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Reversal of endothelial dysfunction by nicotinamide mononucleotide *via* extracellular conversion to nicotinamide riboside

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ABSTRACT

Background: Nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) are effective substrates for NAD synthesis, which may act as vasoprotective agents. Here, we characterize the effects of NMN and NR on endothelial inflammation and dysfunction and test the involvement of CD73 in these effects.

Materials and methods: The effect of NMN and NR on IL1β- or TNFα-induced endothelial inflammation (ICAM1 and vWF expression), intracellular NAD concentration and NAD-related enzyme expression (NAMPT, CD38, CD73), were studied in HAECs. The effect of NMN and NR on angiotensin II-induced impairment of endothelium-dependent vasodilation was analyzed in murine aortic rings. The involvement of CD73 in NMN and NR effects was tested using CD73 inhibitor-AOPCP, or $CD73^{-/-}$ mice.

Results: 24 h-incubation with NMN and NR induced anti-inflammatory effects in HAEC stimulated by IL1β or TNFα, as evidenced by a reduction in ICAM1 and vWF expression. Effects of exogenous NMN but not NR was abrogated in the presence of AOPCP, that efficiently inhibited extracellular endothelial conversion of NMN to NR, without a significant effect on the metabolism of NMN to NA. Surprisingly, intracellular NAD concentration increased in HAEC stimulated by IL1β or TNFα and this effect was associated with upregulation of NAMPT and CD73, whereas changes in CD38 expression were less pronounced. NMN and NR further increased NAD in IL1β-stimulated HAECs and AOPCP diminished NMN-induced increase in NAD, without an effect on NR-induced response. In *ex vivo* aortic rings stimulated with angiotensin II for 24 h, NO-dependent vasorelaxation induced by acetylcholine was impaired. NMN and NR, both prevented Ang II-induced endothelial dysfunction in the aorta. In aortic rings taken from $CD73^{-/-}$ mice NMN effect was lost, whereas NR effect was preserved.

Conclusion: NMN and NR modulate intracellular NAD content in endothelium, inhibit endothelial inflammation and improve NO-dependent function by CD73-dependent and independent pathways, respectively. Extracellular conversion of NMN to NR by CD73 localized in the luminal surface of endothelial cells represent important vasoprotective mechanisms to maintain intracellular NAD.

1. Introduction

Nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) have drawn attention as alternative nicotinamide adenine dinucleotide (NAD) substrates, devoid of side effects for nicotinic acid (NicA), such as "flushing" or hepatotoxicity and side effects of nicotinamide (NA), including sirtuin inhibition. Both NAD substrates, NR and NMN were proposed to be used in sports nutrition as good dietary supplements [9,8] and display numerous beneficial effects in various settings, but their bioavailability and pathways of metabolism towards NAD differs.

NR, detectable in cow milk, milk-derived products and in natural products containing yeast [3] has a good bio-availability and intracellularly is metabolized via NRK1 and NRK2 to NMN, a major precursor of NAD. NR was shown to be effective in restoring the NAD pool both in mice and humans [61]. Numerous studies on NR showed a significant impact of this substrate on NAD content, bioenergetics, and improved regenerative capabilities in various rodent models of disease. For example, NR treatment resulted in increased NAD concentration in a mouse model of respiratory chain III complex deficiency [52],

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improved liver regeneration [46] and restored NAD content in mouse skeletal muscle myotubes [17]. NR treatment also enhanced oxidative metabolism and prevented weight gain in a mouse model of diet-induced obesity [7]. Moreover, NR treatment increased NAD content in the cerebral cortex, thus attenuated cognitive deterioration in a mouse model of Alzheimer's disease [21]. NR was also effective in heart failure, as NR-supplemented diet administrated to murine models of dilated cardiomyopathy or pressure overload-induced heart failure restored myocardial NAD levels and improved impaired cardiac function [15].

In contrast to NR, NMN has a worse bioavailability, as extracellular NMN is unable to pass the endothelial membrane without prior dephosphorylation by CD73 (ecto-5′-nucleotidase) to NR [24] or prior to metabolism to nicotinamide by extracellular CD38 [25,28]. Extracellular nicotinamide in the presence of phosphoribosyl-1-pyrophosphate (PRPP) could be also converted NMN by visfatin as reviewed recently [23,65]. Interestingly, in cardiomyocytes, it was demonstrated that connexin 43 (Cx43) channels are permeable to extracellular NAD [4] suggesting that intracellular transport of NAD and NMN may be cell-type dependent and reliant on various transporters. Intracellularly, NA could be converted by nicotinamide phosphoribosyltransferase (NAMPT) to endogenous NMN in three-step Preiss-Handler pathway [48,20,31], or methylated by nicotinamide N-methyltransferase (NNMT) to 1-methylnicotinamide (MNA) [1]. NR is phosphorylated by nicotinamide riboside kinases (NRK1, NRK2) to endogenous NMN [53,17]. NMN is subsequently transformed to NAD by nicotinamide mononucleotide adenylyltransferases (NMNAT1-3). Apart from involvement in redox reactions, NAD is also substrate for sirtuins (SIRT), poly-ADP-ribose polymerases (PARP) and other NAD-dependent enzymes resulting in release of endogenous NA.

In numerous studies, NMN unequivocally afforded NAD-dependent beneficial effects. For example, NMN improved muscular contractile function in mouse age-related models of muscle dysfunction [17,45], restored cardiac NAD content in mouse model of ischemia-reperfusion [63], improved metabolic balance in type 2 diabetes mice [66], improved NAD content and survival in rat models of hemorrhagic shock [55] and had a protective effect in β-amyloid oligomer-induced rat model of Alzheimer's disease [62]. In some previous reports, the effects of NMN and NR were compared [49,17], but in most of these studies, either NMN or NR was characterized. Still, the role of ecto-enzymes CD73 and CD38 in NMN-induced effects has not been fully characterized, so it is not clear whether NMN-triggered beneficial effects are NR- or NA-dependent and what metabolic enzymes are involved.

Despite numerous studies on the beneficial effects of NR and NMN in various models, there is still a paucity of data as regards the effects of NMN and NR on endothelial function. NMN treatment had a beneficial effect in various mouse models of age-related vascular pathologies in line with the gradual fall in NAD content in aging [35,13]. These studies demonstrated that NMN restored endothelium-dependent vascular function and mitigated oxidative stress in age-related model [51], rescued angiogenic capacity in aged cerebrovascular endothelial cells [35] and restored fenestration-like phenotype of liver sinusoidal endothelial cells (LSECs) isolated from old mice [30]. NR was also shown to be effective to improve vascular function. NR improved endothelium-dependent relaxation of isolated rat mesenteric arteries in ischaemia-reperfusion model [60]. Beneficial endothelial effects of NR was also shown in a mouse model of endotoxaemia, in which model NR restored NAD contents in lung and heart as well as decreased ROS production and apoptosis in isolated endothelial cells [29].

In the present work, we aimed to characterize the endothelial profile of action of NMN in comparison to NR in cellular and vascular models of endothelial inflammation, with particular attention to the involvement of extracellular conversion of NMN in these effects. Our research demonstrated, that both NMN and NR modulated intracellular NAD content in the endothelium, inhibited endothelial inflammation and improved NO-dependent function. The important finding of this work was to show that NMN effects on endothelium were mediated by CD73-dependent conversion of NMN to NR.

