Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2007/07/02/M610357200.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 34, pp. 24574–24582, August 24, 2007

Elevation of Cellular NAD Levels by Nicotinic Acid and Involvement of Nicotinic Acid Phosphoribosyltransferase in Human Cells*[S]

Received for publication, November 7, 2006, and in revised form, June 28, 2007 Published, JBC Papers in Press, June 29, 2007, DOI 10.1074/jbc.M610357200

Nobumasa Hara[‡1], **Kazuo Yamada**[‡], **Tomoko Shibata**[§], **Harumi Osago**[‡], **Tatsuya Hashimoto**[‡], **and Mikako Tsuchiya**[‡]
From the Department of [‡]Biochemistry and [§]Center for Integrated Research in Science, Shimane University Faculty of Medicine, 89-1, Izumo, Shimane 693-8501, Japan

NAD plays critical roles in various biological processes through the function of SIRT1. Although classical studies in mammals showed that nicotinic acid (NA) is a better precursor than nicotinamide (Nam) in elevating tissue NAD levels, molecular details of NAD synthesis from NA remain largely unknown. We here identified NA phosphoribosyltransferase (NAPRT) in humans and provided direct evidence of tight link between NAPRT and the increase in cellular NAD levels. The enzyme was abundantly expressed in the small intestine, liver, and kidney in mice and mediated [^{14}C]NAD synthesis from [^{14}C]NA in human cells. In cells expressing endogenous NAPRT, the addition of NA but not Nam almost doubled cellular NAD contents and decreased cytotoxicity by H_2O_2. Both effects were reversed by knockdown of NAPRT expression. These results indicate that NAPRT is essential for NA to increase cellular NAD levels and, thus, to prevent oxidative stress of the cells. Kinetic analyses revealed that NAPRT, but not Nam phosphoribosyltransferase (NamPRT, also known as pre-B-cell colony-enhancing factor or visfatin), is insensitive to the physiological concentration of NAD. Together, we conclude that NA elevates cellular NAD levels through NAPRT function and, thus, protects the cells against stress, partly due to lack of feedback inhibition of NAPRT but not NamPRT by NAD. The ability of NA to increase cellular NAD contents may account for some of the clinically observed effects of the vitamin and further implies a novel application of the vitamin to treat diseases such as those associated with the depletion of cellular NAD pools.

NAD serves as a coenzyme in cellular redox reactions and is, thus, an essential component of metabolic pathways in all living cells. Numerous recent studies have demonstrated that NAD plays important roles in a variety of biological processes in mammals, such as cell survival and apoptosis (1–9), differentiation (10, 11), and metabolism of carbohydrates (12) and fat (13) through the activity of a longevity factor NAD-dependent histone/protein deacetylase SIRT1. Changes in the cellular NAD level would, thus, have a significant impact on mammal physiology, including humans, and NAD biosynthesis reactions should be tightly regulated; however, the mechanisms regulating the cellular content of NAD remain to be determined.

Mammalian NAD biosynthesis is accomplished through either the *de novo* pathway from tryptophan or salvage pathway from nicotinamide (Nam)[2] and nicotinic acid (NA) (Fig. 1) (14). In the salvage pathway Nam is recycled to NAD by two enzymes, Nam phosphoribosyltransferase (NamPRT, also known as pre-B-cell colony-enhancing factor (15), or visfatin (16)), and Nam mononucleotide (NMN) adenylyltransferase, which convert Nam to NMN and NMN to NAD, respectively. Although Nam has been thought to represent the main precursor of the salvage synthesis to keep cellular contents of NAD constant in mammals (17), the supplementation of Nam does not seem so effective in elevating cellular NAD contents beyond the basal level (18).

NA, the other substrate of the salvage pathway, is converted by NA phosphoribosyltransferase (NAPRT) to NA mononucleotide (NaMN), which is then converted into NA adenine dinucleotide (NaAD), and lastly into NAD (Fig. 1). In mammals, which lack nicotinamidase (17), NA seems to be derived primarily from the extracellular sources. Contrary to Nam, exogenously added NA has been clearly shown to be a better precursor in NAD biosynthesis than Nam and markedly increases NAD levels in mammalian tissues including liver, kidney, and heart in classical studies (19–22). The tissue-specific increase in NAD levels by the addition of NA seems to correlate well with relatively high NAPRT activities in these tissues (23), suggesting an important role of the enzyme in the NA-induced increase in cellular NAD levels. However, whether the enzyme indeed mediates the increases in cellular NAD levels and the role of the NA pathway in regulating biological processes through altering cellular NAD contents remain largely unknown, since the enzyme NAPRT has not been identified molecularly in mammals.

In the present study we identified and characterized human NAPRT molecularly and showed using short interfering RNA (siRNA) knockdown of NAPRT that the enzyme is essential for

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental methods and Figs. 1 and 2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB242230.

[1] To whom correspondence should be addressed. Tel.: 81-853-20-2120; Fax: 81-853-20-2120; E-mail: nhara@med.shimane-u.ac.jp.

[2] The abbreviations used are: Nam, nicotinamide; NA, nicotinic acid; NAPRT, NA phosphoribosyltransferase; NaMN, NA mononucleotide; NaAD, NA adenine dinucleotide; NamPRT, Nam phosphoribosyltransferase; NMN, Nam mononucleotide; PRPP, 5-phosphoribosyl 1-pyrophosphate; ESI-MS, electrospray ionization mass spectrometry; RT, reverse transcription; siRNA, short interfering RNA.

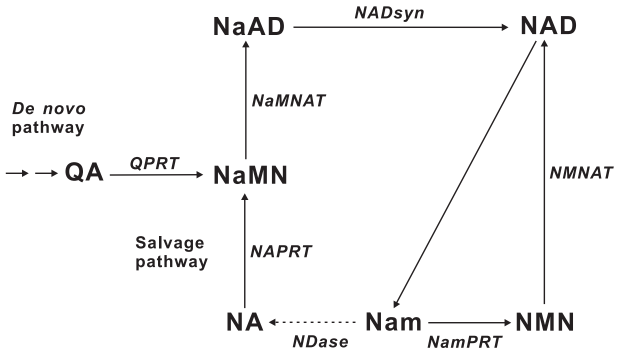

FIGURE 1. **Metabolic pathways of NAD biosynthesis.** *QA*, quinolinic acid; *QPRT*, quinolinic acid phosphoribosyltransferase; *NMNAT*, NMN adenylyltransferase; *NaMNAT*, NaMN adenylyltransferase; *NDase*, nicotinamidase. *NADsyn*, NAD synthetase. *A broken arrow* indicates that the gene encoding nicotinamidase has not been identified in mammals.

the effects of NA to elevate NAD contents in human cells and protect the cells against oxidative stress. Given the critical roles of NAD in regulating cell functions, the strong capability to increase cellular NAD contents of NA, which has long been used for the treatment of hyperlipidemia (24), may have clinical relevance.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham Biosciences. [*carboxyl*-^{14}C]NA (50 mCi/mmol) and [*carbonyl*-^{14}C]Nam (50 mCi/mmol) were from American Radiolabeled Chemical Inc. (St. Louis, MO). NA and Nam were from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. NAD and 5-phosphoribosyl 1-pyrophosphate (PRPP) were from Oriental Yeast (Tokyo, Japan) and Sigma, respectively.

