNAT. Reportedly, NaPRT is the rate-limiting enzyme of the mammalian NAD-rescue pathway [21]. When NMN was added to the culture medium for 1 h, its intracellular contents increased in both cells types (Fig. 3C), indicating that the nucleotide readily permeates the plasma membrane. Accordingly, NAD contents increased in both HeLa and U937 cells exposed to NMN (Fig. 3D). We also measured the subcellular distribution of NMN in these cell lines. As shown in Fig. 2E, NMN was significantly more concentrated in the mitochondrial fraction than in the cytosolic or nuclear ones. However, when subcellular NMN levels were expressed as % of total cellular content, the nucleotide amount appeared higher in the nuclear fraction (Fig. 3F).

3.3. Pharmacological modulation of intracellular NMN contents

It has been repeatedly reported that, in condition of massive DNA damage, hyperactivation of the NAD-consuming enzyme poly(ADP-ribose) polymerase (PARP)-1 leads to depletion of the nucleotide pool and cell death [22]. The cell's capability to face PARP-1-dependent NAD depletion by activating the NAD-rescue pathway is therefore central to cell survival. Yet, how NMN concentrations vary when PARP-1 hyperactivates is still unknown. As shown in Fig. 4A and B, exposure of U937 cells to the prototypical PARP-1 activator MNNG [23–25] only marginally increased NMN contents despite massive NAD depletion. The increase of NMN and depletion of NAD were prevented by the PARP-1 inhibitor phenanthridinone (PHE). These findings suggested that the rate of PARP-1-dependent NAD utilization exceeded that of NMNAT-mediated NAD resynthesis. However, the possibility of a compartmentalized pool of NMN not readily convertible into NAD must also be considered. Additionally, massive ATP depletion that typically follows PARP-1 activation could differently affect the activity of the ATP-dependent enzymes NaPRT and NMNAT. Gallotannin has been reported to inhibit NMNAT [26]. In keeping with this finding, we found that NMN contents increased in cells exposed to gallotannin (Fig. 4C). Evaluating the effect of the latter in cells undergoing PARP-1 hyperactivation indicated that the gallotannin- and PARP-1 activation-dependent increases of NMN were additive (Fig. 4C). FK866 is a recently identified inhibitor of NaPRT [15,16]. It is unknown, however, whether FK866 also inhibits NMNAT. We report here that FK866 similarly reduced both NAD and NMN contents in U937 cells (Fig. 4D and E), confirming its ability to inhibit NaPRT activity. However, increases in NAD contents prompted by the addition of NMN to the culture medium were proportionally similar in the presence or absence of FK866 (Fig. 4F), indicating that the drug does not affect NMNAT activity.

3.4. Effect of NMN or NAD on PARP-1-dependent cell death

It has been reported that strategies aimed at increasing intracellular NAD contents provide cytoprotection under different pathological conditions (see Refs. [2,3,21,27] for reviews). Hence, to evaluate the potential cytoprotective properties of NMN, we analyzed various cell death parameters of HeLa and U937 cells undergoing NAD and ATP depletion because of PARP-1 activation. As shown in Fig. 5A and B, both NMN and NAD were able to counteract the reduction in NAD and ATP contents in HeLa and U937 cells exposed 1 h to the PARP-1-activating agent MNNG. Twenty-three hours later, MNNG-challenged cells appeared shrunken and joined in clusters of dead cells. Conversely, those exposed to MNNG

apparatus described in (E). (G) Effect of the temperature of the step 2 of the derivatization procedure on efficiency of NMN derivatization. (H) Effect of duration of step 1 or 2 of the derivatization procedure on efficiency of NMN derivatization. (I) NMN calibration curve. Fluorescence of NMN is linear up to 2500 pmol injected. In G-I, bars/points represent the mean ± S.E.M. of three experiments conducted in duplicate.

