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Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes

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ABSTRACT

Circulating NAMPT (PBEF/visfatin) has pleiotropic functions and is secreted from adipocytes. Since it is doubtful whether serum levels can be explained by secretion from adipocytes alone, we asked whether hepatocytes are also able to liberate NAMPT. Using HepG2 cells and primary rat and human hepatocytes, release of NAMPT into the cell culture supernatant was found to occur constitutively in a time-dependent manner. In primary human hepatocytes, secretion within 24 h was far higher than the cellular content, but was neither influenced by inhibitors of secretion nor by glucose, insulin or TNFα. As determined by size exclusion chromatography, HepG2 lysates and supernatants primarily contained the dimeric form of NAMPT which exhibited similar *in vitro* specific enzymatic activity. In primary human hepatocytes, secreted NAMPT was less active. Our results demonstrate that human hepatocytes are a potential source of circulating NAMPT.

Introduction

In mammals, the rate-limiting step in NAD biosynthesis starting from nicotinamide is catalysed by nicotinamide phosphoribosyltransferase or NAMPT, which transfers a phosphoribosyl group onto nicotinamide yielding nicotinamide mononucleotide (NMN) [1]. NAMPT is a regulator of intracellular NAD levels and consequently influences the activity of NAD consuming enzymes [2].

Also known as PBEF or visfatin, circulating NAMPT exerts pleiotropic actions. It has been described as an extracellular NMN producing enzyme [3], with NMN eliciting protective cellular responses [3,4]. Dimerisation is essential for NAMPT to be enzymatically active [5] and NAMPT has been shown to occur as dimer in human serum [6].

Additionally, NAMPT has been reported to function as a cytokine. Its serum concentration has been shown to be increased in a number of immunological and metabolical disorders [7]. In most of these studies it remained unclear whether enzymatic activity of NAMPT is necessary for its cytokine-like action. However, two studies recently demonstrated that NAMPT acts independent of its NMN biosynthetic activity [8,9].

Differentiated adipocytes are a source of circulating NAMPT [3,10–12], which is possibly released through a non-classical pathway which is blocked neither by brefeldin A and monensin, inhibitors of the ER–Golgi secretory pathway, nor by glibenclamide [11], an inhibitor of ABC-dependent secretion [13]. NAMPT release was reported to be influenced by insulin, glucose and TNFα [10,14]. It is not known whether adipose tissue is the major source for NAMPT in humans or if there are other organs or tissues capable of releasing significant amounts of this protein. Interestingly, a recent study found NAMPT serum levels to be lower in patients with non-alcoholic steatohepatitis than in patients with simple steatosis or obese healthy controls [15]. Therefore, we aimed to characterize NAMPT expressed in and released from hepatocytes and asked whether NAMPT from hepatocytes may retain its enzymatic activity outside the cells.

Materials and methods

Materials. Cell culture media, supplements and antibiotics were obtained from PAA (Cölbe, Germany) or Invitrogen (Karlsruhe, Germany). Brefeldin A, monensin, glibenclamide, glucose, insulin

Abbreviations: ABC, ATP binding cassette transporter; ATP, adenosine triphosphate; BSA, bovine serum albumin; cpm, counts per minute; ER, endoplasmic reticulum; FBS, fetal bovine serum; kDa, kilo Dalton; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; PBEF, pre B-cell colony enhancing factor; PBS, phosphate buffered saline; PRPP, 5-phospho-1-ribosyl pyrophosphate; SEC, size exclusion chromatography; TNFα, tumour necrosis factor α.

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and chemicals were purchased from Sigma–Aldrich (München, Germany); TNFα was from CellSystems® (St. Katharinen, Germany).

Cell culture of HepG2 and primary rat/human hepatocytes. HepG2 cells were maintained in MEM or in RPMI1640 for glucose stimulation supplemented with 10% FBS and 2 mmol/l glutamine. For experiments, HepG2 cells were seeded at 3×10^5 cells/well or 5×10^5 cells/well in 6-well plates for immunoblot or measurement of enzymatic activity, respectively, and grown in serum-containing medium for 24 h. After washing with PBS, medium was changed to serum-free medium +0.01% BSA.

