Endogenous NAMPT dampens chemokine expression and apoptotic responses in stressed tubular cells

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Grant numbers and sources of support FIS 06/0046, FIS PS09/00447, PI081564, ISCIII-RETIC REDinREN/RD06/0016/0003, RD06/0016/0004, RD12/0021/0001 and 0002, Comunidad de Madrid (Fibroteam S2010/BMD-2321, S2010/BMD-2378). Programa Intensificación Actividad Investigadora (ISCIII/Agencia Laín-Entralgo/CM) to AO.

Con formato: Inglés (Reino Unido)

Running Title: Endogenous NAMPT and stressed kidney tubular cells.

Abstract

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease and identification of new therapeutic targets is needed. Nicotinamide phosphoribosyltransferase (NAMPT) is both an extracellular and intracellular protein. Circulating NAMPT is increased in diabetics and in chronic kidney disease patients. The role of NAMPT in renal cell biology is poorly understood. NAMPT mRNA and protein were increased in the kidneys of rats with streptozotocin-induced diabetes. Immunohistochemistry localized NAMPT to glomerular and tubular cells in diabetic rats. The inflammatory cytokine TNFα increased NAMPT mRNA, protein and NAD production in cultured kidney human tubular cells. Exogenous NAMPT increased the mRNA expression of chemokines MCP-1 and RANTES. The NAMPT enzymatic activity inhibitor FK866 prevented these effects. By contrast, FK866 boosted TNFα-induced expression of MCP-1 and RANTES mRNA and endogenous NAMPT targeting by siRNA also had a proinflammatory effect. Furthermore, FK866 promoted tubular cell apoptosis in an inflammatory milieu containing the cytokines TNFα/IFNγ. In an inflammatory environment FK866 promoted tubular cell expression of the lethal cytokine TRAIL. These data are consistent with a role of endogenous NAMPT activity as an adaptive, protective response to an inflammatory milieu that differs from the proinflammatory activity of exogenous NAMPT. Thus, disruption of endogenous NAMPT function in stressed cells promotes tubular cell death and chemokine expression. This information may be relevant for the design of novel therapeutic strategies in DN.

Key words: diabetes, NAMPT, apoptosis, inflammation, kidney.

1. Introduction

Diabetic end-organ damage contributes to at least 233,000 yearly deaths in the US alone. Diabetic nephropathy (DN) is the most common cause of end-stage renal disease [1, 2]. Failure of current therapeutic approaches to successfully prevent progression of DN in a sizable proportion of patients must be ascribed to an incomplete understanding of the molecular pathways contributing to DN progression. The diabetic microenvironment induces apoptosis in various organs and apoptosis is believed to contribute to the gradual loss of renal function and renal cell mass in DN [3-5]. Renal tubular epithelial, endothelial and interstitial cell apoptosis is observed in DN[6-8]. DN is also characterized by an inflammatory response [9, 10]. Both apoptosis and inflammation may be interrelated. Thus several cytokines, such as TNFα and TRAIL regulate both apoptosis and inflammation in kidney cells in a diabetic milieu[10].

Nicotinamide phosphoribosyltransferase (NAMPT)/visfatin/pre-B-cell colony-enhancing factor (PBEF) was originally identified as a secreted growth factor for early B cells [11]. Visfatin was described as an adipokine with insulin mimetic action that improved glucose tolerance [12]. However, the original description of visfatin activity was retracted because of the irreproducibility of the results. NAMPT was described as an intracellular enzyme in the mammalian salvage pathway of nicotinamide adenine dinucleotide (NAD)+ that catalyzes the synthesis of nicotinamide mononucleotide (NMN) from nicotinamide and phosphoribosylpyrophosphate (PRPP) [13]. NAMPT is now the official name for gene and protein approved by the HUGO Gene Nomenclature Committee [14, 15]. The NAMPT gene encodes a 491 amino acid protein with a predicted molecular weight of 52 kD. The crystal structure of NAMPT suggested a homodimeric enzyme [15]. Additional non-enzymatic NAMPT functions include proinflammatory effects on leukocytes and tumor cells [16, 17], pro-angiogenic effects on endothelial cells [18] and proliferative actions on vascular smooth muscle cells [19]. FK866 is now considered a competitive inhibitor of NAMPT enzymatic activity that reduces NAD+ levels and promotes apoptosis of tumor cells[15] [20]~~FK866 is now considered a competitive inhibitor of NAMPT enzymatic activity that reduces NAD+ levels and promotes apoptosis of tumor cells[15] [20]~~. In this regard, studies are underway to validate NAMPT as a target for the development of new anticancer agents.