2. Materials and methods

2.1. Cells and animals

Eahy.926 endothelial hybrid cell line (ATCC® CRL-2922™, Manassas, VA, USA) and Human Aortic Endothelial Cells (HAECs, CC-2535, Lonza, Basel, Switzerland) were used to study involvement of CD73 and CD38 in NMN metabolism and to analyze, effects of NMN and NR on NAD content and on endothelial inflammation induced by TNFa or IL1β. For preliminary studies due to easier maintenance and faster growth rate Eahy.926 line was used, while major part of experiments were performed on primary HAEC line, used as a representative endothelial *in vitro* model to examine the extracellular metabolism of NAD substrates in human endothelium. Eahy.926 and HAEC lines was used up to the fifth passage to avoid the phenotype changes during late passaging. Cells reaching over ~90% confluence were used for the experiments, grown on EBM-2 medium (CC-3156, Lonza, Basel, Switzerland) or DMEM medium (ATCC® 30–2006, Manassas, VA, USA), containing glucose 1 g/L, L-glutamine 1 mM and 20% FBS.

12–16 weeks – old C57Bl/6J control mice (Jackson Laboratories, Bar Harbor, ME, USA) and age-matched CD73$^{-/-}$ mice, developed in Heinrich-Heine-Universität, Düsseldorf, provided by Gdansk Medical University [36] were used for *ex vivo* studies of vascular function. After transportation, animals (only females) were randomly allocated to control and experimental groups and placed in individual cages with independent ventilation system, hosting up to five animals. Animals were kept in quarantine for 1 week, having an unlimited access to water and chow diet. Two or three mice a day were sacrificed (in the morning and at midday) to isolate thoracic aorta for 24 h incubation and subsequent wire myograph measurements. All animal procedures conformed to the ARRIVE standards and EU Directive 2010/63/EU for animal experiments and all experimental procedures were approved by the First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow.

2.2. Measurement of extracellular metabolism of nicotinamide mononucleotide (NMN) in Eahy.926 cells and HAECs

Eahy.926 cells (ATCC, Manassas, VA, USA), cultured in the 24-well plates, were incubated with substrates for CD73 (AMP and NMN) or for CD38 (only NMN) at the concentration range 1–5 mM. The enzymatic reaction was carried at 100% of confluence in incubation medium (1 ml of Hanks solution) with or without 5 μM EHNA as adenosine deaminase inhibitor, 5 μM nucleoside transport inhibitor (NBTI) and ecto-5′nucleotidase inhibitor – Adenosine 5′-(α,β-methylene)diphosphate (AOPCP; 50 μM), all reagents were purchased from Sigma Aldrich, Saint Louis, MO, USA. Michaelis Constant, Vmax and reaction kinetics were extracted from a graphic presentation of experimental points. Medium samples were taken for HPLC measurement of extracellular adenosine, NA and NR, according to the methodology described by Kutryb-Zajac et al. [38]. After washing with cold PBS, cells were frozen in −80 °C for protein concentration measurement following solubilization in 0.5 M NaOH, using Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MO, USA) and Synergy4 multiplate reader (BioTek, Winooski, VT, USA).

To confirm the data from Eahy.926 cells HAEC line (HAECs, CC-2535, Lonza, Basel, Switzerland) were cultured in 24-well plates and treated with AOPCP (50 μM) for 24 h. After incubation, cells were washed and placed in Krebs buffer for 2 h-nicotinamide starving, then NMN 100 μM was applied for 1 h-incubation. The concentration of NMN was chosen after initial assessment of Km reaction for adenosine release by CD73, to induce the NR production by this enzyme from NMN. NBTI was not used since nucleotide uptake was considered a

marginal and not affecting NMN bio-availability during short-time incubation with this substrate. Effluent samples were taken at time points 0, 30, 60 min. and frozen for further NR and NA measurement, according to the methodology described previously [43]. After sample collection, cells were frozen for protein concentration measurement.

2.3. Assessment of endothelial inflammation in HAECs by immunofluorescent staining of ICAM1 and von Willebrand factor

To study the effect of NR and NMN supplementation on the ICAM1 and vWF expression of TNFα- and IL1β-stimulated HAEC line (CC-2535, Lonza, Basel, Switzerland), cells were plated in 96-well format on black Corning multiplates with clear bottom, then supplemented with NR, NMN, or NA (100 μM/24 h) and stimulated with TNFα (10 ng/ml/24 h) or IL1β (Sigma Aldrich, Saint Louis, MO, USA) used at the same concentration. After 24 h incubation, cells were fixed with a 4% formalin solution (10 min), washed with PBS, then incubated with a blocking solution containing 5% normal goat serum (Jackson Immuno, Cambridgeshire, UK) and 2% filtered dry milk (Gostyn, Poland) were to minimalize non-specific binding of antibodies. For indirect immunohistochemical detection of intercellular adhesion molecule 1 (ICAM1) and von Willebrand factor (vWF), cells were incubated overnight with mouse anti-ICAM1 monoclonal Ig, (Thermo Fisher Scientific, Waltham, MO, USA), rabbit anti-vWF polyclonal Ig (Abcam, Cambridge, UK). After rinsing in PBS (Thermo Fisher Scientific, Waltham, MO, USA), HAEC cells were treated with different secondary antibodies; Alexa Fluor 647-conjugated goat-anti-mouse for ICAM1 and Alexa Fluor 488-conjugated goat-anti-rabbit for vWF (Jackson Immuno, Cambridgeshire, UK). For nuclei counterstaining, Hoechst 33258 solution (Sigma Aldrich, Saint Louis, MO, USA) was applied. Images of immunostained cells were taken using CQ1 confocal quantitative image cytometer (Yokogawa, Musashino, Tokio, Japan) and CQ 1.04 software, then analyzed automatically by Columbus 2.4.2 software (Perkin Elmer, Waltham, MA, USA) to assess mean fluorescence specific for immunostained cells. For data normalization, each immunostaining ($n = 6$) was performed using cells with similar confluence ($\geq 90\%$), the same primary and secondary antibody concentration and constant incubation time for each staining step. As a negative control, cells treated only with secondary antibodies were used to estimate the background signal.

2.4. Measurements of changes in NAD contents in HAECs

HAECs (CC-2535, Lonza, Basel, Switzerland) were cultured in 96 well format at the density $12–15 \times 10^4$ for 24 h. After the required confluence was reached (80–100%), cells were supplemented with 100 μM of NMN (Sigma Aldrich, Saint Louis, MO, USA) or NR (ChromaDex, Irvine, CA, USA). CD73 activity was inhibited by 50 μM AOPCP. For pro-inflammatory activation of HAEC line, IL1β (Sigma Aldrich, Saint Louis, MO, USA) was used at a concentration 10 ng/ml for 24 h. TNFα was also used as a pro-inflammatory stimulator, but as TNFα –stimulated cells display inconsistent results of intracellular NAD content after 24 h-incubation, IL1β was preferably used. Cells were washed with PBS (pH = 7.4), treated with 40 μl of cold 0.4 M $HClO_4$ (Chempur, Piekary Slaskie, Poland) and frozen at −80 °C for at least 24 h. After thawing on ice, $HClO_4$ cell extracts were centrifuged (14000 pm/10 min/4°C). The supernatant was collected and neutralized to ph = 6.5 using 3 M K_3PO_4, kept on ice for 15 min, centrifuged and frozen for further HPLC-RT analysis as described previously [56]. Protein deposit remaining after removing the supernatant was resuspended in 30 μl of 0.5 NaOH (Chempur, Piekary Slaskie, Poland) and used for BCA protein concentration assay.

