Transient downregulation of protein *O-N*-acetylglucosaminylation by treatment of high-dose nicotinamide in human cells

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Abstract

Nicotinamide at millimolar concentrations affects cell survival in various conditions, and is being utilized therapeutically in many human diseases. However, the effect of an acute treatment of nicotinamide at such high dose on gene expression and cellular metabolism has rarely been determined previously. In this study, we found that levels of *O-N*-acetylglucosamin(*O*-GlcNAc)ylated proteins including Sp1 acutely decreased upon treatment of 10 mM nicotinamide. Concomitantly, Sp1 protein level decreased rapidly through accelerated proteasome-mediated proteolysis. Co-treatment of glucosamine or 2-deoxyglucose, which inhibits protein deGlcNAcylation, effectively blocked the decrease induced by nicotinamide. Interestingly, the decline in the levels of Sp1 and protein *O*-GlcNAcylation was only transient lasting for two days post treatment, and this pattern matched closely the rapid fluctuation of the cellular [NAD$^+$]. Our results suggest a possible link between cellular nicotinamide metabolism and protein *O*-GlcNAcylation, and an existence of cellular [NAD$^+$] homeostasis.

Keywords: acetylglucosamine; cyclin-dependent kinase inhibitor p21; glycosylation; NAD; nicotinamide; SIR1 protein, human; Sp1 transcription factor

Introduction

Nicotinamide, a derivative of vitamin B3, is converted to NAD$^+$ through the salvage pathway once taken up in cells (Liu *et al.*, 1982). Administration of nicotinamide positively affects survival of a variety of cell types (Li *et al.*, 2004). Recent studies suggest promising therapeutic effect of nicotinamide in a wide variety of models of human diseases such as inflammatory vulgaris, diabetics, psoriasis, and bullous pemphigoid (Li *et al.*, 2004). It also protects brain cells from oxidative damage caused by reperfusion after ischemic infarction (Mokudai *et al.*, 2000), and neuronal and vascular cells against nitric oxide, anoxia, trauma, endotoxic shock, or oxygen/glucose deprivation (Maiese and Chong, 2003). Application of nicotinamide as a possible therapeutic for human diseases requires a thorough understanding on myriad of its effects and action mechanisms at molecular and cellular levels. However, underlying mechanisms for most of the reported effects of nicotinamide appear to be more complicated than initially presumed, and have rarely been clarified. For example, protection of cells from oxidative stress during reperfusion has been believed to be attributed to the nicotinamide-driven supply of NAD$^+$ which would be otherwise depleted by heavy poly(ADP-ribosyl)-ation of nuclear proteins by PARP-1 (Mokudai *et al*, 2000). However, recent studies indicate that nicotinamide at high doses might exert cytoprotective effect in ways independent of NAD$^+$ supply. It directly inhibits PARP-1 (Virag and Szabo, 2002) and activates Akt (Chong *et al.*, 2004) and thereby may prevent FOXO3a-mediated apoptosis (Skurk *et al.*, 2004). It also has been reported to prevent cell death by directly modulating mitochondrial membrane potential and downregulating caspase- 8, caspase-1 and caspase-3 activities (Kolb and Burkart, 1999).

Nicotinamide also inhibits the activity of SIRT1, an NAD$^+$-dependent histone deacetylase (HDAC) (Luo *et al.*, 2001) responsible for deacetylation of p53 (Vaziri *et al.*, 2001), FOXO proteins (Brunet *et al.,* 2004), and PGC-1 (Rodgers *et al.*, 2005), and thereby affects cellular stress response and metabolism. Furthermore, nicotinamide treatment has been reported to induce GAPDH and glucose-6-posphate dehydrogenase possibly through upregulation of cellular NAD$^+$ level (Yan *et al.*, 1999).

These findings suggest that nicotinamide may affect glucose metabolism.

Protein *O*-GlcNAcylation is a modification by a single N-acetylglucosamine at serine or threonine residues in a variety of proteins including nuclear pore complex proteins, cytoskeletal proteins, and transcription factors such as Sp1 (Hart, 1997). UDP-GlcNAc, the substrate of *O*-GlcNAcylation, is synthesized *de novo* from glucose via the hexosamine biosynthetic pathway. Therefore, protein *O*-glycosylation level may be affected by cellular glucose utilization and possibly be modulated by cellular NAD^+ level.

