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 \(^{14}\text{C}\)NAD synthesis from \(^{14}\text{C}\)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 \(\text{H}_2\text{O}_2\). 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 nucleotide (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 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-[32P]dCTP (3000 Ci/mmol) was purchased from Amersham Biosciences. [carboxyl-14C]NA (50 mCi/mmol) and [carboxyl-14C]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 PRPP as indicated in standard reaction mixtures (50 mM) containing 50 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 [14C]NA (50 mCi/mmol) and PRPP as indicated in standard reaction mixtures (50 μl) containing 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 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 NAPRT activity, purified recombinant NAPRT was incubated with [14C]Nam (50 mCi/mmol) and PRPP in the presence or absence of NAD in the 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 [14C]NA and 0.3 mM PRPP in standard

---

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

---

Human NAPRT and NAD Biosynthesis

-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 [14C]NA (50 mCi/mmol) and PRPP as indicated in standard reaction mixtures (50 μl) containing 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 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 NAPRT activity, purified recombinant NAPRT was incubated with [14C]Nam (50 mCi/mmol) and PRPP in the presence or absence of NAD in the 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 [14C]NA and 0.3 mM PRPP in standard
Human NAPRT and NAD Biosynthesis

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 [14C]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-32P]dCTP. Mouse NAPRT cDNA fragment was amplified from Balb/c mouse tissue total RNA by reverse transcription (RT)-PCR using primers 5′-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 recombinant human NAPRT and purified on the recombinant protein. Western blot analysis was performed in triplicate. 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. 3A). In contrast, HepG2 cells transfected with human NAPRT cDNA exhibited NAPRT activity as well as a protein with a molecular mass of 52 kDa in HepG2 cells transfected with vector (Fig. 3A). 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 [14C]NA and determined the amounts of [14C]-labeled compounds formed in these cells. Analysis of the radioactive compounds in cell extracts by TLC...
revealed that the amount of [14C]NAD was significantly higher in NAPRT cDNA-transfected cells than in those transfected with the vector (Figs. 3, B and C). [14C]NaAD was observed only in cells expressing human NAPRT (Figs. 3, B and C). Under these conditions, [14C]NaMN was not detected. The small amount of [14C]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. 4A, 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. 4B). In rat tissues Western blot analysis revealed the existence of NAPRT protein in the kidney and liver (Fig. 4C). 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. 4C). 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. 4C), 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/10⁶ 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. 6C) and NaAD (Fig. 6D) 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. 6C). 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.
(\(V_{\text{max}}\), \(K_m\), and \(V_{\text{max}}/K_m\)) 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 \(V_{\text{max}}\) 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 \(V_{\text{max}}\); 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 \(\mu\)M for hog liver NAPRT (36); \(K_m\) for Nam = 0.92 \(\mu\)M for recombinant mouse NamPRT (18); \(V_{\text{max}}\) = 53 and 21 pmol/min/\(\mu\)g for human erythrocyte NAPRT (31) and recombinant mouse...
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 mM, from 0.5 to 6 mM, from 0.5 to 6 mM, 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 (μM) | V_max (pmol/min/μg) | V_max/K_m (pmol/min/μM) |
|-------|----------|---------------------|-------------------------|
| Variable Nam |          |                     |                         |
| 0     | 1.13 ± 0.09 | 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                    |

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 disagree-

ments might arise from differences in enzyme purity and assay conditions.

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. 7A). As shown in Fig. 7A, 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. 7A). 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. 7A). 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 knockout cells in the presence or absence of NA. As shown in Fig. 7B, 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 depletion 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
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 were 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 be 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 concentra-
tion of Nam has not yet been determined, it is difficult to evaluate the effects of the increased \( K_{m} \) value on NamPT activity in vivo. Our results support an important role of NAPRT for the cellular NAD increase.

We 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 adenyltransferase (46) and NAD synthetase (25) in the small intestine and NamPT (47) and NMN adenyltransferase (46) in the skeletal muscle. All of the transcripts described above are found in the liver and kidney (25, 46, 47), whereas quinolinate 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., Divecchia, N., Lennieux, 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. (2005) Cancer Res. 65, 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., Isabatan, A., Imai, S., and Gupta, M. P. (2005) J. Biol. Chem. 280, 43121–43130
10. Fulco, M., Schultz, R. L., Jezi, S., King, M. T., Zhao, P., Kashiyawa, 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, I. T., Liner, 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., Akio, M., Ohzubou, T., Kitahara, S., Yamashita, S., Makishima, M., Funahashi, T., Yamanaka, S., Hiratsuka, R., Matsuura, 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. Biol. Chem. 270, 1455–1461
23. Shibata, K., Hayakawa, T., and Iwai, K. (1986) Agric. Biol. Chem. 50, 170–174
24. Hori, M., Makishima, M., Funahashi, T., Yamanaka, S., Hiramatsu, R., Matsuzawa, K., Tanabe, E., Takagi, T., Akiyoshi, M., Ohtsubo, T., Kihara, S., Yamashita, S., Satoh, K., Matsuki, Y., Murakami, M., Ichisaka, T., Murakami, H., Wa-