Primary rat hepatocytes were isolated from male Sprague–Dawley rats (Medizinisch-Experimentelles Zentrum der Universität Leipzig) as described previously [16] and seeded at 1.25×10^6 cells/well into collagen-coated 6-well plates in Williams' MediumE containing 2 mmol/l glutamine, 10^{-7} mol/l dexamethansone, 100 IU penicillin/100 μg/ml streptomycin and 10% FBS. After 2–4 h cells adhered and medium was changed to serum-free medium. Primary human hepatocytes were supplied by the "HepatoSys" program according to the guidelines of the "Charitable state controlled foundation HTCR (Human Tissue and Cell Research)" with written informed consent of patients [17] and isolated from suitable liver material. Hepatocytes were seeded at 10^6 cells/well in 6-well plates in medium as specified above.

The ToxiLight® Cytotoxicity BioAssay kit (Lonza, Basel, Switzerland) was applied according to manufacturer's instructions to quantify the release of adenylate kinase into the medium as a measure of cell death by bioluminescence. Maximal release was determined by measuring the activity of adenylate kinase in the supernatant of totally lysed cells.

Incubation with secretion inhibitors, insulin, TNFα and glucose. After culture in serum-containing medium for 24 h, cells were washed with PBS and medium was changed to serum-free medium alone or containing secretion inhibitors (brefeldin A, monensin or glibenclamide), insulin, TNFα or vehicle at the concentrations indicated. For incubation with different glucose concentrations, HepG2 cells were cultured in serum-containing RPMI1640 with 11 mmol/l glucose for 24 h. Cells were washed with PBS and medium was changed to serum-free medium with glucose concentrations ranging from 2.75 to 44 mmol/l. Medium with 11 mmol/l D-glucose and 33 mmol/l L-glucose served as high osmotic control, while medium with 2.75 mmol/l D-glucose and 8.25 mmol/l L-glucose was used as low osmotic control.

After the indicated times, supernatants were taken off, centrifuged and frozen at −20 °C. After lyophilisation, supernatants were reconstituted in PBS to a 10-fold concentration. Plates were washed once with PBS (4 °C) and stored at −80 °C.

Immunoblot. NAMPT was detected in lysates, supernatants and size exclusion chromatography eluate fractions by using a monoclonal antibody (OMNI379, Axxora, Lörrach, Germany) as described previously [6].

ELISA. NAMPT in lysates and supernatants was quantified using the human intracellular NAMPT and the human extracellular NAMPT/PBEF/visfatin ELISA (AdipoGen, Seoul, South Korea), respectively, according to manufacturer's instructions.