There is scarce information on NAMPT and kidney disease. High glucose increases NAMPT expression in cultured rat mesangial cells, and murine podocytes and tubular cells [21, 22]. Exogenous extracellular NAMPT increased the synthesis of pro-fibrotic molecules in these renal cell types through a mechanism

requiring actin polymerization and/or glucose uptake since it was inhibited by the actin polymerization inhibitor cytochalasin B. In a genetic rat model of type 2 diabetes, plasma NAMPT levels were increased in the early stages of DN and positively correlated with microalbuminuria. Glomerular and tubular cell NAMPT was increased in this model of DN. As a whole, these data suggested a deleterious action of NAMPT on renal cells that might contribute to the pathogenesis of DN [21, 22]. Mesangial cell NAMPT enzymatic activity may be inhibited by FK866 [21]. However the role of NAMPT enzymatic activity in renal cells beyond regulation of glucose uptake was not addressed.

We now aimed at unraveling the role in kidney cells of the increased endogenous NAMPT observed during kidney injury. We describe increased local kidney NAMPT in a type 1 diabetes model and in cultured tubular cells exposed to TNFα. We also describe the interplay between endogenous NAMPT and inflammatory cytokines in the regulation of chemokine expression and cell death. The data suggest a cytoprotective, anti-inflammatory role of inflammation-induced endogenous NAMPT in tubular cells.

2. Materials and methods

2.1 Animal model

Diabetes was induced in six 6-week-old rats (WKY, Criffa, Barcelona, Spain) by a single intraperitoneal injection of 50mg/Kg streptozotocin (Sigma, St. Louis, MO, USA) in 0.01 M citrate buffer pH 4.5. Six control rats received the vehicle. Rats were sacrificed at 6 weeks following induction of diabetes. Kidneys were perfused in situ with cold saline before removal. One kidney was fixed in buffered formalin, embedded in paraffin and used for immunohistochemistry, and the other kidney was snap-frozen in liquid nitrogen for RNA and protein studies of renal cortexes [23]. Insulin (Insulatard NPH, Novo Nordisk, Denmark) was administered at a dose of 1-4 IU s.c. weekly to prevent death from 7 days after administration of streptozotocin, once all animals had blood glucose levels >400 mg/dl. Twenty-four hour albuminuria was assessed by ELISA (Celltrend, Luckenwalde, Germany). **Table 1** presents animal model data. All studies were performed in accordance with the European Union normative.

2.2 Immunohistochemistry

Immunohistochemistry was carried out in 5 µm thick paraffin-embedded rat kidney sections. The primary antibody was a mouse monoclonal anti-NAMPT (1:200, Enzo life science). Sections were counterstained with Carazzi`s hematoxylin. Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. Image quantification was carried out with ImageProPlus

software (MediaCybernetics, Bethesda, MD) as previously described [24]. This software allows selecting and calculating the area of pixels with similar color. Results are shown as percentage of positive stained area versus total area from 10 fields per kidney (x200 magnification).

2.3 Cell culture

HK-2 human proximal tubular epithelial cells (ATCC, Rockville, MD) were grown on RPMI 1640 (GIBCO, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite, and 5 ng/mL hydrocortisone in 5% CO_2 at 37°C as previously described [10]. For experiments cells were rested in serum-free media for 24 h prior to the addition of stimuli and throughout the experiment. To assess the effect of different concentrations of glucose, cells were grown in DMEM 5 mM or 25 mM glucose (Lonza) with the same additives [25].

2.4 Cell stimulation and NAMPT targeting.

Exogenous ~~extracelullar~~extracellular NAMPT (eNAMPT) was purchased from Enzo Lifesciences. The protein is produced in HEK3 cells and secreted using a proprietary signal peptide. The purity is ≥95% and the endotoxin content is < 0.1EU/µg protein (LAL-test). Concentrations used (10-200 ng/ml range) are indicated in the figure legends. Wild-type and mutant NAMPT (R392A, S200D and H247E) mutants (a kind gift from C.Wolberger) [26] were used at 100 ng/ml. R392A retains 36% of the original enzymatic activity showed by NAMPT, S200D 12% and H247E 8.50%~~–~~. NAMPT siRNA (ID 10135;Trilencer-27, SR306835 Origene). Cells were transfected with lipofectamine. FK866 (1-10nM; Axon 1279, AxonMedchem) was always added 90 minutes before other stimuli. 50 ng/ml of TNFα and 5 ng/ml of IFNγ (Peprotech, UK) were used.

Con formato: Resaltar

2.5 NAD/NADH assay

Total cell NAD was assayed by a commercial NAD/NADH quantification kit following manufacturer's instructions (Abcam, Cambridge, UK). Briefly, cells were washed in cold PBS and lysed. Fifty microliters cell lysate were used for the kit protocol and for protein quantification. All results were corrected by the protein content of each sample.