In a previous study, we found that nicotinamide treatment causes a decrease in p21WAF1 expression (Kang *et al*., 2006). We report here that this attenuation of p21WAF1 expression was at least in part due to nicotinamide-mediated downregulation of Sp1 at protein level. In the nicotinamide-treated cells, *O*-GlcNAylation of cellular proteins including Sp1 was transiently reduced. Concomitant changes in the levels of protein *O*-GlcNAcylation and cellular $[NAD^+]$ suggests a possible link between protein *O*-GlcNAcylation and cellular NAD^+ or nicotinamide metabolism.

Materials and Methods

Chemicals and cell culture

N-acetyl-L-leucinal-L-leucinal-L-norleucinal (ALLN), nicotinamide, SB600125, and 2-deoxyglucose were purchased from Sigma Chemical Co (St. Louis, MO). NIH-H460 cells were purchased from ATCC, and maintained in DMEM containing 10% FBS (Biowhitaker, Walkersville, MD). Normal human fibroblasts explanted from a new-born foreskin was provided by Dr. Sang Chul Park (Seoul Nat'l University, Korea).

Western blotting analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease-inhibitor cocktail (Sigma). 10 or 20 μg protein was applied to Western blotting using antibodies to either p21WAF1 (C-19, HRP; Calbiochem, La Jolla, CA), ERK 1/2(K-23), and Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA), and *O*-GlcNAc residue in *O*-GlcNAcylated proteins (RL-2; Affinity BioReagents, Golden, CO).

RT-PCR

Total RNA was isolated using TRIzol reagent (In-vitrogen, Carlsbad, CA) following the manufacturer's protocol. A total of 5 μg RNA was converted to cDNA by using MuLV reverse transcriptase and oligo(dT) primer (Promega, Madison, WI), and 1/40 volume of the cDNA reaction was applied to PCR using primers for Sp1, IGF-1, and β-actin. The primer sequences will be provided upon request.

Measurement of total $[NAD^+]$ and [NADH]

To determine cellular NAD^+ content, a previously published protocol was used (Wosikowski *et al*., 2002). Briefly, 1×10^6 cells were lysed by adding 200 μl cold $HClO_4$ solution (0.5 M). After 15 min on ice, the extracts were neutralized by adding 61 μl 2 M $KOH/0.2$ M K_3PO_4 (pH 7.5) and spun at 13,000 *g* for 3 min. 10 μl of supernatant or NAD^+ standard diluents were mixed with 150 μl reaction solution, and after incubation for 30 min at 37^oC, absorbance at 450 nm was measured. Reaction solution was composed of 16 μl WST-1, 70 μM 1-methoxy-5methyl-phenazinium methyl sulfate, 20 IU alcohol dehydrogenase (Sigma), 0.32 M ethanol in 64 mM Gly-Gly buffer (pH 7.4). To determine cellular NADH content, cells were lysed in 200 μl 0.02 N NaOH solution containing 0.5 mM L-cysteine for 10 min at 60^oC. 30 μl of the neutralized extract or NADH standard diluents was incubated with 130 μl reaction solution for 60 min at 37^oC, and absorbance at 450 nm was measured.

