Supplementation of nicotinic acid and its derivatives up-regulates cellular NAD$^+$ level rather than nicotinamide derivatives in cultured normal human epidermal keratinocytes

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Research Article

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Abstract

Nicotinamide dinucleotide (NAD^+) is an important component for various biological processes in mammalian cells, such as energy production, redox state maintenance, and gene regulation. In most mammalian cells, NAD^+ is produced by vitamin B3, including nicotinamide (NAM) and nicotinic acid (NA). Recently, NAD^+ up-regulation therapy has attracted attention for suppressing the aging processes, called rejuvenation. Although various enzymes participate in the NAD^+ production pathway, some enzymes are lacking in particular cells. Therefore, it is thought that the suitable material for NAD^+ production varies with the types of cells. However, the optimization of the NAD^+-precursor for use in topical formulations has rarely been considered. In this study, we asked which precursor is suitable for application against human skin keratinocytes. As a result, NA supplementation 1.3-fold up-regulated intracellular NAD^+ level significantly, even with a nicotinamide phosphoribosyltransferase inhibitor, FK866, and its metabolites NA mononucleotide also increased NAD^+ level by1.5-fold with 100 μM application. Surprisingly, NAM and its derivatives could not up-regulate cellular NAD^+ levels in keratinocytes. The NA supplementation also up-regulated mitochondrial superoxide dismutase (SOD2), which indicates the effect for mitochondria. NA also alleviated rotenone-induced mitochondrial ROS accumulation. These results suggest that NA can be used for topical application for skin rejuvenation.

Key Points

- Nicotinamide dinucleotide (NAD^+)-precursor and related compounds were supplemented to the culture medium of normal human epidermal keratinocyte.
- Nicotinic acid (NA) but not nicotinamide supplementation, could up-regulate cellular NAD^+
- NA is a useful ingredient for supplements and cosmetics, especially for topical applications for skin rejuvenation.

Introduction

Nicotinamide adenine dinucleotide (NAD^+) is one of the essential materials for cellular maintenance, such as energy metabolism, redox regulation, and gene regulation [5, 7]. The low NAD^+ levels are linked to multiple diseases, including metabolic and neurodegenerative diseases, and now correlate with inflammation and aging, called inflammaging [7]. Primarily, NAD^+ synthetic pathway starts from an amino acid, tryptophan (TRYP), called the de novo pathway (Fig. 1A). TRYP is sequentially metabolized by seven enzymes, but some enzymes are inactivated in accordance with human organs [4]. All the enzymes that participate in this pathway are active only in the liver, so the peripheral cells like keratinocytes are considered that they cannot use the *de novo* pathway. NAD^+ produced by the liver is thought to be delivered to peripheral cells by the circulation of the bloodstream. Because NAD^+ is a large and hydrophilic molecule, it is difficult to penetrate into cells. Recently, it has been reported that the extracellular NAD^+ is degraded to nicotinamide (NAM) by membrane-bound enzyme, cluster of

differentiation (CD) 73, and that NAM is used as a material for intracellular NAD^+ production [7, 18]. Generally, NAD^+ production from NAM, called the primary-salvage pathway, is the mainstream of NAD^+ production in peripheral cells. In addition to the primary-salvage pathway, the Preiss-Handler pathway is also important for cellular NAD^+ metabolic pathways. This pathway starts from nicotinic acid (NA). NA is metabolized by nicotinic acid phosphoribosyltransferase 1 (NAPRT1) to nicotinic acid mononucleotide (NAMN) and merged into the *de novo* pathway. The produced NAD^+ is used by many proteins that participate in the various biological processes, such as sirtuins, CD38, and poly(ADP-ribose) polymerases (PARPs), which result in the liberation of NAM (So, the pathway is called salvage). Nicotinamide riboside (NR) is an additional salvage precursor with a two-step [3] or three-step [1] to form NAD^+ (Fig. 1A). This pathway is called the NRK pathway [4, 19]. To date, oral supplementation of NMN and NR has been well studied and commercially available for health maintenance [7]. The availability of these three NAD^+-precursors, NAM, NA, and NR, depends on the cell type [3]. However, the precursor that is efficient for the up-regulation of cellular NAD^+ levels to apply to the epidermal keratinocytes has not been fully understood.

In this study, we asked which NAD^+-precursor is suitable for topical application in promoting NAD^+ on the epidermal keratinocytes.