2.6 Cell death and apoptosis

Apoptosis was quantified by flow cytometry of DNA content, as previously described [27]. Adherent cells were pooled with spontaneously detached cells and stained in 100 µg/mL propidium iodide, 0.05% NP-40, 10 µg/mL RNAse A in PBS at 4°C for >1 hour. This assay permeabilizes the cells and allows

entry of propidium iodide in all cells, dead and alive. Nuclear fragmentation and the consequent reduction in DNA amount (hypodiploid cells) is one of the main characteristics of apoptotic cells. The percentage of apoptotic cells with decreased DNA content (A_0) was counted. Exposure to a lethal cytokine cocktail (50 ng/ml TNFα, and 5 ng/ml IFNγ, Peprotech, UK) present in the diabetic milieu for 24h was used as a proinflammatory lethal stimulus[10]. Cytokine concentrations were based on prior experience and dose-response studies from our lab [28, 29]. Cells were seeded at a density of 25,000/ml (12-well plates for flow cytometry and coverslips for cytofluorescence) and grown for 24 h. Thereafter, they were rested in serum-free medium for 24 h and stimuli were added to subconfluent cells. Cells were also seeded in 96-well plates, serum depleted for 24 hours, stimulated and then assayed for cell viability with the MTS-PMS assay (Promega).

2.7 Western blot

Proteins extracts were isolated with lysis buffer and western blots were performed as described previously [27]. Membranes were incubated overnight at 4ºC with anti-NAMPT antibody (1:2,000, EnzoLife Science), which recognizes the intracellular and extracellular forms of human NAMPT, anti-TRAIL (1:1,000, R&D systems), or mouse anti-β Actin monoclonal antibody (1:5,000, Abcam) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000, Amersham, Aylesbury, UK). Blots were developed with the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham).

2.8 Reverse transcription and real time PCR

RNA was isolated using TRIzol reagent. Reverse transcription of RNA and real time quantitative-PCR was performed as previously described. Pre-developed TaqMan reagents were used for RANTES and MCP-1 (Applied Biosystems, Darmstadt, Germany), and normalized for 18S ribosomal RNA. Quantification of templates and relative expression for each gene was achieved following the model $R=2e(-\Delta\Delta Ct)$. Serial dilutions of kidney or cellular cDNA were included in all PCR runs and served as standard curve. All measurements were performed in duplicate. Controls consisting of bidistilled H_2O were negative in all runs.

2.9 ELISA

The ELISA kit for MCP-1 (R&D, Minneapolis, MN; USA) was carried out following manufacture's instructions in HK2 cell culture supernatants. Final wavelength was measured at 450nm and corrected by 540 nm wavelength.

2.10 Statistical analysis

Results are expressed as mean ± S.E.M. of at least three independent experiments. Significance at the $p<0.05$ level was assessed by non-parametric Mann-Whitney test for two groups of data and Kruskal-Wallis or one-way analysis (ANOVA) with a Bonferroni post hoc correction for analysis of differences between three or more groups by SPSS software (SPSS Inc., Chicago, IL).

3. Results

3.1 Increased local kidney NAMPT in rat type I diabetes

Rats with streptozotocin-induced diabetes or non-diabetic vehicle-injected controls were sacrificed at 6 weeks. At the time of sacrifice, diabetic rats had developed albuminuria (**Table 1**). Whole kidney NAMPT protein (**Fig. 1A**) and mRNA (**Fig. 1B**) were increased in the diabetic kidney. Immunohistochemistry localized NAMPT expression to glomerular and tubular cells (**Fig. 1C,D**).

3.2 TNFα increases NAMPT in cultured human tubular cells

We focused on tubular cells in search of factors potentially driving NAMPT expression in DN. TNFα is a multifunctional cytokine involved in DN progression [9, 30]. TNFα increased NAMPT mRNA expression at three hours in cultured human tubular epithelial cells. There was a non-significant trend towards increased NAMPT mRNA levels in cells cultured with high glucose concentrations at 24 (**Fig. 2A**) and 48 hours (data not shown). Similar results were observed at the protein level. High glucose tended to increase NAMPT while TNFα significantly increased cell-associated NAMPT expression after 48 hours of stimulation (**Fig. 2B**), while increased levels of soluble NAMPT were already evident at 24 and 48h following TNFα stimulation (**Suppl Fig. 1**). No differences in protein expression were observed at 3h (not shown). TNFα also increased NAD, suggesting an increased NAMPT enzymatic activity after 24 hours of stimulation, an effect abrogated by the NAMPT enzymatic activity inhibitor FK866 (**Fig. 2C**). However, we cannot exclude that TNFα modulates nicotinamide nucleotide adenylyltransferase (NMNAT), the enzyme directly responsible for NAD generation.