Results

Nicotinamide-induced downregulation of Sp1 activity

Previously, we have shown that acute treatment of 10 mM nicotinamide downregulates p21WAF1 expression in both normal fibroblasts and cancer cells (Kang *et al*., 2006) as demonstrated with H460 cells in this study (Figure 1A). This downregulation of p21WAF1 was independent of p53 since it occurred in SKOV3 cell, a p53-null carcinoma line (HI Lee, unpublished data). Since the background level expression of p21WAF1 is maintained by Sp1 (Biggs *et al*., 1996), we checked the level of Sp1 expression (Figure 1A). Indeed, Sp1 protein level got lower upon the treatment of 10 mM nicotinamide. And, the expression of IGF-1 receptor, an Sp1-dependent gene, was also suppressed suggesting that Sp1 was functionally downregulated by the treatment. The status of the Sp1 protein level was further checked in a time course study for 72 h in H460 cells. Treatment of 10 mM nicoti-
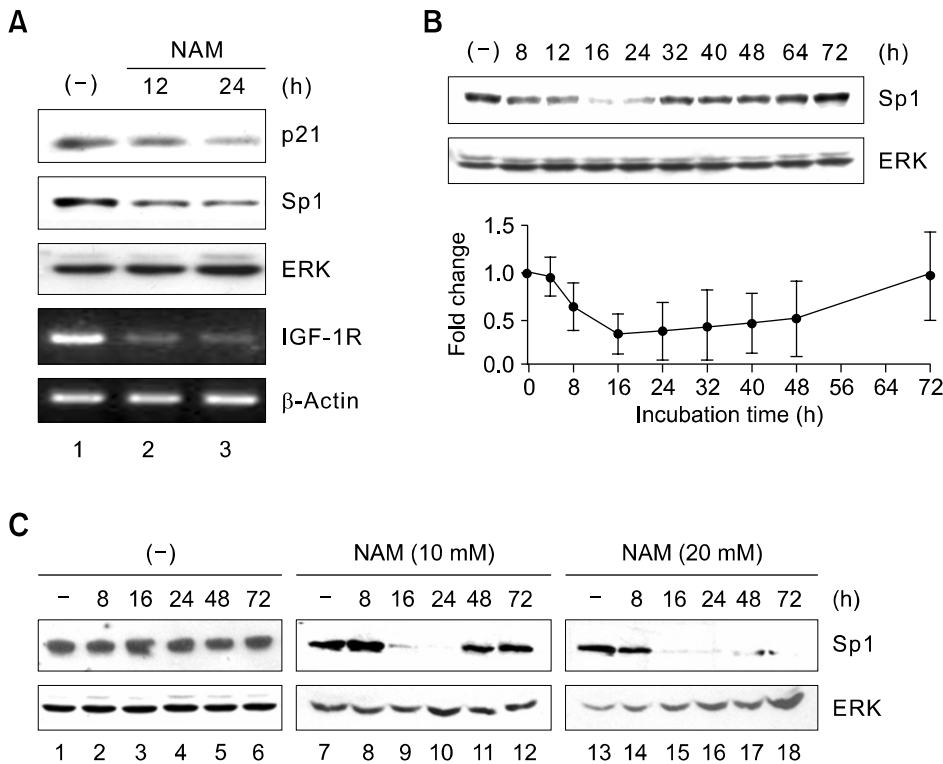
A

B

C

Figure 1. Decreased Sp1 level in cells treated with nicotinamide. (A) H460 cells were treated with 10 mM nicotinamide for 12 or 24 h, and processed either for Western blotting analysis of p21WAF1, Sp1, and ERK proteins, or for RT-PCR using primers for IGF-1 receptor and β-actin. (B) Cells treated with 10 mM nicotinamide, collected at the indicated time points, and extracts were applied to Western blotting for Sp1 and ERK proteins. In the graph below, the band intensities relative to that of untreated cells (fold change) were plotted. The values were from 3 independent experiments. (C) Cells were either mock-treated or treated with nicotinamide at the concentration either of 10 or 20 mM, collected at the indicated time points, and applied to Western blotting analysis.

namide apparently induced a gradual decrease in Sp1 protein level from as early as 8 h post treatment (Figure 1B). Interestingly, the level reached the lowest point (approximately 30-40% of the untreated-cell level) in 16-24 h, and thereafter, increased steadily recovering the level of the untreated cells in around 72 h. On average of three independent experiments, Sp1 level was maintained below 50% from 16 to 48 h post treatment (Figure 1B, lower panel). The severity of the effect appeared to be dose dependent. While the Sp1 level in the cells treated with 10 mM nicotinamide was restored in 72 h, that in the cells treated with 20 mM nicotinamide was not (Figure 1C). And, addition of 10 mM nicotinamide anew at 24 h point was unable to prevent the Sp1 level from returning to the background level (JA Han, unpublished result), suggesting that this recovery in Sp1 protein level is not due to a decrease in the supply of nicotinamide. These results together suggest that nicotinamide treatment induces a substantial but transient decrease in the level of Sp1 protein. Sp1 level did not show such fluctuation in H460 cells cultured in the absence of extra nicotinamide added [Figure 1C (-)].