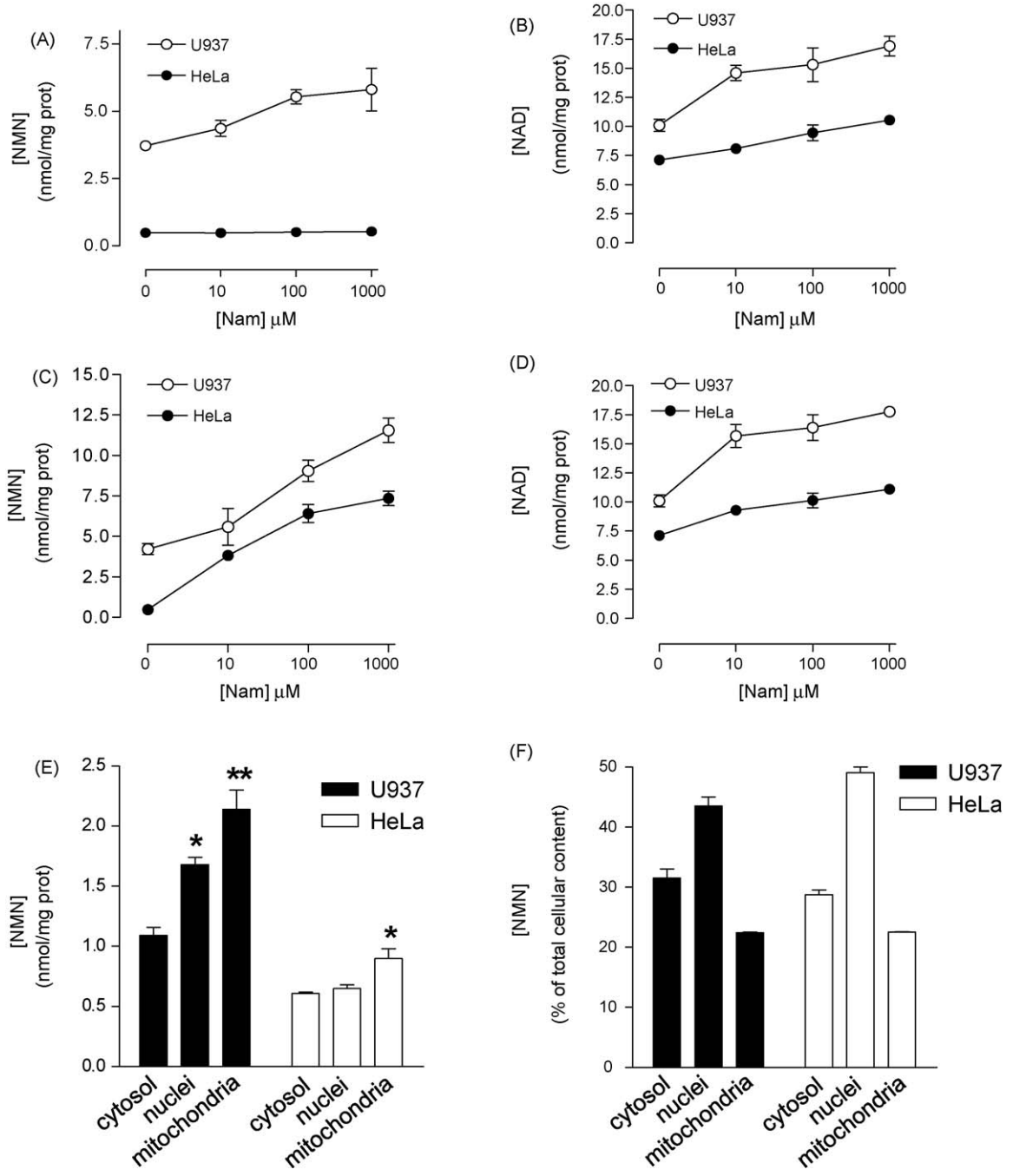

Fig. 3. NMN and NAD contents in HeLa and U937 cells. NMN and NAD contents were measured in HeLa and U937 cells under control conditions or after exposure for 1 h to different concentrations of nicotinamide (Nam) (A and B) or NMN (C and D). (E and F) Contents of NMN in cytosol, nuclei and mitochondria of HeLa or U937 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Cytosol (ANOVA + Tukey's post hoc test). In A–E, each point/bar represents the mean ± S.E.M. of three experiments conducted in duplicate.