3.3 Exogenous NAMPT is proinflammatory in tubular cells

In order to assess the role of NAMPT in tubular cells we added exogenous NAMPT to the cells. Exogenous NAMPT did not modulate human tubular cell viability (**Fig. 3A**), apoptosis (**Fig. 3B**) or proliferation (**Fig. 3C**). However, NAMPT dose-dependently increased the mRNA expression of the

chemokine MCP-1 (**Fig. 4A**). This effect is similar to that previously reported in murine tubular cells and podocytes and human vascular smooth muscle cells [22, 31].

3.4 Exogenous NAMPT proinflammatory activity depends on its enzymatic activity

Previously described actions of exogenous NAMPT on kidney cells were thought to be a cytokine-like activity dependent on its now retracted insulin-mimetic properties promoting glucose entry into the cells [21, 32]. However, in cultured human tubular cells inhibition of NAMPT enzymatic activity by FK866 decreased the proinflammatory actions of exogenous NAMPT on the mRNA expression of the chemokines MCP-1 (**Fig. 4B**) and RANTES (**Fig. 4C**). We confirmed the requirement for the enzymatic activity for the proinflammatory effects using specific NAMPT mutants. Cells stimulated with NAMPT mutated in the catalytic site failed to upregulate the mRNA expression of MCP1, RANTES and IL-6 (**Fig. 4D**). These two sets of independent experiments suggest dependence of the proinflammatory action of exogenous NAMPT on its enzymatic activity.

3.5 Inhibition of endogenous NAMPT magnifies chemokine expression

Since cellular NAMPT was increased in tubular cells cultured in an inflammatory milieu and in tubular cells in vivo, we next explored whether endogenous NAMPT expressed in response to TNFα might regulate inflammation through its enzymatic activity. TNFα induction of MCP-1 mRNA in tubular cells peaks at 3h and later decreases towards baseline at 24h (**Fig. 5A**). When endogenous NAMPT activity was inhibited by FK866, TNFα proinflammatory effects were prolonged in time and increased MCP-1 mRNA was still observed at 24h (**Fig. 5A**). Endogenous NAMPT inhibition also prolonged peak expression of RANTES, a gene whose expression in response to TNFα increases later and is more persistent than that of MCP-1[24](**Fig. 5B**). Endogenous NAMPT inhibition of cytokine-induced chemokine expression was confirmed when assessing secreted MCP-1 by ELISA. TNFα increased MCP-1 secretion in tubular epithelial cells, and this increment was higher when endogenous NAMPT was inhibited (**Fig. 5C**). We next studied the effect of knocking down NAMPT expression by siRNA. siRNA-mediated knock-down of NAMPT resulted in increased RANTES mRNA and a trend towards increased MCP-1 mRNA, both spontaneously and in the presence of TNFα (**Fig. 5D,E**). Thus, endogenous renal tubular cell NAMPT in response to TNFα appears to limit the proinflammatory action of TNFα. Furthermore, the spontaneous proinflammatory action of knocking down endogenous NAMPT suggests that part of the anti-inflammatory action of endogenous NAMPT may be enzyme activity-independent. In

conclusion, under the conditions explored endogenous and exogenous NAMPT have opposite effects on chemokine expression.

3.5 Endogenous NAMPT may modulate cell death and tubular cell proliferation

Since tubular cell apoptosis is a feature of DN[4], we next studied the modulation of apoptosis by endogenous tubular cell NAMPT by inhibiting its enzymatic activity. FK866 alone increased tubular cell proliferation after 24h (**Fig. 6A**), but did not modify the apoptotic rate at 24 or 48h in the absence of an inflammatory milieu (**Fig. 6B**). However, in an inflammatory microenvironment composed of TNFα and IFNγ similar to what may be found in DN [9], the inhibition of endogenous NAMPT increased the apoptotic rate at 48h (**Fig. 6C**). Thus, the increase in endogenous NAMPT in response to an inflammatory milieu has an antiapoptotic action.

3.6 Inhibition of endogenous NAMPT enhances TNFα/IFNγ-induced expression of the lethal cytokine TRAIL

We next addressed the relationship between proinflammatory cytokines, endogenous NAMPT and lethal cytokine expression. A proinflammatory milieu composed of TNFα and IFNγ increased expression of TRAIL, the most upregulated lethal cytokine in DN, as previously observed by our group[10]. Inhibition of endogenous NAMPT enzymatic activity by FK866 did not modulate TRAIL expression by itself, but increased cytokine-induced TRAIL upregulation (**Fig. 7**). TRAIL had been previously shown to promote tubular cell death in an inflammatory milieu [10].