Cell Culture—Human hepatoma HepG2, kidney epithelia HEK293, and cervical carcinoma HeLa cells were cultured in Eagle's minimum essential medium (Sigma) and human promyelocytic HL60 cells in RPMI1640 medium (Sigma). These media were supplemented with 10% fetal bovine serum and antibiotics.

Cloning and Expression of Human NAPRT and NamPRT cDNAs—Full-length human NAPRT cDNA was determined from a candidate sequence in a public data base (GenBank™ accession number AAH06284) and 5′- as well as 3′-flanking parts of the sequence obtained using rapid amplification of cDNA ends (see supplemental methods). Human NAPRT cDNA and the coding region of human NamPRT were ligated into pET22b (Novagen, Madison, WI) and pET15b (Novagen) to produce C- and N-terminal His$_6$-tagged proteins, respectively. The recombinant proteins expressed in *Escherichia coli* BL21 (DE3) cells were purified with His-Bind resin (Novagen) (25). To express human NAPRT in eucaryotic cells as a fusion protein with a His$_6$ tag, human NAPRT cDNA cloned into pcDNA3His$_6$ (25) (pcDNA3-NAPRT) was transfected into the cells as described (25). Sequences of expression plasmids and PCR fragments were confirmed by entire sequencing in both directions.

Knockdown of NAPRT—siRNAs specific for human NAPRT were synthesized against two regions of human NAPRT cDNA (nucleotides 829–1634 and 445–935 for NAPRT siRNA1 and -2, respectively) using X-tremeGENE siRNA Dicer kit (Roche Applied Science). Hypoxanthine-guanine phosphoribosyltransferase specific siRNA, generated using the hypoxanthine-guanine phosphoribosyltransferase template included in the kit, was used as control siRNA. NAPRT and hypoxanthine-guanine phosphoribosyltransferase siRNAs (at final concentrations of 13–26 nM) were transfected into HEK293 cells using X-tremeGENE siRNA transfection reagent (Roche Applied Science). Two days after transfection cells were subjected to assays as indicated. The control siRNA reduced hypoxanthine-guanine phosphoribosyltransferase, but not NAPRT, transcript levels in HEK293 cells (data not shown).

Preparation of Lysates from Culture Cells and Animal Tissues—Cultured human cells were collected and sonicated in buffer containing 0.5 M NaCl, 20 mM Tris-Cl$^-$ (pH 7.5), and 10% glycerol. Tissues were removed from female Wistar rats, washed with 0.9% NaCl, 10 mM Tris-Cl$^-$ (pH 7.5), 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, and protease inhibitor mixture (Roche Applied Science), and homogenized in the buffer. After centrifugation of these homogenates, the supernatants (whole cell lysates or tissue extracts) were subjected to enzyme assay or Western blot analysis as described below.

Enzyme Assays—Unless otherwise stated, NAPRT activity was determined by measuring the formation of NaMN from NA and PRPP using thin layer chromatography (TLC). Enzyme preparations were incubated with [^{14}C]NA (50 mCi/mmol) and PRPP as indicated in standard reaction mixtures (50 μl) containing 50 mM Tris-Cl$^-$ (pH 7.5), 10 mM MgCl$_2$, 2.5 mM dithiothreitol, 1 mM ATP, and 25 μg of bovine serum albumin. After incubating at 37 °C for the indicated times, the reaction was terminated by heating in a boiling water bath for 60 s. Proteins were removed by centrifugation, and reaction products were separated on silica gel sheets (Merck) using an isobutyric acid-5% ammonium hydroxide-water mixture (66:10:19, v/v/v) as a solvent and visualized and quantified using a bio-imaging analyzer BAS 2000 (Fujifilm, Tokyo, Japan).

In some cases recombinant human NAPRT was incubated with 1 mM NA, quinolinic acid, or Nam in the presence of 0.6 mM PRPP in standard reaction mixture at 37 °C for 2 h. The reaction product (NaMN or NMN) was quantified by electrospray ionization mass spectrometry (ESI-MS) as described below.

For kinetic analyses, purified recombinant NAPRT was incubated with the specified concentrations of NA and PRPP in standard reaction mixture with or without NAD as indicated at 37 °C, and the amount of NaMN formed was determined by TLC assay. Kinetic parameters were determined by analysis of a Lineweaver-Burk plot of the initial rate of NaMN synthesis.

For determination of NamPRT activity, purified recombinant NamPRT was incubated with [^{14}C]Nam (50 mCi/mmol) and PRPP in the presence or absence of NAD in the same standard reaction mixture as for NAPRT assay, and NMN formation was determined by TLC assay.

Determination of Molecular Mass of Catalytically Active Human NAPRT—Purified recombinant human NAPRT was electrophoresed on non-denaturing polyacrylamide gel as described previously (25). Gel sliced into 2-mm pieces was incubated with 50 μM [^{14}C]NA and 0.3 mM PRPP in standard reaction mixture (100 μl) at 37 °C for 3 h. The amount of NaMN formed in the reaction mixture was determined by TLC assay.

Determination of NAD Synthesis by Human Cells—Cellular contents of NAD and related compounds were simultaneously determined by ESI-MS analysis, as described previously (26), using a triple quadrupole mass spectrometer (API3000, Applied Biosystems, Foster City, CA).

Based on NAD contents and packed volumes of human cells, basal cellular concentrations of NAD were calculated as 503 ± 104 μM for HEK293 cells, 546 ± 46 μM for HeLa cells, and 597 ± 90 μM for HL60 cells (mean ± S.D. of three separate experiments). HepG2 cells transfected as indicated were incubated with 1 μCi/ml [^{14}C]NA for 6 h. After incubation, pyridine nucleotides were extracted from each cell pellet as described (7), separated on silica gel sheets using the solvent as described above, and quantified by BAS 2000.

mRNA Analysis—NAPRT gene expression was determined in various Balb/c mouse tissues by Northern blot analysis (25) using NAPRT cDNA probe (corresponding to amino acids 200–521 of mouse NAPRT in supplemental Fig. 1) labeled with [α-^{32}P]dCTP. Mouse NAPRT cDNA fragment was amplified from Balb/c mouse tissue total RNA by reverse transcription (RT)-PCR using primers 5′-GTG AGG TGA ATG TCA TTG GC-3′ (sense) and 5′-ACA GTG CGA CCG GAT ACA CT-3′ (antisense).

Western Blot Analysis—Polyclonal anti-human NAPRT antibodies were generated by immunizing a mouse with purified recombinant human NAPRT and purified on the recombinant enzyme blotted to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). NAPRT was immunodetected with anti-human NAPRT and peroxidase-conjugated anti-mouse IgG (MBL, Nagoya, Japan) antibodies, as described previously (27). Protein loading was assessed using rabbit anti-actin (Sigma) and anti-rabbit IgG (MBL) antibodies.

Determination of Cytotoxicity—HEK293 cells untransfected, mock-transfected, or transfected with hypoxanthine-guanine phosphoribosyltransferase or human NAPRT siRNAs were seeded at 2 × 10^4 cells/well in 96-well culture plates and allowed to adhere overnight. Subsequently, the cells were pretreated in Eagle's minimum essential medium with or without exogenously added NA or Nam as indicated and then further incubated in the presence or absence of H_2O_2 together with or without added NA or Nam. After incubation, WST-1 reagent was added to the wells according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan) to determine cellular activity (28). After 2 h of incubation, absorbance at 450 nm was measured via a microtiter plate reader. Cytotoxicity was calculated as described (29). Each assay was performed in triplicate.