plus NMN or NAD in part conserved their healthy morphology (Fig. 5C). Accordingly, MTT reduction and LDH release assays showed that both NMN and NAD partially counteracted cell death 24 h after PARP-1 hyperactivation (Fig. 5D and E). NMN- or NAD-treated cells died 36 h after MNNG exposure (not shown), indicating that nucleotide cytoprotection was only transient.

3.5. NMN content in mouse organs and effect of FK866

To our knowledge, the impact of FK866 on NMN contents *in vivo* has not been reported. We therefore injected the drug in mice (100 mg/kg, i.p.) and measured NMN contents in various organs at different times after injection. Reportedly, mouse NaPRT activity is high in liver, intermediate in heart, and under detectable levels in brain [11]. Accordingly, we found that NMN contents were higher in the liver than in the heart (6.7 ± 0.3 and 4.4 ± 0.2 nmol/mg tissue, respectively). However, the brain contents of NMN (3.86 ± 0.1 nmol/mg tissue) were similar to those of the heart. Importantly, injection of FK866 reduced NMN contents in the three organs after 4 and 8 h. At 16 h from the injection, NMN contents in the organs were still lower than control with a tendency to return to basal values (Fig. 6).

4. Discussion

In the last several years we have witnessed a renewed interest in NAD biology and basic biochemistry of pyridine nucleotide