4 Discussion

The main findings of our study are that NAMPT is upregulated by inflammatory mediators in human tubular cells and that endogenous NAMPT enzymatic activity plays a key role in protecting tubular cells from adverse consequence of an inflammatory environment such as cell death and a prolonged inflammatory response. This is consistent with the hypothesis that upregulation of endogenous NAMPT during kidney injury might be a compensatory protective mechanism that requires NAMPT enzymatic activity to protect from apoptosis and quench the inflammatory tubular cell response. This effect is in contrast to the previously described proinflammatory effect of exogenous NAMPT, that we have also observed and determined to be dependent on NAMPT enzymatic activity [31]. The involvement of the enzymatic activity is in line with a prior report in mesangial cells, where exogenous NAMPT actions on glucose transport were prevented by FK688 [21]. However, these observations differ from findings in

other organs, since in a lung carcinoma epithelial cell line NAMPT overexpression increased expression of inflammatory cytokines via a nonenzymatic mechanism [17]. Our experimental design differs from the latter in that we assessed the role of endogenous NAMPT following stimulation of its expression by a pathophysiologically relevant stimulus, as opposed to the enforced genetic-mediated expression of the gene.

There are controversial results on NAMPT and diabetes, including the retraction of the initial report of its insulin mimetic activity [12]. Mice lacking NAMPT develop impaired glucose tolerance and defective insulin secretion, which are restored by administration of exogenous NAMPT [33]. NAMPT-mediated NAD biosynthesis regulates glucose homeostasis and maintenance of high circulating NMN levels by NAMPT is critical for normal β cell function [33]. However, NAMPT plasma levels have been reported increased, unchanged or decreased in diabetic patients [34-37] and are increased in CKD patients [38, 39]. In experimental diabetes, plasma and renal NAMPT were elevated in type 2 diabetes rats with DN [22]. We have now confirmed increased tubular NAMPT in a type 1 diabetes DN model and provide evidence for a role of locally synthesized NAMPT in tissue protection in the presence of a cell environment that recapitulates the diabetic kidney injury milieu. This protective action of endogenous NAMPT, in the absence of enforced overexpression, differs from the proinflammatory role described up to now for exogenously added or for the enforced overexpression of NAMPT.

Once demonstrated increased NAMPT in diabetic kidneys, the next step was to identify the factors responsible for this high expression. High glucose concentrations upregulate NAMPT synthesis in rat mesangial cells, murine podocytes and tubular cells [21, 22]. We also observed a trend toward increased NAMPT in human tubular cells exposed to a high glucose concentration, although the effect was milder than the one described in murine cells [22]. In contrast to the failure of angiotensin II to upregulate NAMPT in rat and murine renal cells [21, 22], we now report that TNFα upregulates NAMPT in human tubular cells. TNFα is upregulated in human and rat DN [9, 30]. Cytokines also upregulated expression of NAMPT in rheumatoid arthritis synovial fibroblasts [40]. Thus, inflammation, as metabolic stress does, upregulates NAMPT in tubular cells. However, the role of this stress-induced NAMPT upregulation in tubular cell biology had not been previously characterized.

Initial attempts to unravel the role of NAMPT on cultured renal cells relied on the addition of exogenous NAMPT. Exogenous NAMPT induces production of profibrotic molecules in rat mesangial cells and murine podocytes and tubular epithelial cells [21, 22]. Exogenous NAMPT also increased MCP-1

expression in murine renal epithelial cells [22] and synthesis of several renin angiotensin system components in cultured rat mesangial cells [41]. Exogenous NAMPT increased the synthesis of pro-fibrotic molecules and MCP-1 through a cytochalasin-B sensitive mechanism suggesting dependence on cellular glucose uptake [22]. These observations in kidney cells were consistent with data in other cell systems. Thus, NAMPT activated the transcription factors NF-κB and AP1 and induced cytokine synthesis in rheumatoid arthritis synovial fibroblasts and monocytes. In fibroblasts NAMPT knockdown inhibited basal and TLR ligand-induced production of cytokines [40]. In contrast to the liver and the kidney, immune cells seem to rely exclusively on the NAMPT salvage pathway for NAD biosynthesis [42]. Thus, cells of the immune system express high levels of NAMPT [43]. In immune cells FK866 decreased intracellular NAD and reduced TNF-α synthesis in response to LPS [43]. However, cells displaying low intracellular NAD levels retained the ability to secrete other proteins (such as the chemokine RANTES) in response to the same stimuli. Thus, the role of endogenous NAMPT on the synthesis of inflammatory mediators is cell-type specific: endogenous NAMPT may be required for full activation of the inflammatory response in cells with constitutive high NAMPT and NAD levels, such as immune cells.