RESULTS

Cloning of Human NAPRT cDNA—Based on a candidate sequence of human NAPRT in a public data base (GenBank™ accession number AAH06284), we determined 5′- as well as 3′-flanking parts of the sequence using rapid amplification of cDNA ends and obtained a putative full-length human NAPRT cDNA (GenBank™ accession number AB242230) encoding a highly conserved protein of 538 amino acids (see supplemental Fig. 1). The primary structure of NAPRT did not show significant similarity to the NamPRT.

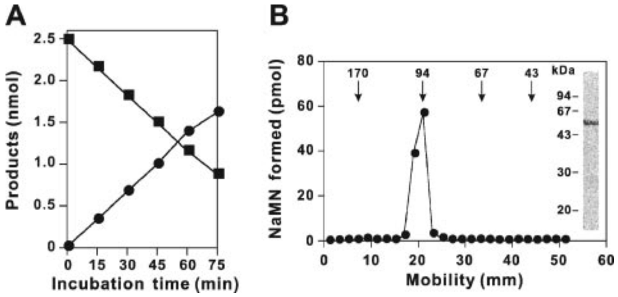

FIGURE 2. **NAPRT activity of recombinant human NAPRT.** *A*, purified recombinant human NAPRT (2 μg) was incubated with 50 μM [^{14}C]NA and 50 μM PRPP for the indicated times, and the amounts of NaMN formed (*circles*) and of remaining NA (*squares*) were determined by TLC assay, as described under "Experimental Procedures." *B*, purified recombinant human NAPRT (3 μg) was subjected to non-denaturing PAGE, and NAPRT activities in gel slices were measured. The proteins used for calibration (positions are indicated by *arrows* with numbers in kDa) were α-macroglobulin (170 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). *Inset* shows SDS-PAGE of the purified recombinant NAPRT (2 μg) on 12.5% gel. Molecular size markers are indicated on the *left*.

Expression and Characterization of Human NAPRT—The protein encoded by the sequence, expressed in *E. coli* as His$_6$-tagged recombinant protein and purified on nickel chelate resin, has a molecular mass of 54 kDa, slightly smaller than the value calculated from the deduced sequence, 59.8 kDa (Fig. 2*B*, *inset*). In human cell lines and rat tissues, antibodies raised against purified recombinant human NAPRT specifically recognized endogenous proteins with a molecular mass of 51 kDa as well as the expressed enzyme (Figs. 3*A* and 4*C*). As shown in Fig. 2*A*, recombinant human protein catalyzed the formation of [^{14}C]NaMN in the presence of 50 μM [^{14}C]NA and 50 μM PRPP, and the amounts of NaMN formed were comparable with those of NA consumption. The omission of either PRPP or Mg^{2+} from the reaction mixture resulted in a complete loss of NaMN synthesis (data not shown). These results indicate that the cDNA encodes NAPRT in humans. Recombinant human NAPRT did not catalyze the formation of NMN or NaMN from Nam or quinolinic acid (data not shown). When purified recombinant NAPRT was fractioned by non-denaturing PAGE and NAPRT activity was determined in gel slices, the activity of human NAPRT had mobility consistent with a protein of 110 kDa (Fig. 2*B*), suggesting that the human enzyme may exist as a homodimer, as described for the native enzyme (30–32).

To investigate whether the human enzyme mediates NAD biosynthesis from NA, we searched for human cell lines where the expression of NAPRT is not detected. We found that this is the case with human hepatoma HepG2 cells (data not shown); NAPRT protein levels and its enzymatic activity were very low in HepG2 cells transfected with vector (Fig. 3*A*). In contrast, HepG2 cells transfected with human NAPRT cDNA exhibited NAPRT activity as well as a protein with a molecular mass of 52 kDa (Fig. 3*A*). The expressed protein was uniformly distributed throughout cells (see supplemental Fig. 2), indicating the localization of human enzyme in cytosol. We cultured these transfected HepG2 cells in the presence of [^{14}C]NA and determined the amounts of ^{14}C-labeled compounds formed in these cells. Analysis of the radioactive compounds in cell extracts by TLC

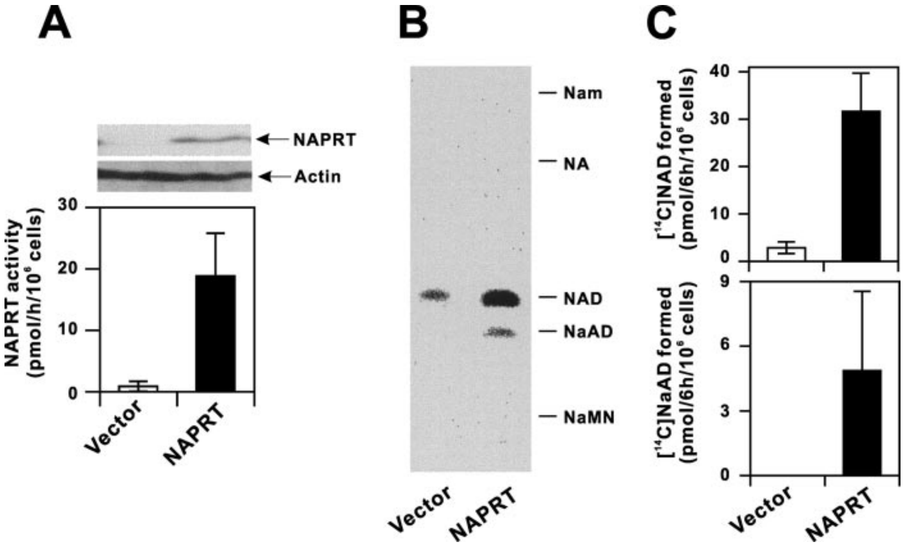

FIGURE 3. **Effects of forced expression of NAPRT on cellular NAD biosynthesis in the presence of NA in human cells.** HepG2 cells were transfected with either pcDNA3His$_6$ (*Vector*) or pcDNA3-NAPRT (*NAPRT*), as indicated. *A*, whole cell lysates from these cells were subjected to Western blot analysis (*top*) with anti-NAPRT or anti-actin antibodies as indicated. The lysates from 2.6–5.5 × 10^5 cells were incubated with 50 μM [^{14}C]NA and 0.3 mM PRPP for 2 h. The amount of NaMN formed was determined by TLC assay (*bottom*). *B* and *C*, HepG2 cells transfected as indicated (1.8–2.4 × 10^6 cells on 6-well dishes) were incubated in the presence of 20 μM [^{14}C]NA (0.75 ml) for 6 h. After incubation, pyridine nucleotides were extracted from these cells, separated by TLC (*B*), and quantified (*C*), as described under "Experimental Procedures." The positions of radiolabeled metabolites (*B*) and amounts of NAD (*C*, *top*) and of NaAD (*C*, *bottom*) formed are shown. Data in this and the following figures represent the mean ± S.D. of at least three separate experiments unless otherwise stated.

revealed that the amount of [^{14}C]NAD was significantly higher in NAPRT cDNA-transfected cells than in those transfected with the vector (Figs. 3, *B* and *C*). [^{14}C]NaAD was observed only in cells expressing human NAPRT (Figs. 3, *B* and *C*). Under these conditions, [^{14}C]NaMN was not detected. The small amount of [^{14}C]NAD observed in vector-transfected cells probably reflects a trace of NAPRT activity in the original cells. All these observations indicate that the cDNA identified here indeed encodes NAPRT protein in human cells and that the enzyme mediates the NAD biosynthesis step from NA in the cells.