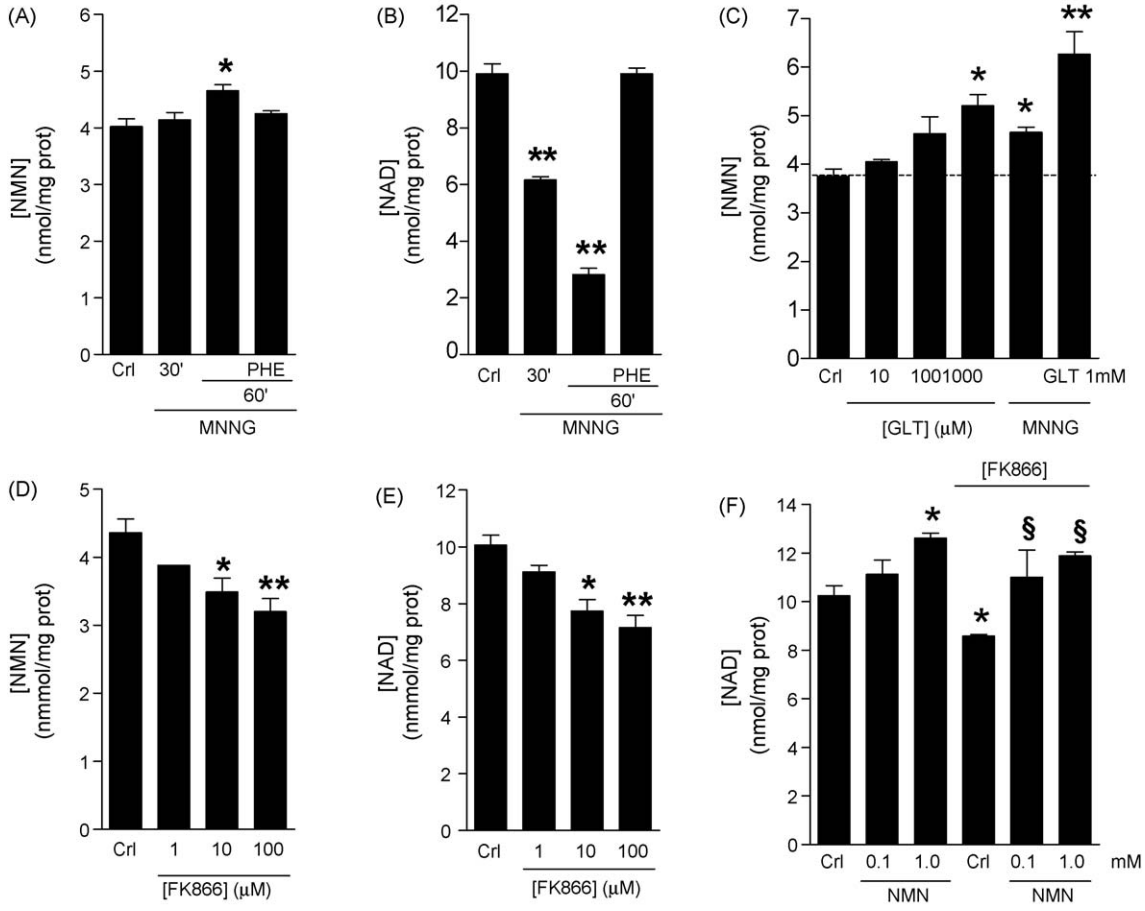

Fig. 4. Effect of PARP-1 activation, gallotannin or FK866 on NMN or NAD contents in U937 cells. The NMN (A) or NAD (B) contents were measured in cells under control conditions and at different times after exposure to the PARP-1-activating agent MNNG (100 μM) in the presence or absence of the PARP-1 inhibitor phenanthridinone (PHE, 30 μM). (C) The contents of NMN were measured in cells exposed for 1 h to different concentrations of gallotannin (GLT) in the presence or absence of MNNG 100 μM/1 h. Effect of a 4-h exposure to FK866 on intracellular NMN (D) or NAD (E) contents. (F) NAD contents in cells exposed 1 h to different concentrations of NMN and preincubated 4 h with FK866 (10 μM). Each bar represents the mean ± S.E.M. of at least three experiments conducted in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Crl (ANOVA + Tukey's post hoc test). In (F) $^{§}p < 0.05$ vs. Crl in the presence of FK866 (ANOVA + Tukey's post hoc test).

metabolism [1]. Among the biochemical routes leading to NAD formation, the rescue pathway operated by NaPRT and NMNATs is of pivotal importance to NAD homeostasis in mammals. Indeed, this pathway rescues nicotinamide which is a by-product of several enzymes such as poly and mono-ADP-ribose transferases, sirtuins as well as ADP-ribosyl cyclase, which are responsible for substantial consumption of NAD under constitutive and pathological conditions. However, the intracellular contents as well as subcellular compartmentalization of NMN, the first metabolite of the nicotinamide-dependent NAD-rescue pathway, are in large part unknown. Furthermore, whether NMN formation mediates the signalling properties of extracellular NaPRT/visfatin/PBEF waits to be clearly understood. Current lack of knowledge about the biological relevance of NMN can be ascribed, at least in part, to difficulties in nucleotide measurements in biological samples. Previous studies have quantified NMN by means of complex HPLC systems coupled to UV detection and subsequent mass spectrometry analysis [11,13,28]. Indirect methods or radioactive precursors have been also adopted to follow NMN formation and degradation [6]. In the present study, we report a new method to quantify NMN in biological samples, based on the ability of its N-alkylpyridinium moiety to be transformed into fluorescent compounds.