Accelerated proteolysis of Sp1 in nicotinamide-treated cells

The level of Sp1 mRNA was not affected by

nicotinamide treatment as checked by RT-PCR (Figure 2A) indicating a post-translational mechanism. Sp1 undergoes proteasome-mediated degradation (Han and Kudlow, 1997; Hart, 1997). In fact, when cells were treated with 10 mM nicotinamide together with ALLN, a proteasome inhibitor, Sp1 level did not decrease, and was stably maintained at a level comparable to that of untreated cells (Figure 2B and C). The downregulation in p21WAF1 expression by the nicotinamide treatment was also prevented by the presence of ALLN as shown in Figure 2B. These results suggest that the presence of nicotinamide induces a rapid degradation of Sp1 protein through the proteasome system. Previously, it was reported that the proteasome-mediated Sp1 degradation is accelerated in cells under nutritional stress such as glucose starvation where its *O*-GlcNAcylation of cellular proteins is blocked (Han and Kudlow, 1997; Kudlow and Kennedy, 2006). Therefore, the *O*-GlcNAcylation status of Sp1 was checked. Sp1 protein was immunoprecipitated from the cells treated with 10 mM nicotinamide and then blotted with either anti-Sp1 or anti-*O*-GlcNAc antibody, RL-2 (Figure 2C), and the intensity of the bands was quantified. The bar graph representing the ratio of the intensities of the RL-2 band over the Sp1 band shows that *O*-GlcNAcylation level of Sp1 protein got significantly lower upon nicotinamide
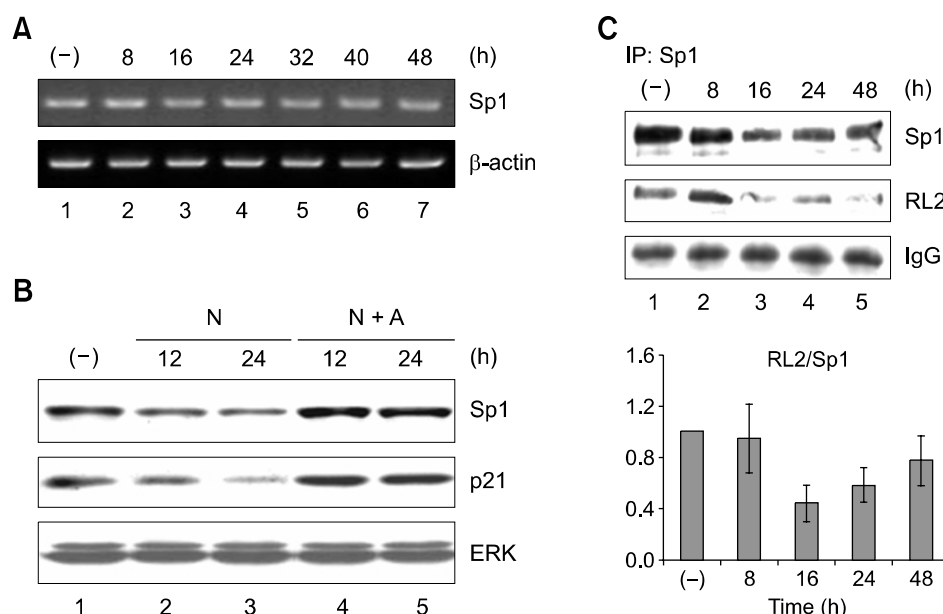
Figure 2. Sp1 downregulation by proteasome-mediated degradation in cells treated with nicotinamide. (A) H460 cells were treated with 10 mM nicotinamide and collected at indicated time points. RNA was isolated and applied to RT-PCR using primers for Sp1 or β-actin. (B) Cells were treated with 10 mM nicotinamide (N) alone or with 25 μM ALLN (N+A) for 12 or 24 h. Cell lysates were applied to Western blotting for Sp1, p21WAF1, and ERK. (C) Cells were treated with 10 mM nicotinamide, collected at the indicated time points, and lysed and applied to immunoprecipitation using the antibody to Sp1. The immunoprecipitates were applied to Western blotting for Sp1, O-GlcNAc (RL-2), or murine IgG. In the graph below, the ratio of the intensities of the RL2 band over that of Sp1 band was calculated, and the values normalized by the value from the untreated cells were plotted. The measurements were made from 2 independent experiments.

treatment reaching the lowest point in 16 h, and thereafter, steadily increased.