2.5. Assessment of the expression of extracellular metabolism enzymes by immunofluorescent staining and Western Blot in HAECs

Immunofluorescent staining of NAMPT, CD73 and CD38 were performed in HAECs plated in 96-well format (Corning, NY, USA), supplemented with 100 μM NMN or NR, after incubation with IL1β (10 ng/ml/24 h). Cells were fixed with a 4% formalin solution (10 min), washed with PBS, then incubated with a blocking solution containing 5% normal goat serum (Jackson Immuno, Cambridgeshire, UK) and 2% filtered dry milk (Gostyn, Poland). Before NAMPT immunostaining cells were slightly permabilized with TritonX-100 (0,1% in PBS for 5 min) to improve antibody binding. The following primary antibodies were used; rabbit anti-NAMPT (Thermo Fisher Scientific, Waltham, MO, USA), rabbit-anti-CD38 (Abnova, Taipei, Taiwan) and mouse-anti-CD73 (Merck Millipore, Burlington, MA, USA). For visualization on primary antibody binding sites, Alexa Fluor 488-conjugated goat-anti-rabbit and Cy3-conjugated goat-anti-mouse secondary antibodies (Jackson Immuno, Cambridgeshire, UK) were added for 30 min. Images were taken and analyzed as described above.

For Western Blot analysis of NAMPT, CD73 and CD38 expression in HAEC line, cells were plated in 6-well format (Corning, NY, USA), incubated or not with 100 μM NMN or NR and stimulated with IL1β (10 ng/ml/24 h), then collected using Accutase solution for 5 min. (Thermo Fisher Scientific, Waltham, MO, USA), lysed by M-Per reagent (Thermo Fisher Scientific, Waltham, MO, USA) and frozen in −80 °C. Samples were thawed on ice, spinned down (12000G/10 min/4°C) to remove protein clots, then the protein concentration was measured, using BCA protein concentration assay (Thermo Fisher Scientific, Waltham, MO, USA) and Synergy4 multiplate reader (BioTek, Winooski, VT, USA). WB samples were prepared by combining 30 μg protein with a proper volume of 4x Laemmli buffer, heating (95 °C/5 min.) and frozen in −80 °C. At the day of the analysis, samples were thawed on ice and placed in a 12% FastCast separating gel (BioRad, Hercules, CA, USA). As a protein standard, 5ul of PrecisionPlus Unstained Protein Ladder was used. The separation was performed under 100 V during 1,5h, then proteins were transferred to PVDF membrane during 1 h transfer, under 400 mA, using PowerPac HC Power Supply (BioRad, Hercules, CA, USA). Membranes were blocked with 2% milk solution in TBST (TBS + 0,1% Tween 20) for 1 h, then incubated (overnight, 4 °C) with primary antibodies; mouse anti-CD73 (Merck Millipore, Burlington, MA, USA), rabbit anti-NAMPT (Novus Biologicals, Cenntenial, CO, USA) or rabbit-anti-CD38 (Abnova, Taipei, Taiwan), used at dilutions 1:1000 to 1:5000. After washing with TBST, secondary antibodies were applied for 1 h; goat-anti-rabbit-HRP or goat-anti-mouse-HRP (Santa Cruz Biotechnology, Dallas, Texas, USA), used at recommended concentration 1:5000. As an additional control, mouse anti-β-actin Ig (Santa Cruz Biotechnology, Dallas, Texas, USA) was used to detect β-actin bands at 42 kDa site, followed by goat-anti-mouse-HRP (Santa Cruz Biotechnology, Dallas, Texas, USA). Due to a similar molecular weight of NAMPT and CD38, to avoid band overlapping, β-actin Ig was used only on CD73-immunostained membranes. NAMPT and CD38, total protein load was used as a reference unstained control bands. Protein bands were detected using Clarity Max Western ECL Substrate and ChemiDoc Imaging Station (BioRad, Hercules, CA, USA). Specific bands representing molecular weights 37 kDa, 45 kDa and 70 kDa were detected for NAMPT, CD38 and CD73, respectively. Western Blot assay was repeated three times for each of analyzed proteins.

2.6. Measurement of endothelium-dependent vasodilation in aortic rings

After ketamine/xylazine anesthesia, thoracic aorta was isolated, washed with cold PBS, cleaned of perivascular fatty tissue and divided into three 2-mm-long rings, which was placed in DMEM medium

containing a vehicle, NMN, NR (100 μM) and/or AOPCP (50 μM). To trigger endothelial dysfunction aortic rings were incubated with angiotensin II at a concentration of 100 nM for 24 h. (Sigma Aldrich, Saint Louis, Missouri, USA). Then, each ring was placed separately in DMT 620 M multi-wire myograph chamber (Danish Myo Technology A/S, Aarhus, Denmark) containing Krebs solution (37°C) with constant CO_2 flow and kept for 15 min. under stabilizing conditions. After initial stretching with 30 mM and 60 mM KCl, the endothelium-dependent and endothelial-independent vasodilatory function were measured according to a standardized protocol, in phenylephrine-precontracted vessels using increasing concentrations of acetylcholine or sodium nitroprusside, respectively. Data were acquired and analyzed by LabChart 3.01 software (Danish Myo Technology A/S, Aarhus, Denmark).

2.7. Statistical analysis

Data were analyzed by Prism 6.0 software (GraphPad, CA, USA), using the nonparametric Mann-Whitney test and Kruskal-Wallis One way ANOVA, followed by *post hoc* multiple comparisons Dunn test. Data were shown as mean + SEM (* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

3. Results

3.1. Effect of CD73 inhibition by AOPCP on the extracellular conversion of NMN in Eahy.926 and HAEC cells

In Eahy.926 cells exposed to the increasing concentration of exogenous NMN, the release of NR and NA was increased as measured by HPLC assay (Fig. 1A and 1B). The Michaelis constant and Vmax for NMN → NR reaction was 1.37 mM and 1.138 nmol/ml/min, respectively, while for NMN → NR reaction: 2.29 mM and 0.583 nmol/ml/min, respectively. AOPCP (CD73 inhibitor) incubated for 2 h at a concentration of 50 μM effectively diminished adenosine production from AMP by CD73 (Fig. 1C) as well as NR production from NMN, but had almost no effect on NA production from NMN (Fig. 1D). In HAECs AOPCP (incubated for 24 h) also effectively inhibited conversion of NMN to NR as measured by LC/MS/MS assay (Fig. 1F), resulting in very low concentration of extracellular NR, as compared with NMN-treated group not pretreated with AOPCP (33.54 nmol/g of prot. *vs* 3355 nmol/g of prot, respectively, p ≤ 0.01). NA production was only marginally affected by AOPCP (1147 nmol/g of prot. in NMN/AOPCP-treated group *vs* 1357 nmol/g of prot in NMN-treated group; p ≤ 0.05).

3.2. Effects of NMN and NR on von Willebrand factor and ICAM1 expression in IL1β- and TNFα-stimulated HAEC cells; involvement of CD73

IL1β-induced the upregulation of vWF and ICAM1 in HAECs, and NMN prevented the pro-inflammatory effects of IL1β. The anti-inflammatory effect of NMN was lost in the presence of CD73 inhibitor AOPCP (50 μM). HAECs treated only with AOPCP displayed increased expression of vWF, as compared with untreated control HAECs (p ≤ 0.05). IL1β–induced upregulation of vWF and ICAM1 expression was also reduced in the presence of NR but the anti-inflammatory effect of NR was not modified by AOPCP (Fig. 2).