Tissue Distribution of NAPRT—To evaluate the tissue distribution of NAPRT, Northern blot analysis was performed with total RNA from various mouse tissues. As shown in Fig. 4*A*, a 1.9-kilobase message was detected in the small intestine, liver, kidney, and heart. Consistent with these results, RT-PCR revealed a higher expression of the NAPRT gene in the former three tissues and a moderate expression in other tissues including the heart (Fig. 4*B*). In rat tissues Western blot analysis revealed the existence of NAPRT protein in the kidney and liver (Fig. 4*C*). Corresponding with these results, robust activity of NAPRT was detected in these two tissues, and significant activity was also detected in other tissues including the heart (Fig. 4*C*). Subcellular fractionation in these tissues demonstrated the presence of endogenous NAPRT protein and enzyme activity in the cytosol (data not shown). NAPRT transcript was observed in the small intestine in mice (Figs. 4, *A* and *B*), but we did not detect NAPRT protein in the small intestine in rats (Fig. 4*C*), probably due to proteolytic degradation of the enzyme during the preparation of tissue extracts.

Addition of NA in Culture Medium Increases Cellular NAD Contents—Knowing that human NAPRT mediates NAD biosynthesis from NA, we next investigated whether the induction of NAD biosynthesis from NA could elevate intracellular NAD levels. HEK293 cells were cultured in the presence of exogenously added NA, and the total cellular contents of NAD as well as related compounds were determined by ESI-MS analysis. As shown in Fig. 5, the addition of NA in culture medium markedly increased the total cellular NAD contents in a dose-dependent manner. As low as 1 μM, NA significantly increased the cellular NAD contents. A nearby 2-fold increase beyond the basal level was observed with 5–10 μM NA. The increase in NAD contents correlated well with the accumulation of NaAD in the cells (Fig. 5). In contrast, corresponding doses of added Nam did not significantly increase cellular NAD levels (Fig. 5), and under these conditions, the amounts of NaAD were below the limit of detection. However, when Nam concentration in culture medium was increased to 5 mM, cellular NAD contents were increased to 131 ± 7% that of the control (mean ± S.D. of three separate experiments), and a small but significant amount of cellular NaAD was detected (7.1 ± 2.7 pmol/10^6 cells, mean ± S.D. of three separate experiments).

To determine whether the increase in cellular NAD contents in the presence of NA is mediated by NAPRT activity, we investigated the effects of the knockdown of NAPRT expression on NAD contents in HEK293 cells. Compared with cells transfected with control siRNA, cells transfected with siRNAs specific for NAPRT exhibited significant decreases in NAPRT enzyme activity as well as NAPRT protein (Figs. 6, *A* and *B*). When these cells were cultured in the presence of NA, the magnitude of increase in NAD (Fig. 6*C*) and NaAD (Fig. 6*D*) contents was markedly reduced only in NAPRT siRNA-treated cells. Knockdown of NAPRT expression did not affect basal NAD contents obtained in the absence of added NA (Fig. 6*C*). These observations indicate that cellular NAD contents can be increased by the addition of NA, but not Nam, at the micromolar range in culture medium and that NAPRT activity mediates increases in NAD contents in human cells.

Human NAPRT Is Not Inhibited by NAD—The observation that NA was a better precursor to increase total NAD contents than Nam in HEK293 cells may be explained by the lack of feedback inhibition of NAPRT, but not NamPRT, by NAD (33–35). To examine this assumption, we carried out complete kinetic analyses in the presence or absence of NAD using the recombinant human phosphoribosyltransferases under the same conditions and directly compared kinetic parameters

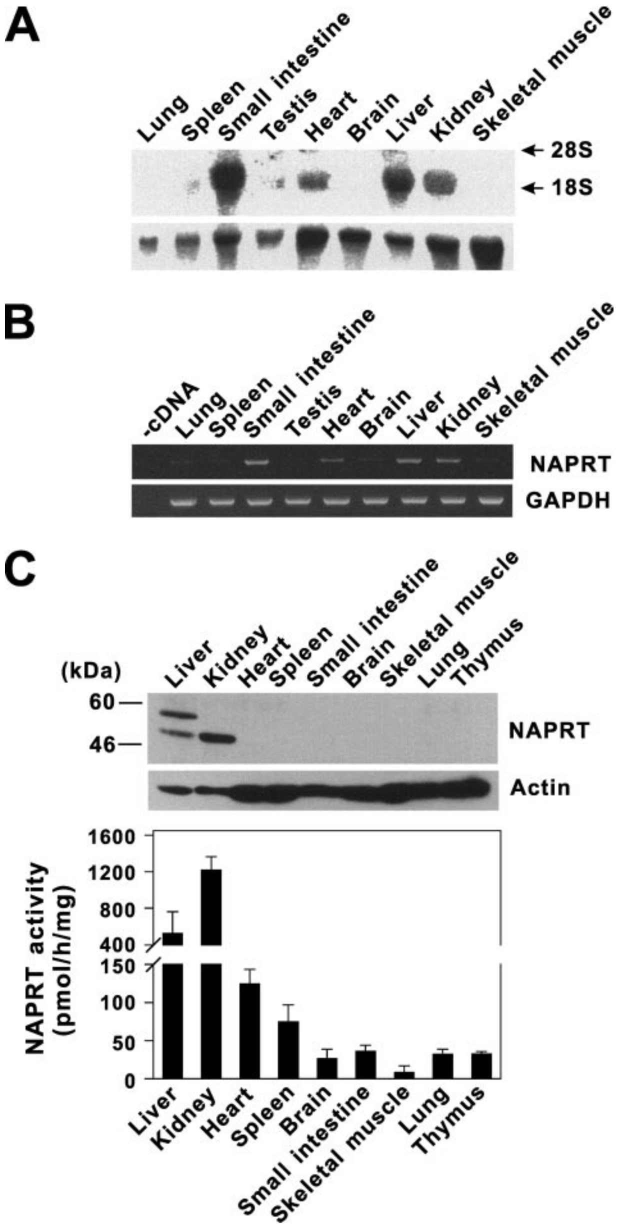

FIGURE 4. **Analysis of NAPRT expression in mouse and rat tissues.** *A*, 10 μg of total RNA from the indicated mouse tissues fractionated on agarose-formaldehyde gel was analyzed using Northern blot hybridization with a radiolabeled mouse NAPRT cDNA (*top*). The blot was then stripped and reprobed with a radiolabeled glyceraldehyde-3-phosphate dehydrogenase cDNA (*bottom*). The positions of 28 and 18 S ribosome RNA are indicated by *arrows*. *B*, RNAs from mouse tissues were subjected to RT-PCR for NAPRT (*top*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, *bottom*). *C*, tissue extracts (70 μg) from rats were subjected to Western blot analysis with anti-human NAPRT antibodies (*top*). The blot was then stripped and reprobed with anti-actin antibodies (*middle*). Molecular weight markers are indicated on the *left*. Tissue extracts (30 μg) from rats were incubated with 50 μM [^{14}C]NA and 0.3 mM PRPP for 1 h, and the amount of NaMN formed was determined by TLC assay (*bottom*).