In cultured mesangial cells exogenous NAMPT increased intracellular NAD concentrations and FK866 significantly inhibited NAMPT-induced NAD biosynthesis, suggesting NAMPT-mediated NAD biosynthesis [21]. In addition, in mesangial cells FK866 decreased exogenous NAMPT-induced glucose uptake, suggesting NAD involvement in NAMPT-mediated glucose uptake. However, the effect of FK866 on cell survival or inflammatory mediator synthesis was not explored. At least some actions of exogenous NAMPT on vascular cells depend on its enzymatic activity [31, 44, 45] and exogenous NAMPT enhanced vascular smooth muscle cell proliferation [19]. However, we observed that exogenous NAMPT did not modulate cell death or proliferation in non-stressed human tubular cells.

Endogenous NAMPT may also regulate cell proliferation and cell death. In endothelial cells NAMPT overexpression increased proliferation and extended replicative lifespan, preferentially during glucose overload [46]. High intracellular NAD levels may act through sirtuins to favor tumor cell survival, especially in response to genotoxic stress [47]. Increased NAMPT activity is a physiological response to replenish NAD levels in cells exposed to stress. Thus, by blocking this response, FK866 decreased intracellular NAD and reduced proliferation and induced apoptosis in human leukemia cell lines [48] and inhibited growth of glioblastoma cells and other tumor cells [14, 49, 50]. This is the basis for a potential

role of NAMPT targeting on cancer therapy. Along the same lines, the NAMPT inhibitor FK866 increased sensitivity to cell death of mouse fibroblasts exposed to genotoxic stress [13, 51], demonstrating that NAMPT regulation of cell viability requires its enzymatic activity and that endogenous NAMPT may be cytoprotective under conditions of cell stress. However there was no prior information on the relationship between the enzymatic function of stress-induced endogenous NAMPT and cell death or inflammatory responses in an inflammatory milieu, as may be found in the kidney during DN. In non-stressed tubular cells inhibition of NAMPT activity with FK866 slightly increased tubular cell proliferation, without modifying the apoptosis rate. This should be interpreted in the context of FK866 as an experimental anti-cancer drug. A potential extrapolation is that no direct kidney toxicity might be expected in normal healthy kidneys when using FK866 therapeutically for cancer. However, the consequences of the therapeutic use of FK866 for the kidney may differ under situations of kidney stress. Thus, tubular cells stressed with the inflammatory cytokines TNFα and IFNγ became dependent on endogenous NAMPT for survival and inhibition of endogenous NAMPT activity increased cell death. The precise contributors to the increased cell death observed were not specifically characterized. However, increased expression of the lethal cytokine TRAIL was observed that coincided in time with the increased amount of cell death. TRAIL had been previously shown to promote death of tubular cells in an inflammatory environment [10]. Furthermore, under these culture conditions, inhibition of endogenous NAMPT prolonged TNFα-induced chemokine expression to 24h. However, only a modest increase in total cell NAMPT was observed at this time point. This observation may have two potential explanations: a) that the trend to increased NAMPT observed at 24 h of stimulation is already functionally significant at 24h or that the clearly increased endogenous extracellular NAMPT contributed to the effect; b) As an alternative, that another unidentified regulator might be responsible for the observation.

5. Conclusion

How do we reconcile the proinflammatory role of exogenous NAMPT with the anti-inflammatory, cell-protective role of endogenous NAMPT in tubular cells in an inflammatory milieu? Any interpretation must take into account two facts: a) The concentration of exogenous NAMPT that promotes profibrotic and inflammatory responses in renal cells is in the 10-100 ng/ml range, as observed in this paper and by other authors [22]. This is the concentration range reported for circulating visfatin/NAMPT in CKD patients [38]. By contrast, extracellular NAMPT released by renal cells is measured in pg, a 1000-fold

lower concentration [21, 22]. We have now confirmed secretion of NAMPT by tubular cells, consistent with previous findings. Thus we are unable to pinpoint the protective effect of endogenous NAMPT to the fact that it is the intracellular form. We cannot discard that besides any difference in concentration; postraslational modifications of renal cell NAMPT explain these differential properties of endogenous NAMPT be it intracellular or secreted. b) The proinflammatory action of exogenous NAMPT has been observed in non-stressed cells, as exemplified by data presented here and by others [21, 22, 52] However, the anti-inflammatory and cell-protective endogenous NAMPT effects in tubular cells are observed when cells are stressed in an inflammatory microenvironment. Thus, our current interpretation is that low amounts of endogenous NAMPT synthesized by tubular cells in response to inflammatory stress help the cells to withstand that stress, while high amounts of exogenously added NAMPT may be detrimental, especially in the absence of stress. Our data and experimental design do not allow to discriminate between effects of endogenous, intracellular NAMPT versus endogenous extracellular (that is, NAMPT secreted by the tubular cells) NAMPT (**Fig. 8**).