Similarly to the effects of IL1β, 24 h-incubation with TNFα also resulted in the upregulation of vWF and ICAM1 in HAECs. Both NMN and NR prevented TNFα–induced upregulation of vWF and ICAM1 and these effects were abolished by CD73 inhibition in NMN-treated HAECs, while in NR-treated HAECs CD73 inhibition had no effect on vWF-specific and ICAM1-specific fluorescence (Fig. 3).

3.3. Effects of NMN and NR on intracellular NAD concentration in HAECs after stimulation with IL1β; effects of CD73 inhibition

NMN or NR raised intracellular NAD content in basal non-stimulated HAECs (Fig. 4). NMN-triggered NAD increase was a CD73-dependent response, since, in AOPCP-treated HAECs, NMN supplementation did not increase significantly NAD content. In contrast to NMN, NR-induced raise of NAD was not modified by AOPCP. Stimulation with IL1β was not linked to the significant fall in intracellular NAD content, rather an increased was noted. CD73 inhibition by AOPCP prevented the rise in NAD induced by NMN in IL1β-stimulated HAECs, however effects of NR on NAD content in this experimental setup was not modified by AOPCP.

3.4. Changes in NAMPT, CD38 and CD73 expression in IL1β-stimulated HAECs

To explain the increase of NAD content in IL1β -stimulated HAECs we analyzed the expression of NAMPT and two ectoenzymes; CD73 and CD38 in this experimental setting by immunocytochemistry and Western Blot. As shown in Fig. 5A, a specific immunofluorescence of NAMPT, the main cytosolic enzyme involved in NAD synthesis was upregulated (p ≤ 0.01) in HAECs stimulated by IL1β. CD73-specific immunofluorescence was higher in IL1β- treated HAECs, as compared with untreated HAECs (Fig. 5B). Similarly, CD73 and NAMPT expression in HAEC stimulated by IL1β were increased as assessed by Western Blot (Fig. 5D). Supplementation with NMN or NR resulted in a decrease of upregulated NAMPT and CD73 expression in IL1β-stimulated HAECs, which was also confirmed by WB assay. Stimulation with IL1β had no significant effect on CD38-specific immunofluorescence (Fig. 5C), while WB analysis shown a minor upregulation of CD38 in IL1β-treated cells and downregulation after co-incubation with NMN or NR. This data suggests the compensatory upregulation of NAMPT, CD73 in response to inflammatory stimulus, might contribute to the preservation of NAD pool in IL1β-stimulated HAECs. NMN or NR treatment resulted in the anti-inflammatory effects, and reverted compensatory upregulation of NAMPT and CD73 expression in IL1β-stimulated HAECs.

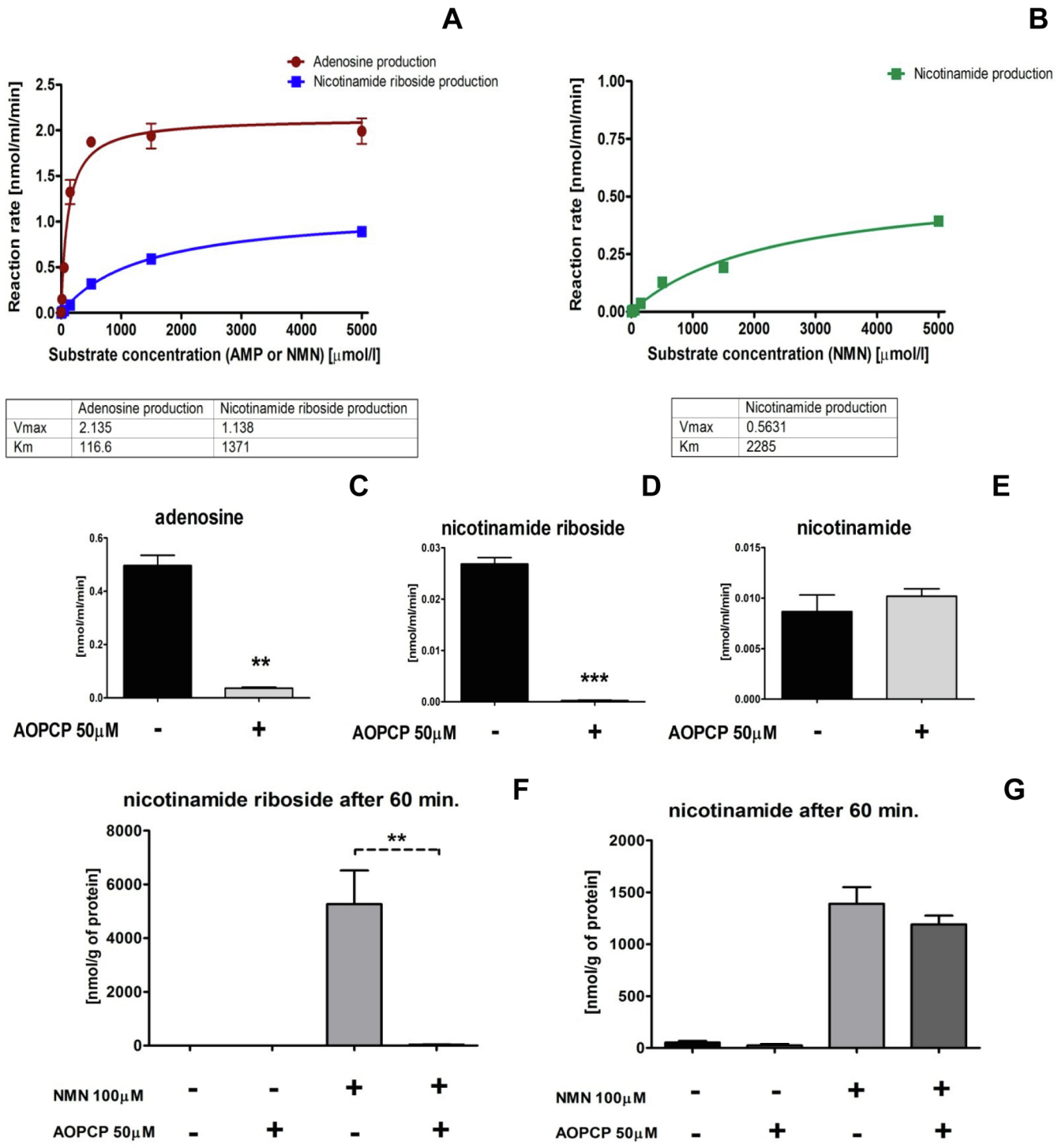
3.5. Effects of NMN or NR on angiotensin II-induced impairment of endothelium-dependent vasodilatory response in aortic rings in wild type and $CD73^{-/-}$ mice

Angiotensin II impaired vasodilatory response to Ach in aorta taken from C57Bl/6 mice, without an effect on SNP-induced vasodilation. Co-incubation with NMN mitigated the impairment of Ach-induced vasodilation induced by Angiotensin II (Fig. 6**A**), and this effect of NMN was abrogated by CD73 inhibitor AOPCP. NMN had no effect on Angiotensin II-induced impairment of vasodilation of aortic rings to Ach in $CD73^{-/-}$ mice (Fig. 6**B**). In contrast to NMN, NR effectively restored response to Ach in Angiotensin II-treated aortic rings isolated both from C57 and $CD73^{-/-}$ mice (Fig. 6**C, D**). SNP-induced vasodilation was similar in magnitude in all experimental groups including NMN- and NR- treated vessels taken from C57Bl/6J and CD73-deficient mice (Fig. 6**E, F**). AOPCP used alone had no significant effect on vasodilatory responses (data not shown).