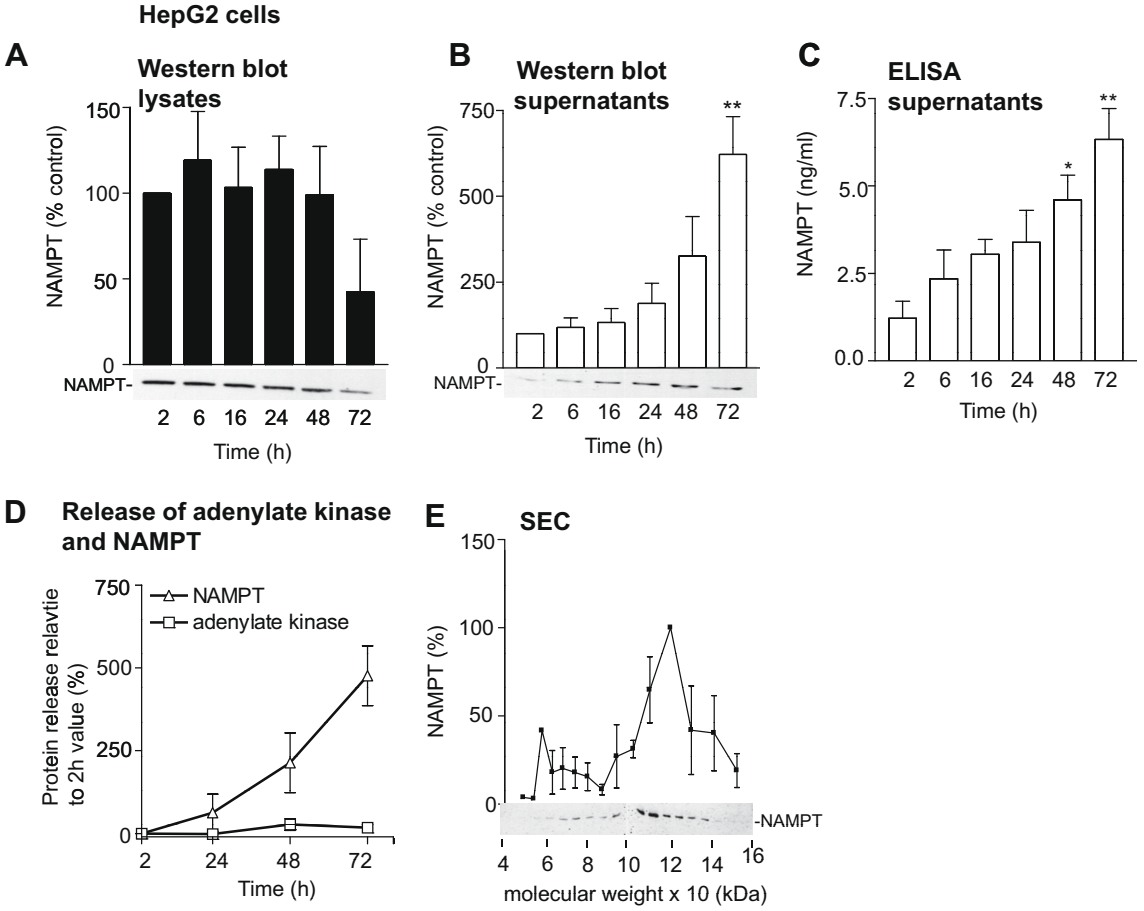

Fig. 1. NAMPT is released into HepG2 culture supernatant in a time-dependent manner. NAMPT content was evaluated densitometrically after immunoblotting in (A) HepG2 lysates and (B) HepG2 supernatants. Data were normalised to 2 h values, which were set 100%. (C) NAMPT concentration quantified by ELISA. (D) Release of NAMPT and adenylate kinase (measure of cell leakage) into the supernatant was monitored by ELISA and enzyme activity, respectively. Differences of NAMPT amount or adenylase kinase activity to the respective reference value at 2 h (set 100%) were plotted for each time point. (E) HepG2 supernatant was fractioned by SEC and analysed densitometrically after immunoblotting. The highest value of each experiment was set 100%. Below graphs in (A, B and E) one representative blot out of three is shown.

Size exclusion chromatography (SEC). HepG2 supernatants were collected after 72 h, frozen and concentrated 10-fold by lyophilisation and reconstitution in PBS + 0.1% BSA. The clarified sample (1 ml) was applied to a Superdex 200 column (16/60) (GE Healthcare, München, Germany) in PBS + 0.1% BSA and eluted with a flow rate of 1 ml/min at 4 °C. The distribution of molecular masses within the eluates of the column was determined by measuring the absorbance of commercially available calibration proteins (Pharmacia-Biotech, Freiburg, Germany). Fractions of 1 ml were frozen, lyophilised, reconstituted in PBS + 0.1% BSA to a 10-fold concentration and subsequently analysed by immunoblotting.

NAMPT enzymatic activity. Measurement of NAMPT enzymatic activity was performed as described previously [18]. For preparation of lysates, 10^7 cells were resuspended in 100 µl 0.01 mol/l $NaHPO_4$ buffer, pH 7.4, frozen at −80 °C for 24 h and thawed at room temperature. Cell debris was removed by centrifugation at 23,000*g*, 90 min, 0 °C. Protamine sulphate solution (1% in $NaHPO_4$ buffer) was added to the supernatant (70 µl/ml supernatant) to precipitate DNA by incubation on ice for 15 min. After centrifugation at 23,000*g*, 30 min, 0 °C, aliquots of the supernatant were stored at −80 °C.