In summary, we have uncovered an anti-inflammatory, cell-protective role of endogenous NAMPT in tubular cells cultured under stress conditions with features of the DN milieu. This information will be useful to study NAMPT as a potential therapeutic target in kidney injury. In addition it raises a note of caution regarding the potential nephrotoxicity of the use of NAMPT inhibitors in other fields of Medicine, such as Oncology, when prescribed to patients with DN [53]. Taking into account the multifunctional nature of NAMPT, the final in vivo result of the therapeutic modulation of NAMPT on kidney injury has to be addressed in experimental animal models that discriminate between tubular and non-tubular sources of NAMPT, such as selective knock-down of NAMPT in tubular epithelium.

Disclosure: The authors state no conflict of interests.

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Table 1. **Rat weight gain and laboratory data at sacrifice**.

	Weight gain (%)	**Systolic BP (mmHg)**	**Glycemia (mg/dl)**	**Urinary albumin (mg/24h)**
Control	76 ±18	112 ± 4	126 ± 2	0.44 ± 0.3
DN	61 ± 25	121 ± 5 *	383 ± 135*	2.31 ± 0.4*

Mean ± SEM of 6 animals per group. * $p<0.05$ vs control

Legends to figures

Figure 1. Kidney NAMPT expression is increased in rats with type 1 diabetes. A) Whole kidney NAMPT protein is increased in rat experimental DN assessed by Western blot. β-actin was used as loading control. B) Kidney NAMPT mRNA levels are significantly increased in rat DN. Values for mRNA were normalized to the housekeeping gene 18s expression. C) NAMPT immunohistochemistry in renal cortex. Quantification and representative image. D) NAMPT immunohistochemistry in renal medulla. Representative image and quantification. Magnification 20X. Neg. con.: negative control for the technique. *p<0.05 vs control. Mean + S.E.M. of 6 animals.

Figure 2. TNFα increases NAMPT expression and activity in cultured human tubular cells. A) NAMPT mRNA expression measured by real time RT-PCR. Cells were stimulated with 50 ng/ml TNFα in normal glucose (5 mM) or high glucose (25 mM) culture medium for 3 and 24 hours. Ribosomal 18S was used as housekeeping gene for normalization. *p<0.05 vs control. B) 50 ng/ml TNFα for 48 hours increased whole cell NAMPT protein as detected by Western blot. β-Actin was used as loading control. Mannitol was added to normal glucose culture medium to control for osmolarity changes. Mean + S.E.M of 3 independent experiments. * p<0.05 vs control. C) NAMPT enzymatic activity measured by a NAD/NADH kit. The amount of NAD+ was higher in HK2 cells stimulated with 50 ng/mL of TNFα for 24 h. This increase was inhibited by the preincubation with the NAMPT enzymatic activity inhibitor FK866. * p<0.05 vs. Control, # p<0.05 vs. TNFα. All experiments are Mean + S.E.M of at least 3 independent experiments.

Figure 3. Exogenous NAMPT does not modulate tubular epithelial cell proliferation or death. Exogenous NAMPT stimulation of HK-2 cells at different concentrations for 24 hours does not change cell viability assessed by MTS-PMS assay (A) nor apoptosis (B) or proliferation (C) measured by flow cytometry analysis of DNA content.

Figure 4. Inhibition of NAMPT enzymatic activity prevents exogenous NAMPT proinflammatory effects in tubular cells. A) Exogenous NAMPT increased in a dose-dependent manner chemokine MCP-1 mRNA expression measured by real-time PCR at 24h. Mean ± S.E.M. of 3 independent experiments.

*p<0.05 vs control. B and C) Tubular cells were incubated with 10 nM FK866 before stimulation with exogenous 100 ng/ml NAMPT for 24 hours. MCP-1 (B) and RANTES (C) gene expression were evaluated by real time RT-PCR. * p<0.05 vs eNAMPT. Mean+S.E.M. of 3 independent experiments. D) Tubular cells were stimulated with 100 ng/ml wild-type NAMPT or enzymatically impaired NAMPT mutants (R392A, S200D, H247E) for 24h. RNA expression for RANTES, MCP-1 and IL-6 was assessed by real time RT-PCR. Mean+S.E.M. of 3 independent experiments.