4. Discussion

Here we demonstrated that NMN inhibited endothelial inflammation and improved NO-dependent function by extracellular conversion via ecto-5′-nucleotidase (CD73)-dependent pathway to NR and by modulation of endothelial intracellular NAD pool. Our results suggest, that extracellular conversion of NMN to NR by CD73 localized in the luminal surface of endothelial cells represents important vasoprotective mechanisms maintaining intracellular NAD and healthy phenotype of endothelial cells.

The salient findings of this work was to demonstrate the anti-inflammatory (inhibition of upregulation of ICAM1, vWF in response to IL1β and TNFα) and vasoprotective (inhibition of the impairment of NO-dependent function in response to Ang II) effects of NMN, that was comparable to the effects of NR in endothelial cells *in vitro* as well as the



	Adenosine production	Nicotinamide riboside production
Vmax	2.135	1.138
Km	116.6	1371

	Nicotinamide production
Vmax	0.5631
Km	2285

Fig. 1. Effects of CD73 inhibition by AOPCP on extracellular metabolism of NMN in Eahy.926 (A-E) and HAEC cells (F, G). Michaelis constant and Vmax of extracellular nicotinamide riboside/adenosine **(A)** and nicotinamide production **(B)** by from exogenous nicotinamide mononucleotide and/or AMP. The rates of adenosine production from adenosine monophosphate (AMP) **(C)**, nicotinamide riboside production from nicotinamide monophosphate (NMN) **(D)** and nicotinamide production from NMN **(E)** on the surface of EA.hy926 cells. The concentration of extracellular nicotinamide riboside **(F)** and nicotinamide **(G)** released by HAEC cells, measured after 24 h-preincubation with CD73 inhibitor AOPCP and 60 min-incubation with nicotinamide mononucleotide (100 μM). n = 6, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

in the aortic rings *ex vivo*. Furthermore, NMN-afforded effects, but not NR-induced effects were absent in the presence of CD73 inhibitor AOPCP or in CD73–deficient mice. To substantiate that the inhibitory effect of AOPCP on NMN response in endothelial cells was indeed due to the inhibition of CD73, we demonstrated that AOPCP (50 μM) effectively diminished NR production from NMN in two types of endothelial cells (Eahy.926, HAECs), but had almost no effect on NA production from NMN suggesting selective inhibition of CD73 without an effect on CD38. The Michaelis Constant and Vmax for NMN → NR reaction was approximately 10x lower then AMP → adenosine value that could point out that the major physiological and pathophysiological role of this enzyme might be linked to AMP, not to NMN

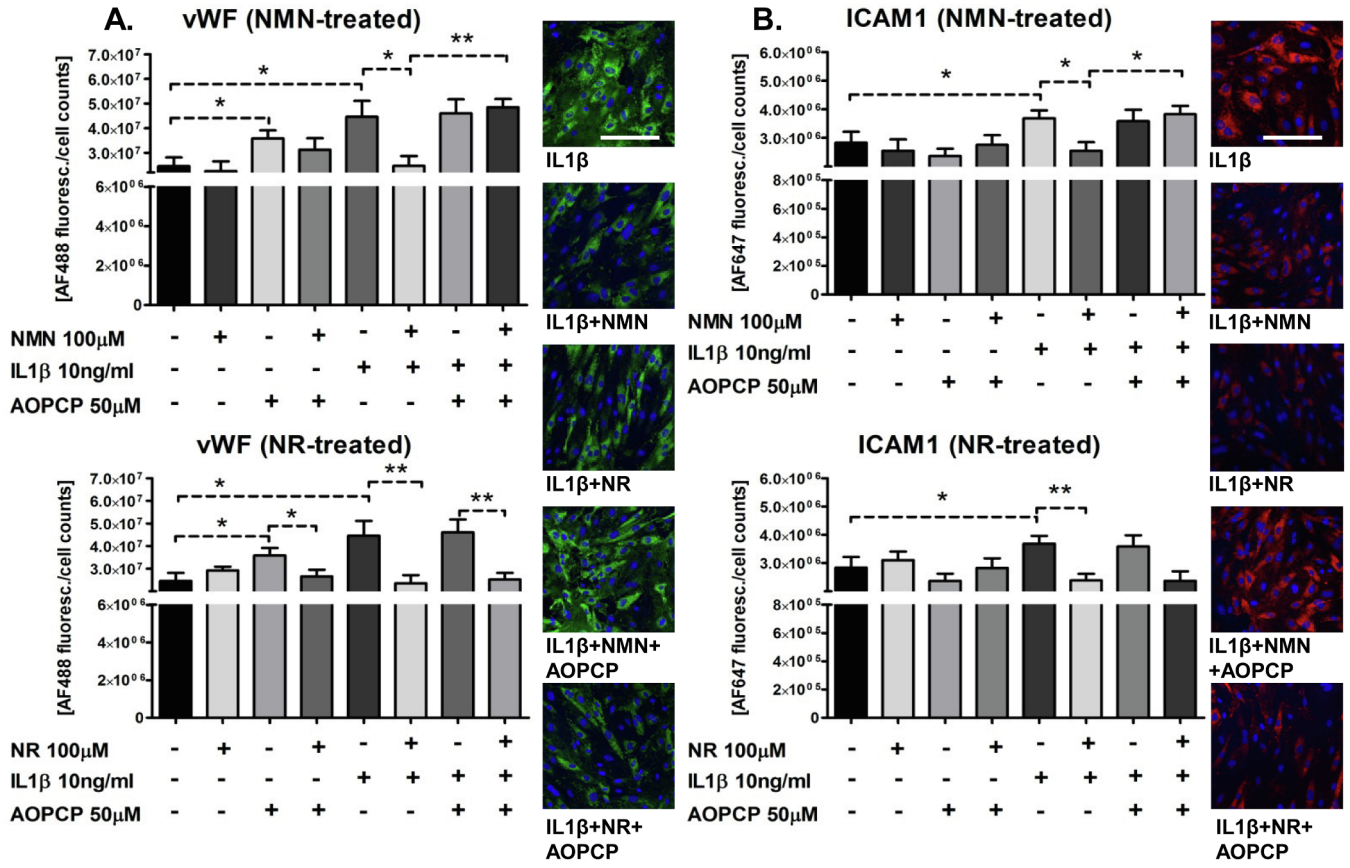


Fig. 2. Effects of NMN and NR on IL1β-induced increase in vWF and ICAM1 expression in HAECs. Expression of vWF in NR and NMN- and NR-treated HAEC cells after 24 h-stimulation with IL1β in the presence or absence of CD73 inhibitor AOPCP, (**A**); expression of ICAM1 in NR- and NMN-treated HAEC cells after 24 h-stimulation with IL1β in the presence or absence of CD73 inhibitor AOPCP, (**B**); n = 6, * $p \leq 0.05$, ** $p \leq 0.01$; white scale bar represents 25 μm.

metabolism. However, the extracellular concentration of AMP was detected in the nanomolar range [22,10]. On the other hand, although this opinion is not univocally accepted [26], the local extracellular concentration of NMN may reach micromolar concentration range as suggested by some authors [54]. Therefore, CD73, a well-known enzyme responsible for the conversion of AMP into adenosine and inorganic phosphate [5] may represent an important regulatory pathway for extracellular NMN metabolism in endothelial cells.