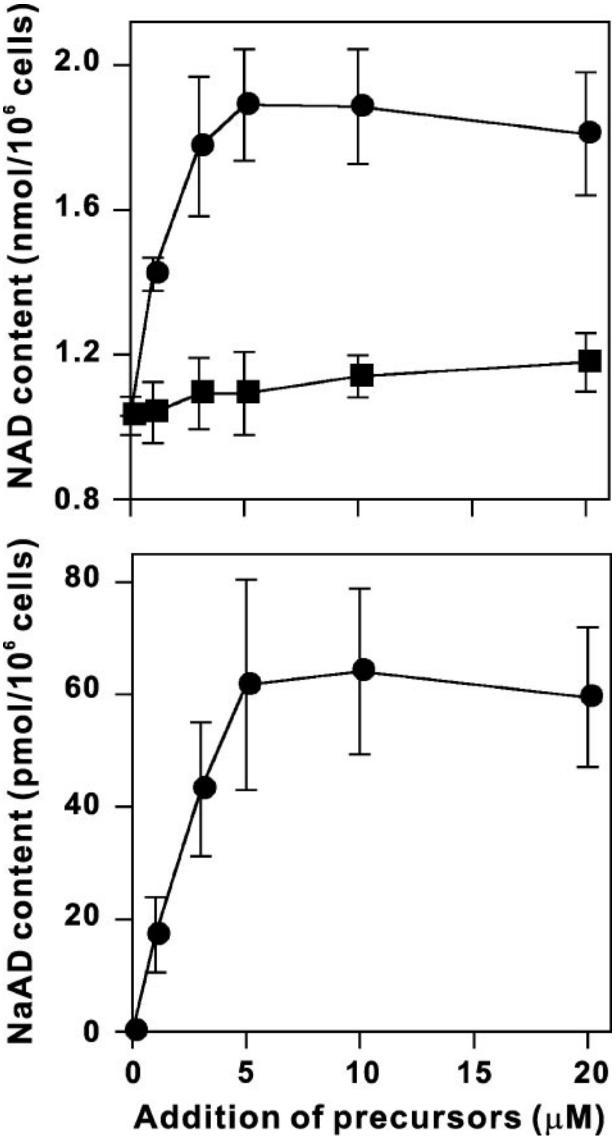

FIGURE 5. **Effects of addition of NA and Nam on total NAD contents in human cells.** HEK293 cells were incubated with indicated concentrations of exogenously added NA (*circles*) or Nam (*squares*) for 6 h. After incubation, total cellular contents of NAD (*top*) and NaAD (*bottom*) were quantified by ESI-MS assay as described under "Experimental Procedures." Amounts of NaAD in the presence of Nam were below the limit of detection (*bottom*). Note that the *abscissa* gives concentrations of NA and Nam added exogenously to the medium, which otherwise contains 8.2 μM Nam.

(V_{max}, K_m, and V_{max}/K_m) for NAPRT reaction with those for NamPRT reaction and the effects of NAD on them. As shown in Table 1, NAPRT activity was not inhibited by NAD, even at 1 mM. On the other hand, NamPRT was markedly inhibited by much lower concentrations of NAD (0.2–0.5 mM), corresponding to basal concentrations of NAD in human cells (see "Experimental Procedures"). Inhibition by NAD was competitive with respect to Nam (21.7-fold increase in K_m for Nam without changing V_{max} in the presence of 0.5 mM NAD) and noncompetitive with respect to PRPP (8.5-fold increase in K_m for PRPP and 5.5-fold decrease in V_{max}; thus, a 46.5-fold decrease in efficiency in the presence of 0.5 mM NAD) (Table 2).

We found differences in some kinetic parameters as well as mechanism of the NAD inhibition between present and previous studies. Although for reactions catalyzed by the recombinant human phosphoribosyltransferases, affinities for NA and Nam as well as maximum rates of catalysis were almost consistent with those reported previously (K_m for NA = 13 μM for hog liver NAPRT (36); K_m for Nam = 0.92 μM for recombinant mouse NamPRT (18); V_{max} = 53 and 21 pmol/min/μg for human erythrocyte NAPRT (31) and recombinant mouse

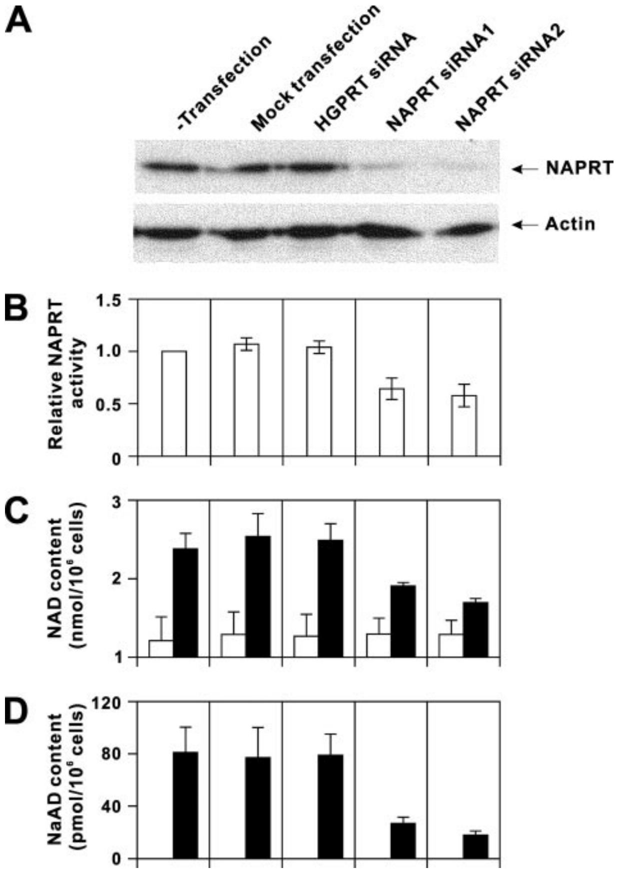

FIGURE 6. **Effects of knockdown of NAPRT expression on NAD biosynthesis from NA in human cells.** *A*, HEK293 cells were not transfected, mock-transfected, or transfected with hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*, control) or human NAPRT siRNAs as indicated. Whole cell lysates from these cells were subjected to Western blot analysis with anti-NAPRT (*top*) or anti-actin (*bottom*) antibodies as indicated. *B*, lysates from 2.3–3.3 × 10^4 cells were incubated with 50 μM [^{14}C]NA and 0.3 mM PRPP for 2 h. The amount of NaMN formed was determined by TLC assay. Activity in the non-transfected cells was set to 1.0, which was 101 ± 29 pmol/h/10^6 cells. *C* and *D*, HEK293 cells transfected as indicated were incubated in the presence (*black bars*) or absence (*white bars*) of 20 μM unlabeled NA for 6 h. After incubation, the total cellular contents of NAD (*C*) and NaAD (*D*) were quantified by ESI-MS assay.

TABLE 1

Effects of NAD on kinetic parameters in NAPRT reaction

Purified recombinant human NAPRT (80 ng) was incubated in the presence or absence of 1 mM NAD with various NA concentrations (from 10 to 70 μM) at a fixed PRPP concentration (0.3 mM) for 30 min. The enzyme (20 ng) was also incubated in the presence or absence of 1 mM NAD with various concentrations of PRPP (from 0.14 to 1 μM) at a fixed NA concentration (40 μM) for 15 min. K_m and V_{max} values represent the mean ± S.D. of three separate experiments. Enzymatic activity was determined using a TLC assay as described under "Experimental Procedures."