For each measurement, 4 ml of cell culture supernatant were concentrated using Amicon® 4Ultra columns with a molecular weight cut-off at 50 kDa (Millipore, Billerica, MA, USA).

Ten microliters of the lysates or concentrated supernatants were added to 50 µl reaction mix (50 mmol/l Tris–HCl pH 7.4; 2 mmol/l ATP; 5 mmol/l $MgCl_2$; 0.5 mmol/l PRPP; 6.2 µmol/l ^{14}C-nicotinamide; American Radiolabelled Chemicals, St. Louis, MO, USA) and incubated at 37 °C for 1 h. The reaction was terminated by transfer into tubes containing 2 ml of acetone. The whole mixture was then pipetted onto acetone-pre-soaked glass microfiber filters (GF/A Ø 24 mm; Whatman, Maidstone, UK). After rinsing with 2 × 1 ml acetone, filters were dried, transferred into vials with 6 ml scintillation cocktail (Betaplate Scint, PerkinElmer, Waltham, MA, USA) and radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter (Wallac 1409 DSA, PerkinElmer). After subtraction of blank values, NAMPT activity was normalised to NAMPT protein as measured by ELISA. A dose curve of HepG2 supernatant showed increasing activity with increasing supernatant concentration, but deviated from linearity above a certain concentration (Supplementary Fig. 1). Thus, activity was measured within the linear range only. Mouse liver lysate at a concentration of 34.5 µg/ml was used as positive control in each assay.

Statistical analyses. For all cell biology experiments, at least three independent cell culture experiments were performed. Measurements of NAMPT enzymatic activity were conducted in triplicates. Unless otherwise indicated, data are presented as mean ± SEM. Significant differences were determined using GraphPad Prism software and unpaired Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

NAMPT is released from HepG2 cells and hepatocytes

Because of the high content of NAMPT in liver extracts [19] we examined NAMPT expression in and release from HepG2 cells and