Figure 5. Inhibition of endogenous NAMPT enzymatic activity magnifies TNFα-induced upregulation of chemokines expression. Time-course of MCP-1 (A) and RANTES (B) mRNA expression induced by 50 ng/ml TNFα in tubular cells assessed by real time RT-PCR. Some cells were prestimulated with 10 nM FK866 before adding TNFα. *p <0.05 vs TNFα alone. Results expressed as mean+S.E.M of 3 independent experiments. C) Secreted MCP-1 was measured by ELISA in HK2 cell supernatants. TNFα for 24 h. increased MCP-1 release. Preincubation with the NAMPT inhibitor FK866 resulted in a higher increase in MCP-1 secretion. * p<0.05 vs. control, # p<0.05 vs. TNFα. D, E) Tubular epithelial cells were transfected with scramble or NAMPT siRNA for 24 hours, and then 50 ng/ml TNFα were added for 24h. MCP-1 (D) and RANTES (E) mRNA were assessed by real time RT-PCR. Results expressed as mean+S.E.M of 3 independent experiments * p<0.03 vs control.

Figure 6. Inhibition of endogenous NAMPT enzymatic activity promotes tubular cell apoptosis in an inflammatory milieu. A) The NAMPT inhibitor FK866 increased the proliferation rate in proximal tubular cells cultured in glucose 11 mM. B) However, FK866 alone did not influence apoptosis. Both processes were assessed by flow cytometry analysis of DNA content. *p<0.05 vs control. Mean+S.E.M. of 3 independent experiments. C) The pro-apoptotic effect of TNFα/IFNγ (50 ng/ml and 5 ng/ml) in a high glucose milieu (25 mM) was increased when NAMPT enzymatic activity was inhibited with 10 nM FK866 for 48 hours. *p<0.05 vs TNFα/IFNγ. Mean+S.E.M. of 3 independent experiments.

Figure 7. Inhibition of endogenous NAMPT further increases TNFα/IFNγ-induced TRAIL lethal cytokine expression. Cells were cultured for 24h with TNFα/IFNγ (50 ng/ml and 5 ng/ml). NAMPT enzymatic activity was inhibited with 10 nM FK866. Western blot of cell lysates. β-Actin was used as

loading control. Results expressed as mean+S.E.M of 4 independent experiments. Representative Western blot. * $p<0.05$ vs TNFα/IFNγ alone.

Figure 8. Hypothetical role of exogenous and endogenous NAMPT on kidney tubular epithelial cell pathophysiology. During kidney injury tubular cell NAMPT is increased. In addition, kidney cells may be exposed to exogenous NAMPT, not originated in tubular cells, that may be derived from the circulation where both the liver and fat tissue are thought to be contributors, or may be released locally from macrophages. This manuscript confirms the proinflammatory action of exogenous, extracellular NAMPT over parenchymal cells described in previous publications. In addition it provides evidence that cultured tubular cells express increased amounts of endogenous NAMPT that can be found both as an extracellular protein in the cell culture supernatants and as an intracellular protein in cell extracts. Evidence derived from siRNA targeting of endogenous NAMPT and from pharmacological inhibition of endogenous NAMPT enzymatic activity suggests an anti-inflammatory, cell-protective role for endogenous NAMPT. The design of the experiments does not allow to independently explore the role of extracellular versus intracellular endogenous NAMPT. The relative contribution of both sources of NAMPT should be addressed in vivo, for example, by selectively knocking down NAMPT in tubular epithelium. Potential explanations for the diverse roles of endogenous vs exogenous NAMPT include: a) a key contribution of intracellular NAMPT to the observed protection by endogenous NAMPT; b) a potential protective role of different molecular forms of extracellular NAMPT produced by tubular cells; and c) a hypothetical role for the concentration of extracellular NAMPT. In this regard, NAMPT concentrations in renal cell supernatants have been reported to be low. The anti-inflammatory and cell-protective endogenous NAMPT effects in tubular cells are most marked when cells are stressed in an inflammatory microenvironment. Thus, intracellular NAMPT or low amounts of (modified?) endogenous NAMPT released by tubular cells in response to inflammatory stress may help the cells to withstand that stress, while high amounts of exogenously added extracellular NAMPT may be detrimental, especially in the absence of stress.

Con formato: Resaltar

Supplementary figure 1. Tubular epithelial cells secrete NAMPT. Cells were stimulated with TNFα (50ng/ml), and then supernatants were collected after 24 and 48h. Representative SDS-PAGE microphotography. 40 ug supernatants were loaded per well.