NAD	K_m	V_{max}	V_{max}/K_m
mM	*μM*	*pmol/min/μg*	
Variable NA			
0	15.5 ± 3.4	18.6 ± 6.0	1.20
1	12.7 ± 3.6	16.9 ± 6.0	1.33
Variable PRPP			
0	0.25 ± 0.06	23.6 ± 1.5	94.4
1	0.22 ± 0.07	22.6 ± 1.6	102.7

NamPRT (18), respectively), affinities for PRPP were much higher than those reported previously (K_m = 2 and 35.7 μM for hog liver NAPRT (36) and rat liver NamPRT (37), respectively). Furthermore, NAD had been shown to be a noncompetitive inhibitor with respect to Nam (38). These disagreements might arise from differences in enzyme purity and assay conditions.

TABLE 2

Effects of NAD on kinetic parameters in NamPRT reaction

Purified recombinant human NamPRT (60 ng) was incubated with various Nam concentrations (from 1 to 10 μM) at a fixed PRPP concentration (0.3 mM) for 7, 15, 20, and 40 min in the presence of 0, 0.2, 0.3, and 0.5 mM NAD, respectively. The recombinant enzyme (12, 30, 60, and 120 ng) was also incubated with various concentrations of PRPP at a fixed Nam concentration (40 μM) for 7, 20, 20, and 30 min in the presence of 0, 0.2, 0.3, and 0.5 mM NAD, respectively. Concentrations of PRPP varied from 0.14 to 1.5 μM, from 0.5 to 6 μM, from 0.5 to 6 μM, and from 1 to 10 μM in the presence of 0, 0.2, 0.3, and 0.5 mM NAD, respectively. K_m and V_{max} values represent the mean ± S.D. of three separate experiments. Enzymatic activity was determined using a TLC assay.

NAD	K_m	V_{max}	V_{max}/K_m
mM	*μM*	*pmol/min/μg*	
Variable Nam			
0	1.13 ± 0.29	33.9 ± 9.3	30.0
0.2	9.60 ± 2.3	32.5 ± 11.4	3.39
0.3	13.3 ± 3.4	33.6 ± 9.8	2.53
0.5	24.5 ± 8.7	30.2 ± 13.4	1.23
Variable PRPP			
0	0.54 ± 0.10	77.6 ± 19.0	143.7
0.2	2.07 ± 0.59	33.6 ± 7.7	16.2
0.3	3.27 ± 1.1	26.0 ± 4.6	7.95
0.5	4.57 ± 0.64	14.1 ± 1.3	3.09

NA-induced Increase in Cellular NAD Contents Reverses Oxidative Stress-induced Cytotoxicity—Our results indicate that total cellular NAD contents can be maintained at elevated levels by the addition of varying concentrations of NA in culture medium. It has been recently reported that strategies which aim to elevate intracellular NAD levels can protect cells from injury (6, 8, 9). Thus, we finally investigated the effects of adding NA to culture medium on stress-induced cell damage in human cells. HEK293 cells were treated with H_2O_2, and the oxidant-induced cytotoxicity was determined by WST-1 reduction activity (28). Cytotoxicity of nearly 60% was observed after treatment with 50 μM H_2O_2 (Fig. 7*A*). As shown in Fig. 7*A*, NA added to culture medium during treatment reversed the H_2O_2-induced cytotoxicity in a dose-dependent manner. In contrast, the addition of corresponding concentrations of Nam to the culture medium did not protect the cells from the stress (Fig. 7*A*). Determination of cellular NAD contents in the presence of the oxidant in combination with added NA or Nam revealed that NA, but not Nam, protected the decrease in cellular NAD contents induced by H_2O_2 in a dose-dependent manner (Fig. 7*A*). To further investigate whether the effect of NA on the oxidant-induced cytotoxicity is mediated by NAPRT activity, H_2O_2-induced cytotoxicity was determined in NAPRT knockdown cells in the presence or absence of NA. As shown in Fig. 7*B*, the reversal of H_2O_2-induced cytotoxicity together with the increase in NAD contents induced by 5 μM NA was significantly suppressed in NAPRT siRNA-treated cells, where significant decreases in NAPRT enzyme activity as well as NAPRT protein were observed (data not shown). These observations indicate that repletion of cellular NAD pools by NA via NAPRT activity mediates the reversal of oxidative stress-induced cell injury, consistent with recent reports indicating that NA protects cells against damage, possibly through NAD increases (39, 40).

DISCUSSION

We demonstrated here that human NAPRT is an essential enzyme to increase cellular NAD levels by the addition of NA

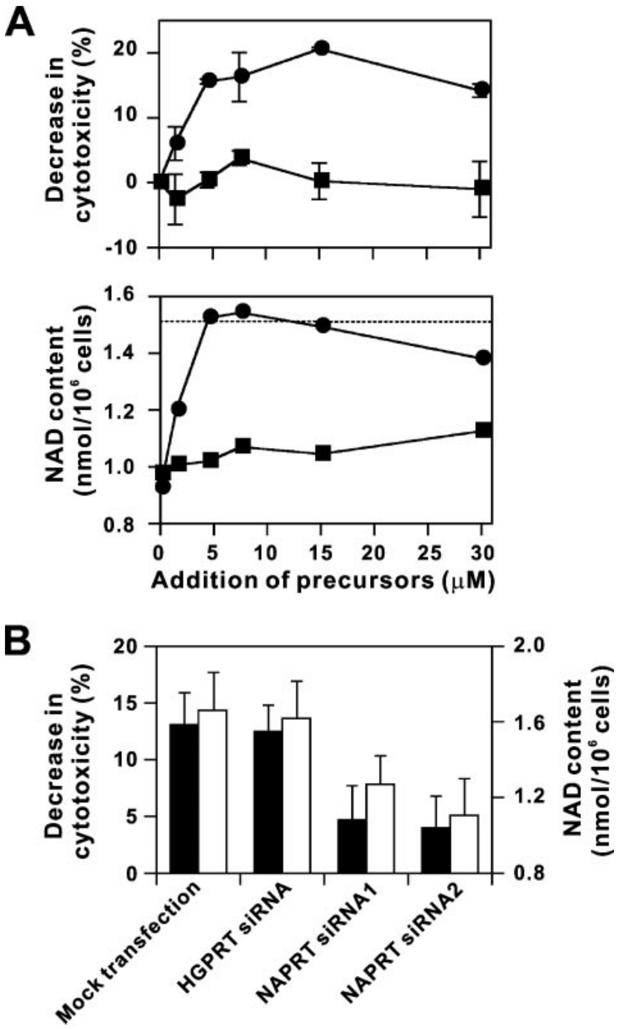

FIGURE 7. **Effects of elevating cellular NAD levels on oxidative stress-induced cytotoxicity.** *A*, HEK293 cells were pretreated in the presence or absence of indicated concentrations of exogenously added NA or Nam for 2 h. After incubation, the cells were cultured in the presence of 50 μM H_2O_2 together with or without the same concentrations of NA (*circles*) or Nam (*squares*) for 3 h. After the addition of WST-1, cytotoxicity was determined as described under "Experimental Procedures" (*top*). Values expressed in the figure are the percent decrease in cytotoxicity. Without added NA or Nam, oxidative stress-induced cytotoxicity was 63 ± 13%. HEK293 cells were similarly treated with 50 μM H_2O_2 in the presence or absence of indicated concentrations of NA (*circles*) or Nam (*squares*) after pretreatment as above. Total cellular NAD contents were determined by ESI-MS assay (*bottom*). The *broken line* indicates basal cellular NAD contents from cells incubated as above but without added H_2O_2, NA, and Nam. Note that the *abscissa* gives concentrations of NA and Nam added exogenously to the medium, which otherwise contains 8.2 μM Nam. Data shown are representative of three separate experiments performed in duplicate. *B*, after pretreatment in the presence or absence of 5 μM NA for 2 h, HEK293 cells mock-transfected or transfected with hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*, control), or human NAPRT siRNAs were cultured in the presence of 30 μM H_2O_2 together with or without 5 μM NA for 3 h. Thereafter, the cells were subjected to determination of cytotoxicity (*black bars*) and NAD contents (*white bars*).