By monitoring the effect of nicotinamide addition to the incubating media of HeLa or U937 cells on their NMN and NAD content, we confirm that, at least in HeLa cells, NaPRT is the rate-limiting step of the rescue pathway [29,30]. Also, evidence that intracellular NMN contents promptly increase when the nucleotide is added to the culture media indicates that plasma membrane is permeable to this nucleotide. These findings suggest that the pharmacologic effects of exogenous NMN in cultured cells and mice [11,13] are due to cellular uptake and changes in NAD contents. Whether NMN permeates the plasma membrane through the putative NAD uptake mechanisms [31] remains to be clarified. We also report that NMN was more concentrated in the mitochondrial fraction than in the nuclear or cytosolic ones. Although we adopted a crude cell fractionation technique with possible subcellular fraction contamination, data suggest high mitochondrial NMN contents. This finding is of particular relevance if we consider that it is still debated whether NaPRT activity is present in the mitochondria. NaPRT can be detected by Western blotting in mitochondria extracted from cultured cells or mouse liver, and the NaPRT inhibitor FK866 reduces mitochondrial NAD content when added to a pure organelle preparation [28]. Overall, these findings suggest that NMN is synthesized in mitochondria by NaPRT. Although the presence of a mitochondrial NaPRT is conceivable if we consider that mitochondria contain a specific NMNAT isoform [26], how NaPRT enters the organelles is unknown given that a mitochondrial-targeting sequence has not been reported [16]. In light of the recent identification of mitochondrial NAD carriers [28,32], the possibility exists that part of the mitochondrial NMN pool is uptaken from the