Nicotinamide-induced downregulation in O-GlcNAcylation of cellular proteins

Whether O-GlcNAcylation of other cellular proteins as well is affected by nicotinamide treatment was determined by directly blotting total cellular proteins with RL-2. The specificity of RL-2 to the O-GlcNAcylated proteins in whole cell extract has been demonstrated in a previous study by the increased intensity of the bands from the cells treated with streptozotocin, an inhibitor of O-GlcNAcase (Kang et al., 2003). Upon the treatment of nicotinamide, O-GlcNAcylation level of total cellular proteins was rapidly and dramatically reduced in parallel to the change of Sp1 protein level (Figure 3A). Notably, the treatment of ALLN, a specific inhibitor of proteasome, increased the levels of the RL-2-reactive cellular proteins and Sp1 in both the untreated (lane 4) and the nicotinamide-treated cells (lane 3) indicating that the nicotinamide-induced decrease in the levels of the O-GlcNAcylated proteins is due to the increased proteasomal degradation of the poorly O-GlcNA-

cylated proteins as is the case with Sp1. The decrease in the level of the O-GlcNAcylated proteins showed a time-course profile that is identical to that of Sp1 (Figure 3B, NAM). Furthermore, the nicotinamide-induced decrease in both the O-Glc-NAcylation level of cellular proteins and the Sp1 level was blocked by co-treatment of 10 mM glucosamine, a key precursor and stimulator of protein O-GlcNAcylation (Konrad et al., 2000) or 45 mM 2-deoxyglucose, a glucose-derivative that has been shown to inhibit protein de-GlcNAcylation (Kang et al., 2003) (Figure 3B, NAM + GA and NAM + 2-DG). This strongly suggests a possibility of a direct interference by nicotinamide in protein O-GlcNAcylation. Previously, Zhang et al. (2003) reported that nutritional stress such as glucose starvation causes poor O-GlcNAcylation of cellular proteins including Rpt2 ATPase, a subunit of proteasome complex, which leads to proteasome activation. Based on this notion, our results suggest a possibility that Sp1 downregulation in nicotinamide-treated cells was caused by proteasome activation through hypo-GlcNAcylation of its Rpt2 ATPase subunit along with other cellular proteins. Meanwhile, both the levels of Sp1 protein and the GlcNAcylated cellular proteins were maintained

A


```
       (−)  N  N + A  A
                           RL2

                           Sp1

                           ERK1/2
        1   2   3    4
```