and, thus, to protect the cells from stress. Our molecular characterization of human NAPRT, including detailed kinetic analysis using the recombinant enzyme and detection of NAPRT message and protein, confirms the previously reported results (23, 30–32, 36) and further provides the firm molecular bases to understand the regulation of NAD biosynthesis in mammals. Using siRNA knockdown procedure together with a recently developed highly specific and sensitive quantification method ESI-MS (26), we for the first time directly demonstrated the tight link between NAPRT and cellular levels of NAD and NaAD, indicating crucial roles of NA and NAPRT in NAD metabolism and also in regulation of cell functions via the NAD level in mammals.

The simultaneous quantification of NAD and related compounds with the mass spectrometry revealed that NA added to the culture medium at the micromolar range results in a marked increase in NAD contents and that the increase is associated with the accumulation of cellular NaAD. Furthermore, knockdown of NAPRT expression by RNA interference reduced the elevation of cellular levels of NAD as well as NaAD. Therefore, we conclude that increases in cellular NAD contents upon NA administration are mediated via the NA pathway and that the enzyme plays an essential role in increasing total cellular NAD contents via the pathway. Because the direct product of NAPRT reaction NaMN was not detected under these conditions, the nucleotide seems to be quickly converted to NaAD by the immediate downstream enzyme NaMN adenylyltransferase.

In contrast with NA, Nam did not significantly increase NAD contents at concentrations where NA increases NAD contents and was required at much higher concentrations to increase NAD contents. The Nam-induced increase in NAD contents might be in part ascribed to the action of NA slightly contaminating in Nam preparations on cellular NAD levels, since a small but significant accumulation of cellular NaAD was observed under these conditions. Indeed, we found that Nam preparation contains up to 0.05% NA in itself (data not shown). In studies using Nam at high concentrations such as more than 10 mM (8, 41, 42), effects of the vitamin could be in part due to the contaminating NA and, thus, need to be interpreted carefully. Although Nam has been believed to the major source of basal NAD biosynthesis in mammals (17), our results, thus, suggest that the vitamin is not an efficient precursor for elevating cellular NAD levels in human cells, consistent with a recent study showing that exogenously added Nam did not increase the cellular levels of NAD without overexpression of NAD-synthetic enzymes such as NamPRT (18).

Exogenously added NA induced a marked increase in cellular NAD contents in human cells, whereas Nam added at the same concentrations did not significantly increase NAD contents. Our direct comparison of kinetic parameters for NAPRT reaction with those for NamPRT reaction seems to give a possible reason why NA is a better substrate to elevate cellular NAD levels than Nam. It is likely that the NAD-insensitive activity of NAPRT allows NA to elevate the cellular NAD level beyond the basal level, whereas the strong inhibition of NamPRT by NAD precludes Nam from being used to synthesize NAD further. The inhibition was due to decreases in both affinity for substrates and the maximum rate of catalysis. The K_m value for PRPP in the NamPRT reaction was increased to 4.6 μM by 0.5 mM NAD. Because the cellular concentration of PRPP in human fibroblasts is estimated to be 0.85 μM (43), NamPRT would be much less active than NAPRT in the intracellular milieu containing a basal NAD level of around 0.5 mM, as estimated in the present study. We also observed an increase in K_m value for Nam by NAD; however, since the cellular concentration of Nam has not yet been determined, it is difficult to evaluate the effects of the increased K_m value on NamPRT activity *in vivo*. Our results support an important role of NAPRT for the cellular NAD increase.

We here showed that NA increases cellular NAD levels from the basal of around 0.5 mM to near 1 mM. Because the K_m value for NAD in the SIRT1 reaction has been reported to be about 0.5 mM (44) and, importantly, unlike Nam, NA does not inhibit SIRT1 (45), such increases in cellular NAD levels by NA would significantly stimulate the deacetylase activity of SIRT1 in the cells and modulate a variety of cell functions. Further investigation will be required to test whether the administration of NA indeed stimulates SIRT1 activity in the cells.

The wide variability of NAPRT expression in rodents suggests the presence of tissue-specific pathways of NAD biosynthesis in mammals. In addition to the liver, kidney, and heart, where high NAPRT activity was detected (23), our Northern and RT-PCR experiments revealed the abundant expression of NAPRT in the small intestine. Recent studies show an abundant expression of NaMN adenylyltransferase (46) and NAD synthetase (25) in the small intestine and NamPRT (47) and NMN adenylyltransferase (46) in the skeletal muscle. All of the transcripts described above are found in the liver and kidney (25, 46, 47), whereas quinolinic acid phosphoribosyltransferase was expressed only in the liver and kidney in mice (data not shown). Taken together, it is likely that NAD biosynthesis occurs mainly from NA in the small intestine and from Nam in the skeletal muscle, whereas in the liver and the kidney salvage pathways from both NA and Nam as well as *de novo* pathway from tryptophan contribute to the synthesis. Although the liver and kidney may use both NA and Nam as a precursor of NAD, our results predict that NAD is synthesized from NA more efficiently than Nam in the tissues. Thus, the tissue-specific expression pattern of the enzymes involved in NAD metabolism now provides molecular basis to the concept that the small intestine, liver, and kidney utilize NA as a major precursor for salvage synthesis of NAD, serving as centers for conversion of NA to NAD, then Nam to supply the amide for peripheral tissues (19, 20).

NA has been used for the clinical treatment of hyperlipidemia (24). Therapeutic efficacy of NA in modulating lipid metabolism is thought to stem from its ability to serve as a ligand for a recently identified G-protein-coupled receptor GPR109A (HM74A or PUMA-G) in adipocytes (48); however, not all the effects of NA on whole body metabolism may be mediated via the receptor, since the vitamin seems to affect tissues lacking the receptor including livers (48, 49). Based on our findings, the treatment of livers with NA would increase cellular NAD contents and activate SIRT1. Because SIRT1 has been implicated in regulating the metabolism of fat (13) and carbohydrates (12), the lipid-lowering and blood glucose-elevating effects of NA (48, 49) could be exerted through the activation of SIRT1 in the tissue. In hearts, oxidative stress-induced depletion of cellular NAD pools results in cardiac myocyte cell death (50), but replenishing the NAD pools protects against cell death (9). Taken together with our findings that NAD repletion by means of NA administration protects human cells against oxidative stress-induced injury, the administration of NA would protect myocytes against cell death via elevating their NAD levels, consistent with the cardioprotective role of NA against ischemia-reperfusion injury (51). Further studies on the actions of NA in energy homeostasis and ischemic injury may provide novel insights for the pathomechanism of diabetes and ischemic heart disease.

In conclusion, our findings indicate that NA is a better substrate for elevating cellular NAD levels than Nam in human cells with endogenous NAPRT and that elevating NAD levels via the NA pathway protects the cells against injury such as by oxidative stress. Our findings, thus, document critical roles of the NA pathway in modulating cellular NAD levels and cell functions in human cells. Our current study will not only deepen the understanding of mechanisms regulating cellular NAD biosynthesis in humans but will also provide some insights into the clinical relevance of NA.