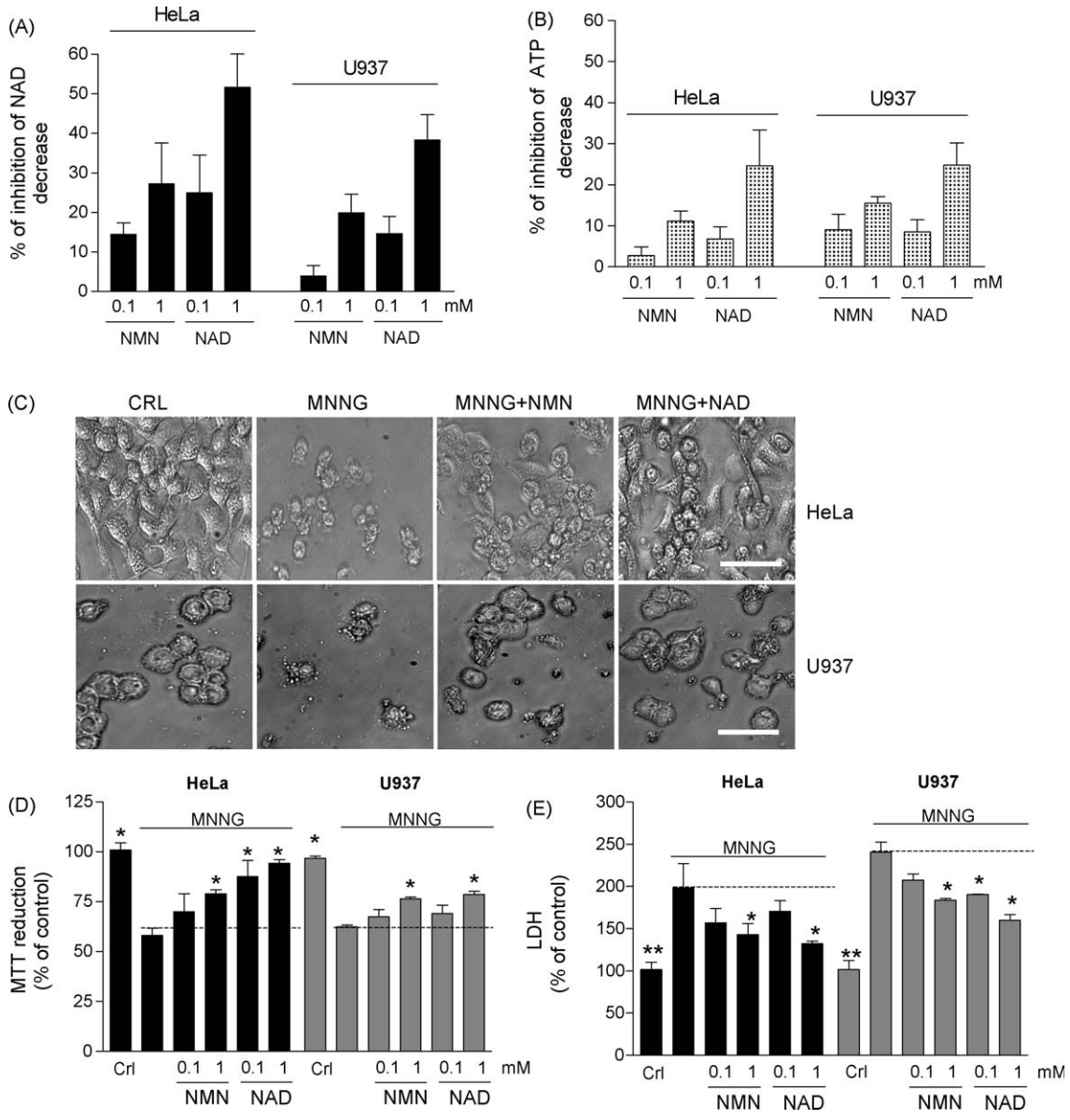

Fig. 5. Cytoprotection form PARP-1-dependent cell death by NMN or NAD. Effect of NMN or NAD added to the incubating media of HeLa and U937 cells (1 h preincubation) on reduction of NAD (A) or ATP (B) contents prompted by 1 h exposure to 100 μM MNNG. (C) Phase contrast visualization of the two cell types under control conditions or 11 h after 1 h exposure to MNNG in the presence or absence of NMN or NAD, both at 1 mM. The two nucleotides were present during and after MNNG exposure. Effect of NMN or NAD added to the incubating media of HeLa and U937 cells on MTT reduction (D) or LDH release (E) evaluated 11 h after 1 h exposure to 100 μM MNNG. The two nucleotides were present during and after MNNG exposure. Each bar represents the mean ± S.E.M. of at least three experiments conducted in duplicate. In (C) representative images of three experiments are shown. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MNNG (ANOVA + Tukey's post hoc test). In (C), bar = 40 or 20 nm for HeLa and U937, respectively.

cytoplasm. Regardless, evidence that mitochondria contain significant amounts of NMN (Fig. 3E), as well as NMNAT3 [26], clearly indicates that at least the last step of the NAD-rescue pathway occurs in these organelles. This makes sense if one consider that NAD-consuming enzymes such as sirtuins and mono-ADP-ribose transferases are present in mitochondria [21,33]. Conversely, the presence of a mitochondrial PARP-1 is still debated [17,34–36].

A large body of evidence identifies PARP-1 as a key player of cell death. It has been repeatedly proposed that the molecular pathway responsible for PARP-1-dependent cell death stems from an excessive consumption and ensuing depletion of NAD pools [22]. It is unknown, however, whether/how PARP-1 hyperactivation affects the kinetics of the NAD-rescue pathway. We show here that the massive depletion of intracellular NAD that follows PARP-1 activation is not accompanied by a concomitant depletion of NMN. Rather, a small but significant increase in NMN content occurred upon PARP-1 activation (Fig. 4A). This finding suggests that NMNAT becomes limiting the rate of the NAD-rescue pathway when the dinucleotide is massively consumed by PARP-1. Also, it indicates that strategies aimed at circumventing the "NMNAT bottleneck" could be of cytoprotective relevance in conditions of hyperactivation of PARP-1. In good agreement with this hypothesis and previous findings [37], we report that exogenous NAD reduced early PARP-1-dependent bioenergetic derangement and cell death. On the one hand this result further corroborates the hypotheses that NAD depletion is causative in this type of cell demise [38], and on the other that NAD permeates through the plasma membrane [31]. Of note, NMN also reduced PARP-1-dependent energy failure and cell death, despite to a lower extent when compared to NAD. This is in keeping with the NMNAT rate-limiting activity for NAD resynthesis during overactivation of PARP-1. We report, however, that protection afforded by NMN or NAD on cells undergoing PARP-