C


```
              NAM
       (−)  0  15  30  60   SB
                               p-JNK

                               Sp1

                               RL2

                               ERK
        1   2   3   4   5
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B


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          NAM              NAM + GA            NAM + 2-DG
   (−)  8 16 24 48 72   (−)  8 16 24 48 72   (−)  8  16 24 48 72  (h)

                                                                    RL2

                                                                    Sp1

                                                                    ERK1/2
    1  2  3  4  5  6     7  8  9 10 11 12    13 14 15 16 17 18
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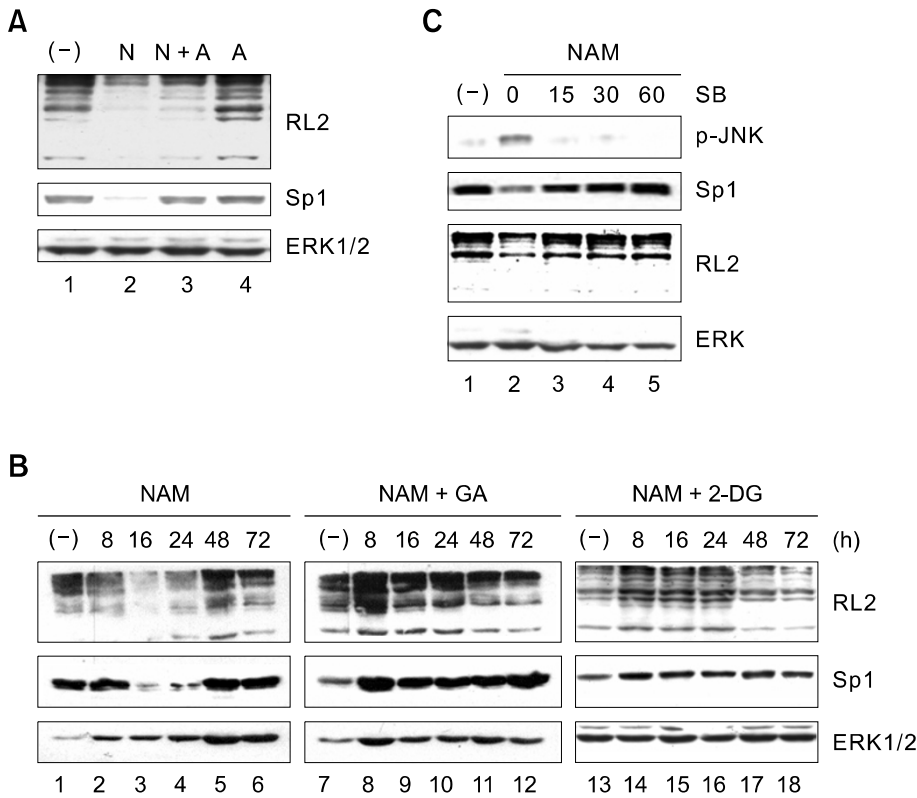
Figure 3. Parallel alterations in the levels of protein *O*-GlcNAcylation and Sp1 in cells treated with nicotinamide. (A) H460 cells were treated with 10 mM nicotinamide (N)(lane 2), 25 µM ALLN (A)(lane 4) alone or together (N + A)(lane 3). After 24 h, cells were lysed and applied to Western blotting for *O*-GlcNAc (RL-2), Sp1 and ERK. (B) Cells were treated with 10 mM nicotinamide alone (NAM) alone (lanes 2-6), or together with 10 mM glucosamine (NAM + GA)(lanes 7-12) or 45 mM 2-deoxyglucose (NAM + 2-DG)(lanes 13-18). At indicated time points, cells were collected, and extracts were applied to Western blotting analysis. (C) Cells were treated with 10 mM nicotinamide alone (lane 2), or together with 15, 30, or 60 mM SB600125 (lanes 3, 4, and 5). After 24 h, cells were lysed and applied to Western blotting using phosphorlated JNK (to show effectiveness of SB600125 treatment), RL-2, or antibodies to Sp1 or ERK.
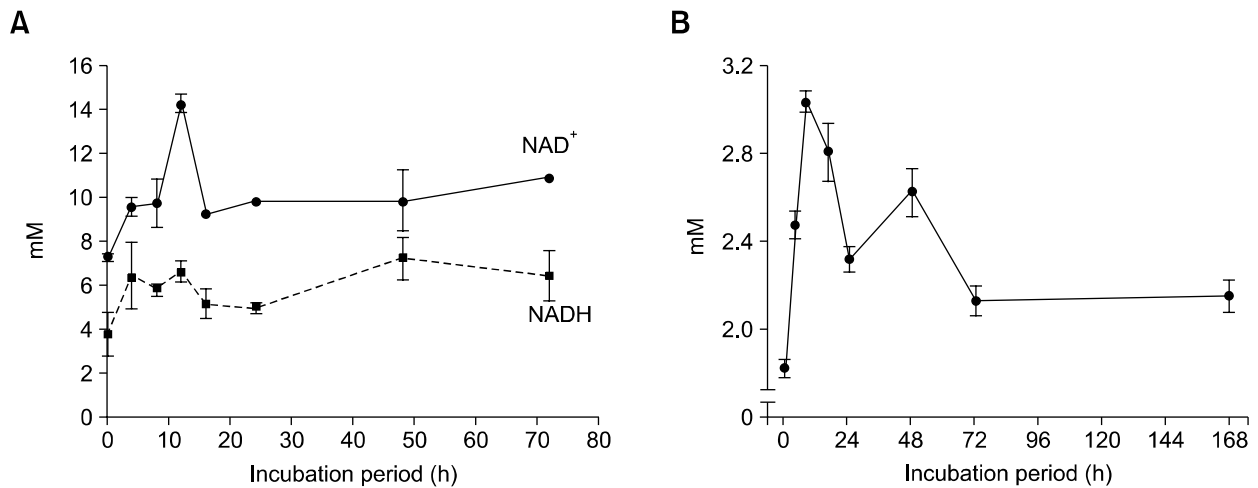
A

B

Figure 4. Rapid up and down of total NAD$^+$ level in cells treated with nicotinamide. H460 cells (A) and normal human fibroblasts (B) were treated with 10 mM nicotinamide. At indicated time points, cells were collected and cellular levels of total NAD+ (—) and NADH (----) were measured. Mean values from the measurement of 3 independent experiments were plotted.

high when cells were co-treated with SB600125, an inhibitor of c-Jun N-terminal kinase (Ventura *et al.*, 2006) (Figure 3C). In this experiment, inhibitors of several kinases involved in various cellular signal transduction pathways were tested in order to determine possible involvement of a signaling pathway. Among the tested, SB600125 substantially blocked the effect of nicotinamide, suggesting an involvement of a JNK-mediated signaling in the effect of nicotinamide.

Rapid fluctuation of NAD$^+$ level upon nicotinamide treatment

Nicotinamide is converted to NAD$^+$ through the salvage pathway (Liu *et al.