REFERENCES

1. Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001) *Cell* **107,** 137–148
2. Vaziri, H., Dessain, S. K., Ng-Eaton, E., Imai, S., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) *Cell* **107,** 149–159
3. Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004) *Cell* **116,** 551–563
4. Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y., Hu, L. S., Cheng, H. L., Jedrychowski, M. P., Gygi, S. P., Sinclair, D. A., Alt, F. W., and Greenberg, M. E. (2004) *Science* **303,** 2011–2015
5. Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A., and Mayo, M. W. (2004) *EMBO J.* **23,** 2369–2380
6. Araki, T., Sasaki, Y., and Milbrandt, J. (2004) *Science* **305,** 1010–1013
7. Hasmann, M., and Schemainda, I. (2003) *Cancer Res.* **63,** 7436–7442
8. Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M. W., and He, Z. (2005) *J. Cell Biol.* **170,** 349–355
9. Pillai, J. B., Isbatan, A., Imai, S., and Gupta, M. P. (2005) *J. Biol. Chem.* **280,** 43121–43130
10. Fulco, M., Schiltz, R. L., Iezzi, S., King, M. T., Zhao, P., Kashiwaya, Y., Hoffman, E., Veech, R. L., and Sartorelli, V. (2003) *Mol. Cell* **12,** 51–62
11. van der Veer, E., Nong, Z., O'Neil, C., Urquhart, B., Freeman, D., and Pickering, J. G. (2005) *Circ. Res.* **97,** 25–34
12. Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., and Puigserver, P. (2005) *Nature* **434,** 113–118
13. Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M. W., and Guarente, L. (2004) *Nature* **429,** 771–776
14. Magni, G., Amici, A., Emanuelli, M., Raffaelli, N., and Ruggieri, S. (1999) *Adv. Enzymol. Relat. Areas Mol. Biol.* **73,** 135–182
15. Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S., and McNiece, I. (1994) *Mol. Cell. Biol.* **14,** 1431–1437
16. Fukuhara, A., Matsuda, M., Nishizawa, M., Segawa, K., Tanaka, M., Kishimoto, K., Matsuki, Y., Murakami, M., Ichisaka, T., Murakami, H., Watanabe, E., Takagi, T., Akiyoshi, M., Ohtsubo, T., Kihara, S., Yamashita, S., Makishima, M., Funahashi, T., Yamanaka, S., Hiramatsu, R., Matsuzawa, Y., and Shimomura, I. (2005) *Science* **307,** 426–430
17. Rongvaux, A., Andris, F., Van Gool, F., and Leo, O. (2003) *BioEssays* **25,** 683–690
18. Revollo, J. R., Grimm, A. A., and Imai, S. (2004) *J. Biol. Chem.* **279,** 50754–50763
19. Collins, P. B., and Chaykin, S. (1972) *J. Biol. Chem.* **247,** 778–783
20. Lin, L.-F. H., and Henderson, L. M. (1972) *J. Biol. Chem.* **247,** 8023–8030
21. Williams, G. T., Lau, K. M., Coote, J. M., and Johnstone, A. P. (1985) *Exp. Cell Res.* **160,** 419–426
22. Jackson, T. M., Rawling, J. M., Roebuck, B. D., and Kirkland, J. B. (1995) *J. Nutr.* **125,** 1455–1461
23. Shibata, K., Hayakawa, T., and Iwai, K. (1986) *Agric. Biol. Chem.* **50,**

3037–3041

24. Carlson, L. A. (2005) *J. Intern. Med.* **258,** 94–114
25. Hara, N., Yamada, K., Terashima, M., Osago, H., Shimoyama, M., and Tsuchiya, M. (2003) *J. Biol. Chem.* **278,** 10914–10921
26. Yamada, K., Hara, N., Shibata, T., Osago, H., and Tsuchiya, M. (2006) *Anal. Biochem.* **352,** 282–285
27. Hara, N., Tsuchiya, M., and Shimoyama, M. (1996) *J. Biol. Chem.* **271,** 29552–29555
28. Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., Ueno, K., and Watanabe, M. (1995) *In Vitro Toxicol.* **8,** 187–190
29. Shingu, T., Yamada, K., Hara, N., Moritake, K., Osago, H., Terashima, M., Uemura, T., Yamasaki, T., and Tsuchiya, M. (2003) *J. Neurosurg.* **98,** 154–161
30. Smith, L. D., and Gholson, R. K. (1969) *J. Biol. Chem.* **244,** 68–71
31. Niedel, J., and Dietrich, L. S. (1973) *J. Biol. Chem.* **248,** 3500–3505
32. Hayakawa, T., Shibata, K., and Iwai, K. (1984) *Agric. Biol. Chem.* **48,** 445–453
33. Dietrich, L. S., Muniz, O., and Powanda, M. (1968) *J. Vitaminol.* (*Kyoto*) **14,** 123–129
34. Keller, J., Liersch, M., and Grunicke, H. (1971) *Eur. J. Biochem.* **22,** 263–270
35. Elliott, G. C., and Rechsteiner, M. C. (1982) *Biochem. Biophys. Res. Commun.* **104,** 996–1002
36. Hayakawa, T., Shibata, K., and Iwai, K. (1984) *Agric. Biol. Chem.* **48,** 455–460
37. Dietrich, L. S., Fuller, L., Yero, I. L., and Martinez, L. (1966) *J. Biol. Chem.* **241,** 188–191
38. Dietrich, L. S., and Muniz, O. (1972) *Biochemistry* **11,** 1691–1695
39. Ogata, S., Okumura, K., and Taguchi, H. (1997) *Biosci. Biotechnol. Biochem.* **61,** 2116–2118
40. Sasaki, Y., Araki, T., and Milbrandt, J. (2006) *J. Neurosci.* **26,** 8484–8491
41. Maiese, K., and Chong, Z. Z. (2003) *Trends Pharmacol. Sci.* **24,** 228–232
42. Alcendor, R. R., Kirshenbaum, L. A., Imai, S., Vatner, S. F., and Sadoshima, J. (2004) *Circ. Res.* **95,** 971–980
43. Rosenbloom, F. M., Henderson, J. F., Caldwell, I. C., Kelley, W. N., and Seegmiller, J. E. (1968) *J. Biol. Chem.* **243,** 1166–1173
44. Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L., Scherer, B., and Sinclair, D. A. (2003) *Nature* **425,** 191–196
45. Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M., and Sinclair, D. A. (2002) *J. Biol. Chem.* **277,** 45099–45107
46. Emanuelli, M., Carnevali, F., Saccucci, F., Pierella, F., Amici, A., Raffaelli, N., and Magni, G. (2001) *J. Biol. Chem.* **276,** 406–412
47. Kitani, T., Okuno, S., and Fujisawa, H. (2003) *FEBS Lett.* **544,** 74–78
48. Offermanns, S. (2006) *Trends Pharmacol. Sci.* **27,** 384–390
49. Karpe, F., and Frayn, K. N. (2004) *Lancet* **363,** 1892–1894
50. Virag, L., and Szabo, C. (2002) *Pharmacol. Rev.* **54,** 375–429
51. Trueblood, N. A., Ramasamy, R., Wang, L. F., and Schaefer, S. (2000) *Am. J. Physiol.* **279,** H764–H771