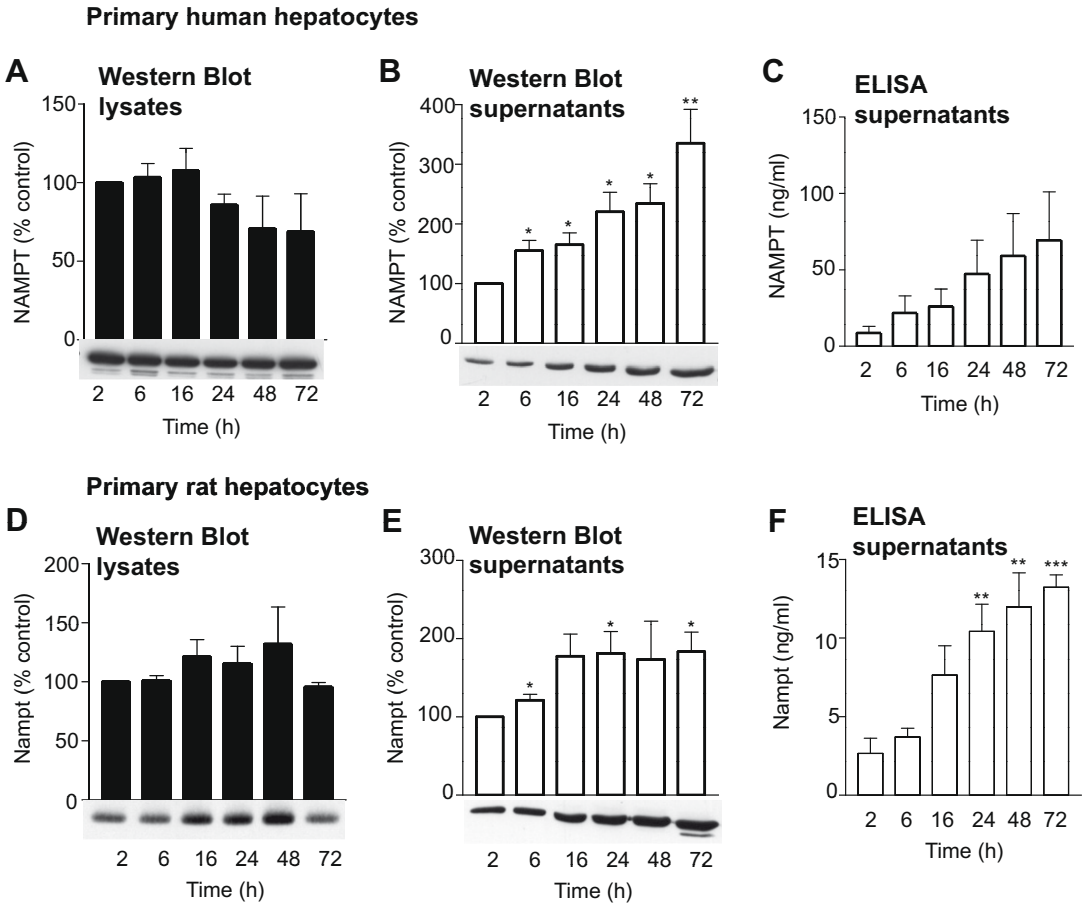

Fig. 2. NAMPT is released from human and rat primary hepatocytes in a time-dependent manner. NAMPT content was evaluated densitometrically after immunoblotting (A, B, D and E). Data were normalised to 2 h values, which were set 100%. NAMPT concentration in the supernatants of (C) human and (F) rat hepatocytes was quantified by ELISA. Below graphs in (A, B, D and E) one representative blot out of three is shown.

primary hepatocytes. NAMPT content in lysates and supernatants of HepG2, rat and human primary hepatocytes were examined after 2, 6, 16, 24, 48 and 72 h of serum-free incubation. In all three hepatocellular models, NAMPT content in lysates did not change significantly over time (Figs. 1A, 2A and D), while it significantly increased in cell culture supernatants as determined by densitometric evaluation of Western blots (Figs. 1B, 2B and E) and by ELISA (Fig. 1C, 2C and F). Cell death monitored by quantifying the activity of adenylate kinase in supernatant of HepG2 cells was found to be neglectable throughout cultivation, while extracellular NAMPT concentration increased continuously (Fig. 1D) indicating release from healthy cells. Similarly, when the amount of NAMPT per million human hepatocytes in lysates and supernatant was compared during a 24 h incubation period, it was clearly obvious that intracellular NAMPT remained almost constant, while secreted NAMPT in the supernatant increased considerably (Supplementary Fig. 2).

Next, we checked whether NAMPT was present as dimer or monomer in culture supernatant (Fig. 1E). HepG2 supernatant was fractionated according to molecular weight by SEC. The greater portion of NAMPT eluted in the fraction corresponding to a molecular weight of 120 kDa which is the approximate molecular weight of the NAMPT dimer. A smaller amount was found in the fraction corresponding to approx. 55 kDa reflecting the molecular weight of the NAMPT monomer.

NAMPT release is independent of classical or ABC-controlled secretion pathways

We tested the impact of brefeldin A, monensin and glibenclamide on NAMPT release from HepG2 cells and primary hepatocytes. NAMPT in lysates and supernatants was detected by immunoblotting. In HepG2 lysates, no changes in NAMPT levels were observed after any of the above mentioned treatments (Fig. 3). Similarly, in HepG2 supernatants no significant down-regulation of release was found with any of the above inhibitors applied for 6 h at various concentrations (Fig. 3A).

Additionally, different concentrations of glucose (Fig. 3B) insulin and TNFα (Fig. 3C) did not significantly alter NAMPT release within an incubation period of 24 h. Similarly, in both rat and human primary hepatocytes, no significant change of NAMPT content in lysates and supernatants in response to glucose, insulin or TNFα was detectable (data not shown).

Hence, NAMPT release from hepatocytes was blocked neither by inhibitors of the classical secretion pathway (brefeldin A, monensin) nor of ABC-dependent secretion (glibenclamide). It was also not influenced by glucose, insulin or TNFα.