*, 1982). Therefore, cellular [NAD$^+$] level is expected to rise upon the addition of nicotinamide in the culture medium. Indeed, total cellular [NAD$^+$] increased rapidly approaching near 2-fold level in 12 h (Figure 4A). However, the level suddenly dropped thereafter, and maintained at levels slightly higher than that of the untreated cells. The reason for this sudden decrease in [NAD$^+$] is not clear, but not an isolated phenomenon of H460 cells since this pattern of rapid up and down of cellular [NAD$^+$] at the early time points of nicotinamide treatment was also found in normal human fibroblasts (Figure 4B). And, it apparently is not due to an increased conversion of NAD$^+$ to NADH since the [NADH] changed in a similar pattern. This change in the [NAD$^+$] roughly preceded that of the Sp1 protein (Figure 1B) and the decrease in O-GlcNAcylation of total cellular proteins (Figure 3B) suggesting a possibility that this change in [NAD$^+$] is associated with the alteration in the levels of protein O-Glc-NAcylation. This pattern of [NAD$^+$] change was reproduced in human normal fibroblasts treated with 10 mM nicotinamide (HT Kang, unpublished result).

Discussion

The results of this study show that nicotinamide treatment induces hypo-GlcNAcylation of cellular proteins including Sp1 and causes proteasome-mediated Sp1 degradation. A preliminary result also indicates a possible involvement of the activity of JNK in this effect of nicotinamide, suggesting a cellular event linked to a stress-signaling pathway. Interestingly, the effect of nicotinamide is transient. With some variation in the severity and the duration in the downregulation of Sp1 level, this transient pattern was also observed in MCF-7 and HeLa cells (data not shown). Possibly due to this transient nature, the effect of nicotinamide itself has not been noticed before, and may not play much significant role in experiments and therapeutic applications.

However, what is note-worthy is the possibility that the nicotinamide metabolism is linked to cellular protein modification, and thereby, to their activity. Earlier, it has been noted that Sp1 stability is regulated by the cellular nutrition level through its O-GlcNAcylation, and therefore, Sp1 has been considered a molecule that links cellular nutritional status and gene expression (Jackson and Tjian,

1988). More recently, however, it has been shown that not the status of O-GlcNAcylation of Sp1 but that of Rpt2 ATPase subunit is what determines the Sp1 stability in cells under nutritional stress (Zhang *et al.*, 2003). Proteasome with a hypo-GlcNAcylated Rpt2 ATPase subunit has high proteasome activity targeting Sp1. Therefore, the decreased level of protein O-GlcNAcylation rather than downregulation of Sp1 may rather be a more profound and direct consequence of the nicotinmaide treatment. And, since an early and transient JNK activation is known to promote cell survival (Ventura *et al.*, 2006), the change imposed in the nicotinamide-treated cells may be a cellular effort to survive under a metabolic stress. The possible role of JNK pathway warrants further investigation.

How does high level nicotinamide cause protein hypo-GlcNAcylation in cells? Protein O-GlcNAcylation is modulated by cellular glucose level (Biggs *et al.