Previously expression of CD73 in endothelial cell membrane was linked to anti-inflammatory, immunosuppressive, vasoprotective or anti-platelet action of adenosine [69,50,42,12,32,39]. Despite the vasoprotective effect of CD73-derived adenosine, this pathway plays also an important role in tumor progression as a potent suppressor of anticancer immune responses [67,2]. Interestingly, mutation in human NT5E gene encoding CD73 triggers recently described genetic malfunction (2011) known as ACDC ("arterial calcification due to deficiency of CD73"), which is reflected by complex phenotype of vascular calcification, arteriomegaly, and tortuosity, and sometimes calcification in small joints [47,33] underscoring the important role of CD73 in vascular homeostasis. Mouse $CD73^{-/-}$ model is criticized as it does not reflect the symptoms of CD73 knockout in humans [33]. However, this differences were ascribed to adenosine-mediated mechanisms; in human blood the half-life of adenosine is <15 s, while in mice the half-life is ≈ 2 min [58,68,16]. In the present work we used human endothelium to study CD73-dependent conversion of NMN to NR but not to study adenosine-dependent mechanisms. Further studies are need to determine the relative importance of CD73-dependent regulation of NAD metabolism in mice and humans.

Nevertheless these limitations, CD73 knockdown in mice seriously affected vascular function. Mierzejewska et al. [44] demonstrated that $CD73^{-/-}$ mice displayed endothelial dysfunction with enhanced adhesion molecules, activation of pro-inflammatory cytokine and impaired L-Arginine metabolism, and these changes progressed with the age of animals. CD73 was also shown to limit endothelial permeability [11], trans-endothelial leukocyte trafficking and immune sequelae of allograft vasculopathy [27]. In all these studies vasoprotective and anti-inflammatory roles of CD73 were uniquely linked to adenosine-mediated mechanisms, for example exerted via A_{2B} receptors [11,27].

In the present study, we provide evidence suggesting that CD73 represents an important pathway that controls the extracellular conversion of NMN to NR before it could be used as the intracellular substrate for NMN (by NRK1 or NRK2) and subsequently for NAD (by NMNAT1-3) in endothelial cells. Thus, the vasoprotective effects of CD73 described previously might be not only linked to adenosine-mediated pathways, but could be also linked to extracellular conversion of NMN to NR by CD73. However, we cannot exclude, that in our experimental system CD73-dependent adenosine signaling in endothelial cells played a role since HAEC cells incubated with AOPCP only displayed a higher expression of von Willebrand Factor (Fig. 2B). The role of CD73 in the conversion of extracellular substrate for endothelial NAD such as NMN converted to NR, seem quite likely not only in aging, a well-known state of NAD deficiency [35,13], but also in various other contexts where vasoprotective effects of NMN or NR were demonstrated [51,30,60,29]. The key element of this concept that yet has to be addressed is the exact source and concentration of NMN in proximity of endothelial luminal surface. That may not be represented by plasma concentration, but by cell surface concentration that may be vastly different as suggested for adenine nucleotides [64].

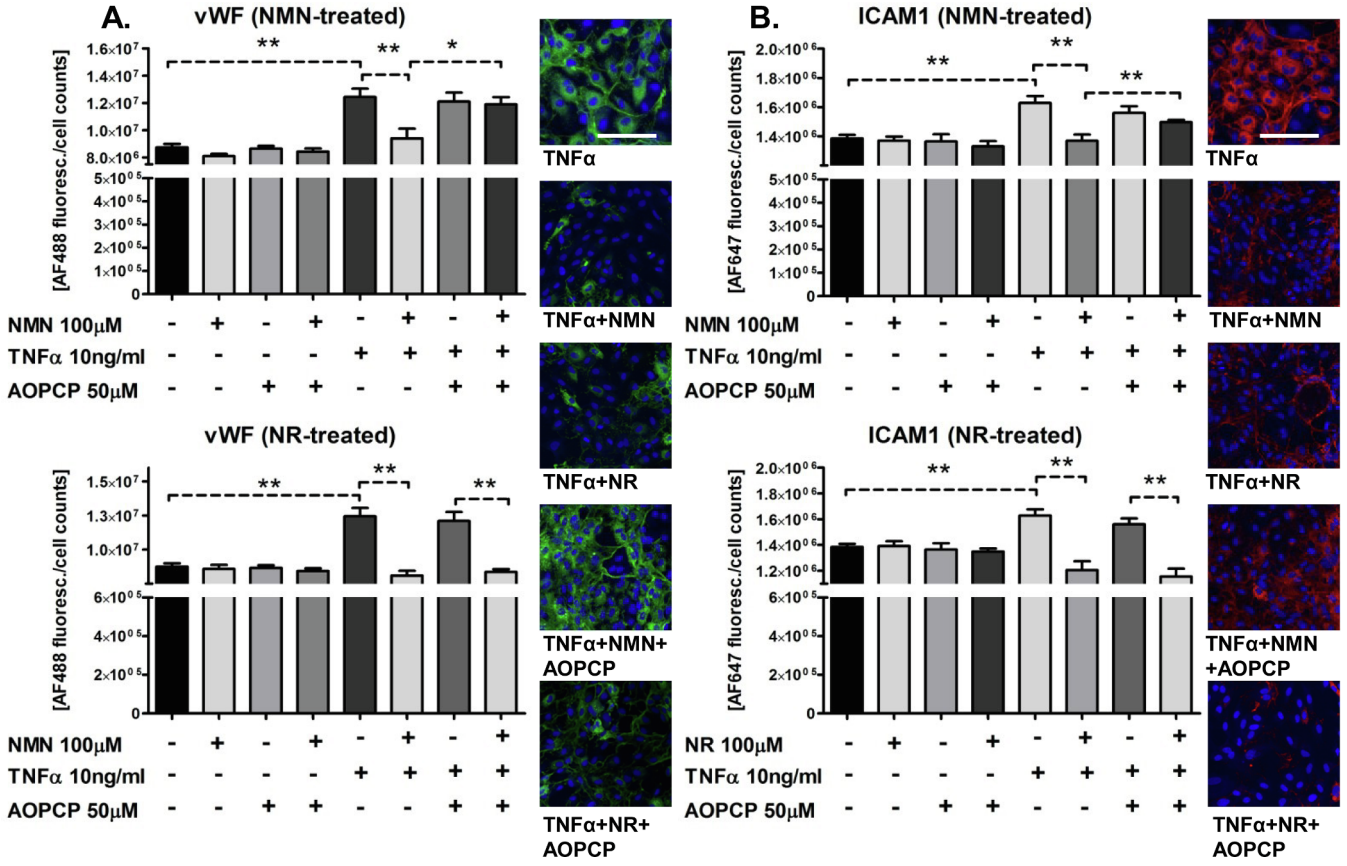


Fig. 3. Effects of NMN and NR on TNFα –induced increase in vWF and ICAM1 expression in HAECs. Expression of vWF in NR and NMN- and NR-treated HAEC cells after 24 h-stimulation with TNFα in the presence or absence of CD73 inhibitor AOPCP, (**A**); expression of ICAM1 in NR- and NMN-treated HAEC cells after 24 h-stimulation with TNFα in the presence or absence of CD73 inhibitor AOPCP, (**B**); n = 6, * $p \leq 0.05$, ** $p \leq 0.01$; white scale bar represents 25 μm.