Hepatocyte lysates and supernatants exhibit in vitro NMN biosynthetic activity

We examined whether released NAMPT retained its enzymatic activity in culture supernatants. NAMPT activity was quantified by measuring formation of the reaction product ^{14}C-NMN and was normalised to the amount of NAMPT (ng) in the reaction sample as determined by ELISA. NAMPT activity was measured in human hepatocyte and HepG2 cell lysates and supernatants after 6 h of cultivation (Fig. 4). Since the activity in unconcentrated culture supernatants was below detection limit, concentrated supernatants with an average NAMPT concentration of 900 ng/ml (human hepatocytes) and 65 ng/ml (HepG2) were used.

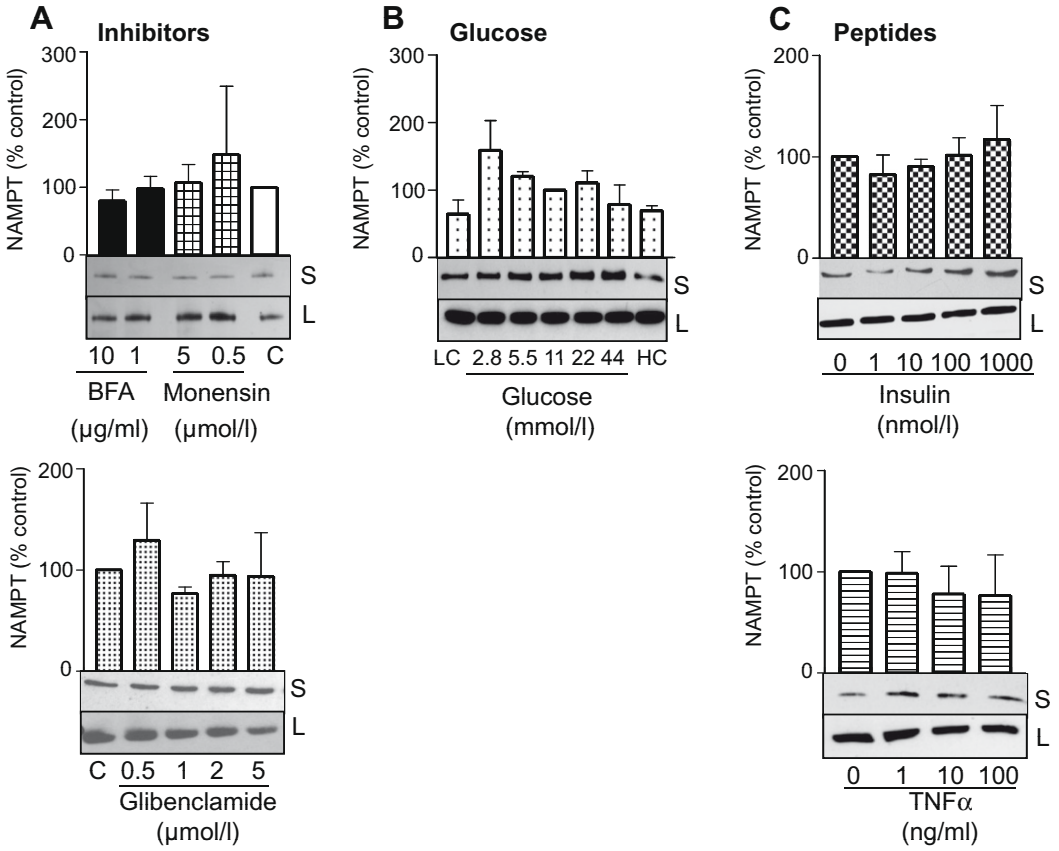

Fig. 3. NAMPT release is independent of classical and ABC-controlled secretion pathways and not regulated by insulin, TNFα and glucose. HepG2 cells were incubated with the indicated doses of (A) brefeldin A, monensin or glibenclamide for 6 h, (B) glucose for 24 h or (C) insulin or TNFα for 24 h. Supernatants were analysed densitometrically after immunoblotting. Data were normalised to (A) vehicle control, (B) glucose content of normal culture medium (11 mmol/l) and (C) normal culture medium values, which were set 100%. Below each graph, one representative blot out of three is shown. S, supernatant; L, lysate; C, vehicle control; LC, low osmotic control; HC, high osmotic control.