*, 1996), and NAD$^+$ is a key molecule regulating glucose metabolism. Therefore, nicotinamide, a precursor of NAD$^+$, may link glucose metabolism to protein O-GlcNAcylation. Upon treatment of nicotinamide, [NAD$^+$] increased quite rapidly reaching a two-fold level in 12 h in H-460 cells. Note-worthily, the level dropped rapidly, and thereafter, was maintained rather steadily. This pattern of [NAD$^+$] change is quite a mirror-image of the changes in the Sp1 level and protein O-GlcNAcylation. Based on these results, a hypothesis may be proposed that an increase in [NAD$^+$] is recognized as a state of poor cellular glucose level, and therefore, cells limit utilization of glucose for protein O-GlcNAcylation. Couple of sketches as for a possible mechanism can be drawn from previous reports. Treatment of 10 mM nicotinamide (or nicotinic acid) has been shown to upregulate GAPDH in Jurkat cells (Yan *et al.*, 1999). High GAPDH activity may drive consumption of glucose through glycolysis rather than through hexosamine biosynthetic pathway (HBP), which converts fructose-6-phosphate to UDP-N-acetylglucosamine for protein GlcNAcylation (Yki-Jarvinen *et al.*, 1997). Or, high level nicotinamide and NAD$^+$ may downregulate HBP by increasing cellular cAMP level through inhibition of cAMP phosphodiesterase (Shimoyama *et al.*, 1975; Dudkin *et al.*, 1983). cAMP and cAMP-dependent protein kinase (PKA)-mediated phsphorylation have been shown to inhibit glutamine:fructose-6-phosphate amidotransferase (GFAT) which produces glucosamine for HBP (Kudlow and Kennedy, 2006). To validate this hypothesis, it needs to be checked if high level nicotinamide indeed causes a decrease in HBP activity.

Why the effect of nicotinamide is transient is another intriguing question. Sp1 downregulation

and protein GlcNAcylation lasted for 36-72 h. And, as protein hypo-GlcNAcylation/downregulation accompanied the increase in cellular [NAD$^+$], their recovery accompanied its decrease, too. If the downregulation was indeed induced by high [NAD$^+$] as postulated above, the effect would lessen when the cellular [NAD$^+$] decreases. Again, a question remains why the [NAD$^+$] declined so rapidly. It is certainly not due to conversion to NADH whose level changed in parallel to that of NAD$^+$. This rapid fluctuation in [NAD$^+$] was observed in many tested cells upon treatment of nicotinamide (HT Kang, unpublished result). And, such a rapid fluctuation in cellular response is not an isolated case. For example, cellular ROS level has been shown to oscillate upon treatment of high-level glucose (Yu *et al.*, 2006). Maybe, cells counteract the increase in [NAD$^+$] and rapidly gain its balance for proper energy metabolism. Abrupt increase in [NAD$^+$] due to exogenous supply would certainly disrupt normal glucose- and energy-metabolism as well as other cellular activities including protein *O*-GlcNAcylation, and therefore, would destroy cellular normality. Therefore, cells may have developed means to rapidly revert from abnormal [NAD$^+$]. This may constitute an important cellular homeostasis function that endows cells proper metabolic as well as stress responses.

Overall, despite a transient nature, nicotinamide treatment causes a significant shift in protein *O*-GlcNAcyaltion and thereby the levels of certain cellular proteins. Further investigation on the action mechanism of nicotinamide would shed light on cellular machinery that regulates the balance of glucose/energy metabolism and protein *O*-GlcNAcyation.

Acknowledgements

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