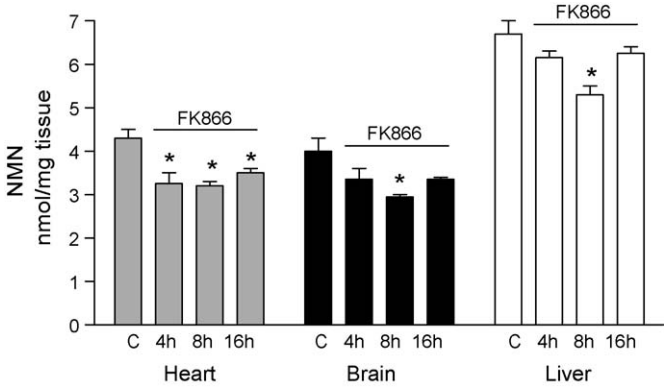

Fig. 6. Effect of FK866 on NMN contents in different mouse organs. NMN was measured in different mouse organs under control conditions (C) and at different times after i.p. injection of FK866 (100 mg/kg). Each bar represents the mean of two experiments ($n = 4$ per group). $^{*}p < 0.05$ vs. C (ANOVA + Tukey's post hoc test).

1 hyperactivation is only transient. This indicates that the partial prevention of NAD/ATP depletion is not compatible with cell survival, and/or that delayed events in addition to nucleotide depletion [i.e. poly(ADP-ribose)-dependent mitochondrial AIF release [39]] contribute to cell demise prompted by PARP-1. Indeed, NMN can improve energy dynamics working as a precursor of NAD but also favour poly(ADP-ribose) formation by the detrimental NMNAT–PARP-1 complex [40].

Despite the emerging complexity and pathophysiological relevance of NAD metabolism, pharmacology of NAD resynthesis is extremely poor, with FK866 being the most studied chemical. Evidence that gallotannin increases intracellular NMN contents (Fig. 4C) is in keeping with prior work showing that gallotannin inhibits NMNAT activity [26]. Although gallotannin is a complex mixture of tannins extracted from oak gall [41], these findings could be of relevance for the identification of the pharmacophore of more potent and specific NMNAT inhibitors. Further, the demonstration that FK866 reduced basal NMN and NAD contents in cultured cells, but did not prevent the increase of NAD in cells exposed to NMN (Fig. 4D–F), indicates for the first time that the drug selectively inhibits NaPRT, having no effects on NMNAT activity. This is of significance if one considers that the chemical is currently in clinical trial for cancer treatment [42].

In keeping with data on tissue distribution of NaPRT expression [11] and activity [43], we report that NMN is more concentrated in the mouse liver than in the heart or brain. Notably, NMN contents in the brain are comparable to those of the heart, despite evidence that NaPRT expression is almost undetectable in the brain [11]. Although we do not know the cell type(s) in which NMN is contained, data suggest active NMN uptake from neural cells. Treatment of mice with FK866 induced long-lasting but mild reduction of NAD contents in liver, heart and brain. Obviously, mild reduction can be primarily ascribed to the pharmacodynamic and pharmacokinetic features of FK866. Indeed, metabolism of FK866 is still to be clearly understood. Data, however, hint that under constitutive conditions NAD consumption and/or recycling through nicotinamide occurs at a slow rate. This hypothesis is consistent with knowledge that NAD half-life in rat liver is about 10 h [44].

In conclusion, the development of a new method for NMM measurement allowed us to gather new information on the biochemistry and pharmacology of NAD biosynthesis. Given that the pathophysiological relevance of NaPRT and NMN is still in large part obscure, the present study helps addressing some of the numerous unanswered questions existing in the field of pyridine nucleotide metabolism and NAD signalling.

Acknowledgments

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