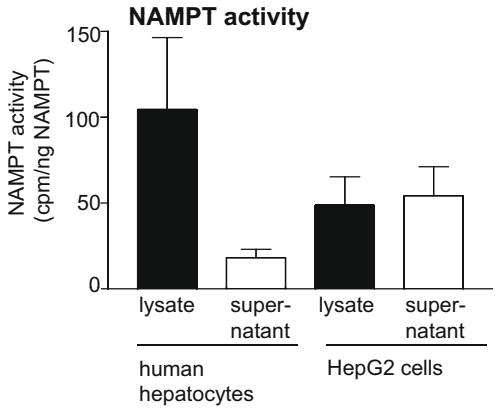

Fig. 4. Lysates and concentrated supernatants of human primary hepatocytes and HepG2 cells exhibit *in vitro* NAMPT enzymatic activity after 6 h of culture. Specific NAMPT activity is depicted in counts per minute (cpm) of the reaction product ^{14}C-NMN per ng of NAMPT.

In human primary hepatocytes NAMPT was shown to be enzymatically active in supernatants, although activity was somewhat lower than in lysates. In HepG2 cells NAMPT activity tended to be equal in supernatants and lysates (Fig. 4). In order to exclude the presence of an inhibitor of NAMPT activity in the supernatant of the cells, we added mouse liver lysate to concentrated HepG2 and human hepatocyte supernatants and measured NAMPT activity in comparison with supernatant or mouse liver lysate alone. As shown in Supplementary Fig. 3, we found no difference in NAMPT activity between mouse liver lysate with or without supernatant.

In summary, NAMPT enzymatic activity was present in lysates and supernatants of hepatocytes.

Discussion

In this study we examined expression, release and enzymatic activity of NAMPT in three different hepatocellular models. NAMPT was found to be released from HepG2 cells as well as primary rat and human hepatocytes. Our findings are in line with two recent studies [20,21] in patients with liver cirrhosis. In these studies, a high abundance of NAMPT protein was found in hepatocytes from normal livers, which decreased in hepatocytes from cirrhotic livers. Likewise, plasma from patients with liver cirrhosis or reduced liver function showed decreased NAMPT levels compared to healthy subjects [20,21]. Taken together, all these findings indicate an important role for human liver tissue in maintaining plasma levels of NAMPT.

NAMPT release from hepatocytes was not blocked by either brefeldin A or monensin as inhibitors of the classical ER–Golgi secretion pathway or by glibenclamide, which inhibits several ABC-dependent pathways of non-classical secretion [13]. These results are in accordance with a former study showing that NAMPT is also not secreted from 3T3-L1 adipocytes via Golgi-derived microvesicles [11]. Thus, the mechanism by which NAMPT is released from adipocytes and hepatocytes remains to be elucidated in further studies.

In contrast to other cell types like adipocytes or the amniotic cell line WISH [10,14], NAMPT release from hepatocytes was not influenced by insulin, glucose or TNFα. Hepatocytes seem to liberate NAMPT in a more constitutive fashion. Other examples for proteins lacking a leader sequence that are secreted constitutively include the metalloendopeptidase EP24.15 and phosphoglucose isomerase/autocrine motility factor/neuroleukin [22,23]. Interestingly, the latter enzyme has a well known dual function, intracellularly in glucose metabolism and extracellularly as a stimulator of growth and motility of tumour cells [23].

Enzymatic activity of NAMPT could be detected in lysates and, with different specific activities, in concentrated cell culture supernatants from HepG2 cells and primary human hepatocytes. Therefore, hepatocytes may release enzymatically active NAMPT into the human circulation. The separation of HepG2 supernatant according to molecular weight revealed that the greater amount of NAMPT occurred as the potential enzymatically active dimer with a minor portion occurring as monomer.

Whether the NMN biosynthetic activity of NAMPT is required for its biological function in serum remains doubtful. A cytokine-like function for extracellular NAMPT has recently been shown, which does not require NAMPT enzymatic activity. For this mode of action, NAMPT is speculated to bind to an as yet unknown receptor [8]. NAMPT released from hepatocytes could possess this cytokine-like activity in addition to an NMN biosynthetic action.

In summary, we have provided evidence that hepatocytes may be a constitutive source of NAMPT contributing to the high level of this versatile factor in human circulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.066.

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