This notion supported by experimental results of this study, stays in line with the recent discovery of NAD-related function of CD73 after the structural and functional analysis of Haemophilus influenzae NAD nucleotidase (NADn), an ortholog of human CD73 capable of processing NMN [19,18]. Indeed, CD73 was previously shown to be involved in the NMN dephosphorylation into extracellular NR to sustain intracellular NAD in various human cancer cells subjected to inhibition of NAMPT [24]. The intrinsic role of CD73 in the NMN-dependent effect on intracellular NAD concentration was confirmed in numerous cancer cell lines (U87, A549, PC3, OVCAR-3, HePG2) as well as in HEK293 cells [37,57,24]. Furthermore, high CD73 expression in the tumor tissue has been linked to poor overall survival and recurrence-free survival in patients suffering from breast and ovarian cancer and this phenomenon could be linked not only to adenosine-dependent pathway [42,59] but

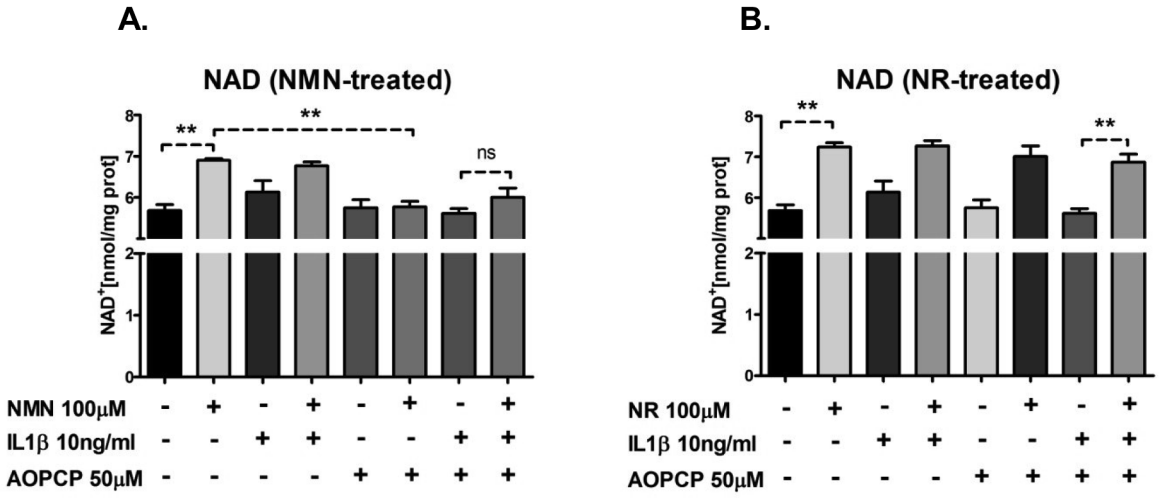


Fig. 4. Effects of NR and NMN on NAD content in HAEC cells, in the presence and absence of CD73 inhibition by AOPCP. Effect of NR (**A**) and NMN (**B**) on intracellular NAD after 24 h-stimulation with IL1β-in the presence or the absence of CD73 inhibitor AOPCP, n = 5, * $p \leq 0.05$, ** $p \leq 0.01$, ns- not statistically significant.

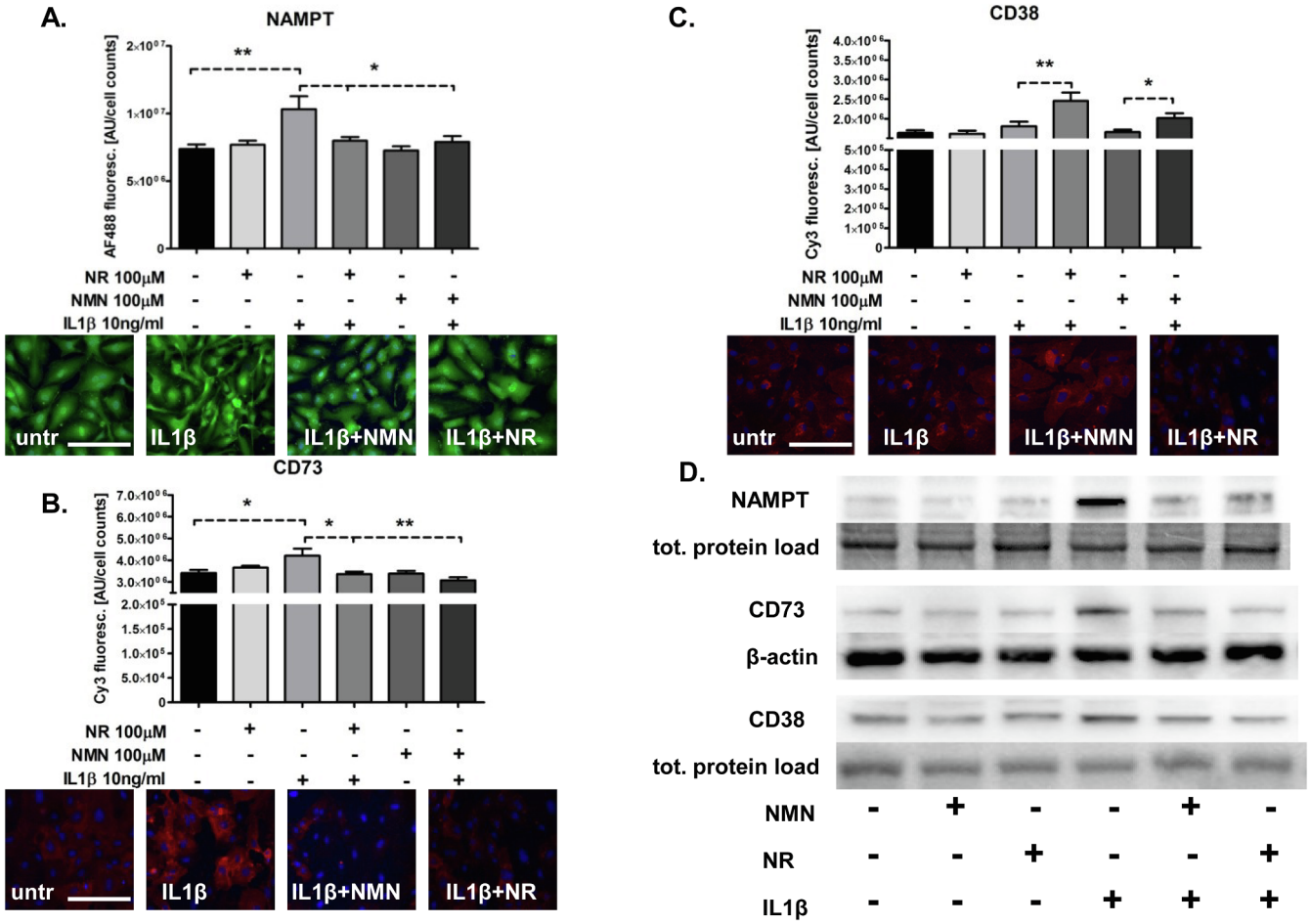


Fig. 5. Upregulation of NAMPT, CD73 and CD38 in HAEC after 24 h-stimulation with IL1β, assessed by immunofluorescent staining and Western Blot assay; results of NAMPT immunofluorescent imaging in IL1β-stimulated cells in the presence or absence of NMN or NR **(A)**; expression of CD73 (5′ectonucleotidase) in IL1β-stimulated cells in the absence or presence of NMN or NR assessed by immunofluorescent imaging **(B)**; results of CD38 imaging after incubation with IL1β in the presence or absence of NMN or NR **(C)**; results of Western Blot analysis of NAMPT, CD73 and CD38 expression after stimulation with IL1β, in a presence or absence of NMN or NR (**D**); n = 6, * $p \leq 0.05$, ** $p \leq 0.01$; white scale bar represents 25 μm.

possibly also to NAD-dependent mechanisms. In the present work, we demonstrated NAD-related function of CD73 in endothelium and ascribed beneficial effects of NMN and NR to NAD-dependent mechanisms described previously in numerous papers in other experimental systems [17,15,51].

It was quite a surprising finding of these studies to show that endothelial inflammation was not associated with NAD deficiency. However, there was an upregulation of NAMPT and CD73, as shown by immunocytochemistry and Western Blot. These results suggest again that intracellular NAD synthesis by NAMPT from nicotinamide and extracellular conversion of NMN to NR represents the two major systems maintaining intracellular NAD in endothelial inflammation. WB assay, but not immunofluorescent imaging, showed a slight upregulation of CD38 after stimulation with IL1β. CD38, identified previously as an main enzyme degrading NMN in mouse tissues *in vivo* [6] seems to play a minor role in NMN conversion in human endothelial cells. These results suggest that pharmacokinetics of NMN *in vivo* is dependent more on NMN uptake by other tissues studied by Camacho-Pereira et al. [6] such as liver, brain skeletal muscle, and spleen, not by endothelial uptake of NMN.

Although in the present work we demonstrated that NMN inhibited endothelial inflammation and improved NO-dependent function by extracellular conversion via ecto-5′-nucleotidase (CD73)-dependent pathway to NR we did not explicitly show that NAD-dependent mechanisms were involved. Obviously, NAD-dependent activation of sirtuins could play a role, for example endothelial SIRT1 that control endothelial homeostasis and vascular functionality by modulating endothelial nitric oxide synthase (eNOS) activity, p53, angiotensin II (Ang II) type 1 receptor (AT1R), forkhead box O1 (FOXO1) or other mechanisms [14].

In summary, we demonstrated the nicotinamide mononucleotide reversed endothelial dysfunction and inflammation by extracellular conversion to nicotinamide riboside via CD73, whereas nicotinamide riboside-induced effects were CD73-independent. Beneficial effects of NMN and NR were comparable and could be both ascribed to NAD-dependent mechanisms, as suggested in previous studies [51,3]. In addition, we demonstrated that endothelial inflammation was associated with the upregulation of NAMPT and CD73, suggesting that intracellular NAD synthesis by NAMPT from nicotinamide and extracellular conversion of NMN to NR, represent the major compensatory systems activated in endothelial inflammation. Altogether, our results point to the extracellular conversion of NMN to NR by CD73 localized in the luminal surface of endothelial cells as important vasoprotective mechanisms to maintain intracellular NAD. Thus, the vasoprotective role of endothelial CD73 cannot be solely attributed to AMP-adenosine dependent mechanisms and the importance of NAD-dependent mechanisms in vascular pathologies where CD73 is altered [34,40,41], needs to be elucidated in further studies.

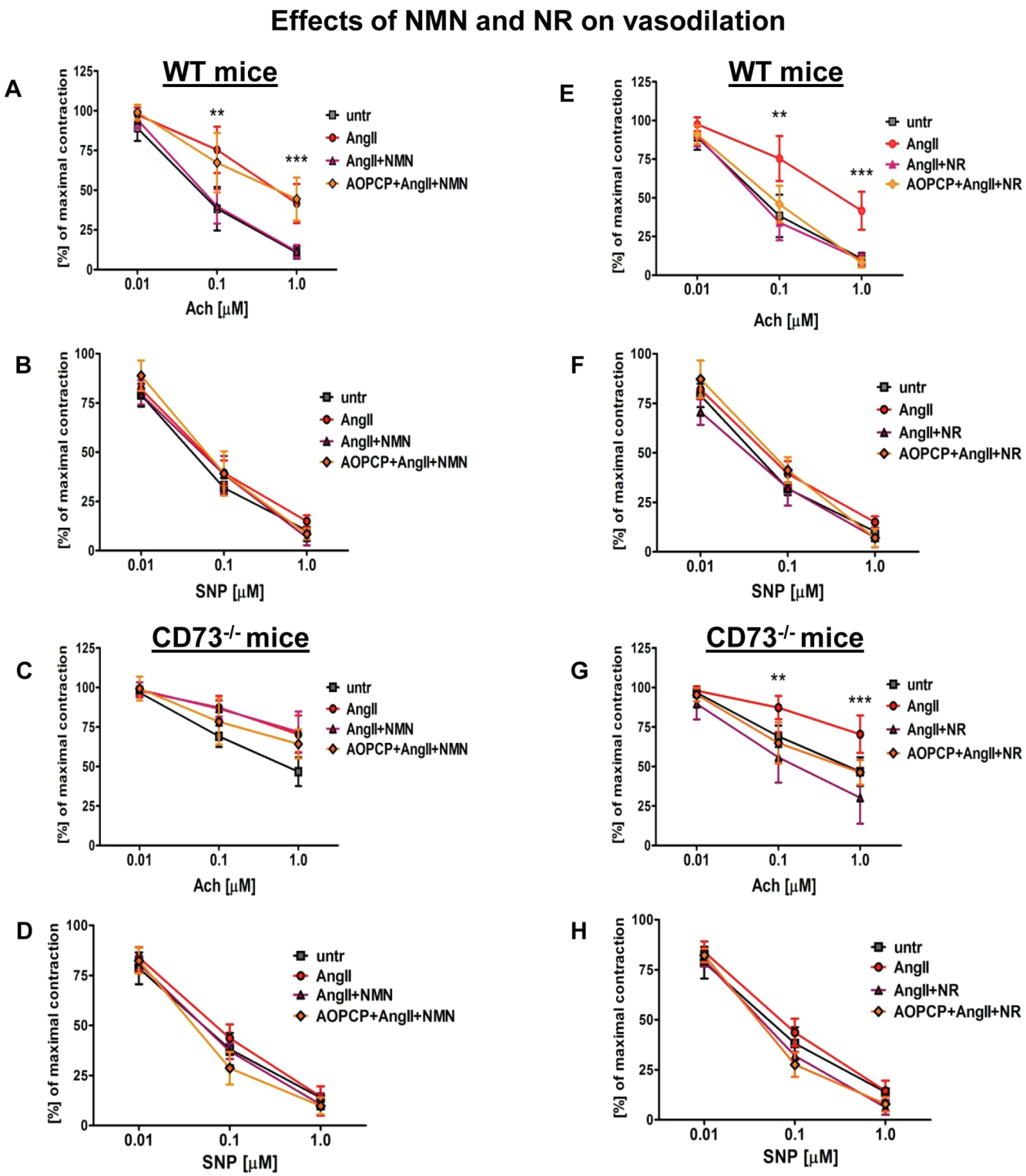


Fig. 6. Effects of NMN and NR on acetylocholine-induced endothelium-dependent vasodilation of aortic rings taken from C57Bl/6J (WT) mice (A,E) and CD73$^{-/-}$ mice (C,G), and incubated with angiotensin II for 24 h. For comparison effects of NMN and NR on endothelium-independent vasodilation induced by sodium nitroprusside was assessed (B, D, F, H); n = 5, ** $p \leq 0.01$, *** $p \leq 0.001$ (AngII-treated *vs* AngII/NMN/NR).

Author contributions

Łukasz Mateuszuk and Stefan Chłopicki concieved and designed the study; Łukasz Mateuszuk, Roberto Campagna, Barbara Kutryb-Zając and Kamil Kuś collected, analyzed and/or interpreted the data; Ewa M. Słominska and Ryszard T. Smolenski provided necessary tools; Łukasz Mateuszuk and Stefan Chlopicki drafted and written the final version of the manuscript; Ewa M. Słominska, Ryszard T. Smolenski and Barbara Kutryb-Zając revised the manuscript; All authors approved the final version of the manuscript.

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