Materials And Methods

Chemical reagents

NA, NAM, and NAMN were purchased from Sigma (St. Louis, USA). NMN, NR, and NAR were purchased from TCI (Tokyo, Japan), Hangzhou J&H (Hangzhou, China), and Tronto Research Chemicals (Tronto, Canada), respectively. FK866 was purchased from BLD Pharmatech Ltd. (Shanghai, China).

Cells and cell culture

Normal human epidermal keratinocytes were purchased from Kurabo (Osaka, Japan) and cultured in a specialized medium (Humedia-KG2) from Kurabo.

Measurement of cellular NAD^+ levels

NHEK cells were subcultured in a 96-well flat-bottom transparent cell culture plate at 5000 cells/well and pre-cultured for one day. Subsequently, the medium was changed to a new medium containing NAD^+-precursors at the indicated concentrations. Six hours later, cells were lysed and the total NAD^+ amount was measured by NAD/NADH-Glo™ Assay (Promega Corp., Madison, USA) according to the manufacturer's protocol. The chemiluminescent signal was measured using SYNERGY HTX (BioTek Instruments, Inc. Winooski, USA).

Immunoblot analysis

The immunoblot analysis was performed as described before [20]. The primary and secondary antibody reactions were performed with an iBind™ Flex Western System (Thermo Fisher Scientific Inc., Waltham, USA). The SOD1, and 2 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, USA). The GAPDH antibody was purchased from proteintech, Inc. (Rosemont, USA). The SOD1 protein was detected using an anti-mouse (NA-931V) IgG secondary antibody (GE Healthcare). The SOD2 and GAPDH proteins were detected using an anti-rabbit IgG secondary antibody (NA-934V; GE Healthcare).

Mitochondrial functions

The 1.25×10^4 cells were inoculated into 8-strip chamber glass plate wells. After preincubation, the cells were treated with 1 μM Rotenone (Cayman Chemical, Ann Arbor, USA) and/or indicated concentrations of NA. Six hours later, the mitochondrial ROS and mitochondrial membrane potential were measured using mtSOX Deep Red and MitoBright LT Green (DOJINDO LABORATORIES, Kumamoto, Japan), respectively. The fluorescence detection was undertaken by FLoid™ Cell Imaging Station (Thermo Fisher Scientific Inc.). The quantification of green and red image pixels was conducted by a python program with Google Collaboratory (Google, Mountain View, USA). The Red/Green ratio was calculated from each pixel.

Statistical analyses

All quantitative data are presented as mean ± standard error (SE). The statistical tests for the difference between two groups or multiple comparisons were conducted using Student's *t*-test (Figs. 3 and 4E), or one-way ANOVA followed by Dunnett's test (Figs. 2, 4B, and C), respectively. The significance level was set as $\alpha = 0.05$.

Results

Nicotinic acid activates NAD^+ production in normal human epidermal keratinocytes.

Mammalian bodies use three metabolic pathways for the production of NAD^+. However, the availability of these pathways is different in cell types (Fig. 1) [3, 22]. To date, the knowledge about which pathway is used by the cultured human keratinocyte is few. To address this question, we evaluated six known NAD^+-precursors (NA, NAMN, NAR, NAM, NMN, and NR) for the NAD^+-production assay. The incubation time was determined as 6 h by preliminary studies (data not shown) and a report by Hara et al. [13], which shows at least 2 h after supplementation of NA can up-regulate NAD^+ levels in HEK293 cells. As shown in Figs. 2A, NA significantly up-regulated the cellular NAD^+ level by 1.3-fold at 10 μM, but high-dose of NA supplementation (30–100 μM) reversed the effect. NAMN also up-regulated the NAD^+ level by 1.5-fold at 30 and 100 μM (but not significant: Fig. 2B). NAR could regulate NAD^+ level slightly positive and showed dose-dependency, 1.1-fold increase at 100 μM (Fig. 2C). These results suggest that the working rate of Preiss-Handler pathway is insufficient in the normal culture medium supplied by the vendor for up-regulation of NAD^+. Contrary, NAM, NMN, and NR could not increase the NAD^+ level (Fig. 2). These results

indicate that the epidermal keratinocytes mainly use the Preiss-Handler pathway under NA-sufficient conditions.

Nicotinic acid recovered FK866-induced NAD^+ depletion in normal human epidermal keratinocytes

To confirm the availability of Preiss-Handler pathway, cellular NAD^+ levels were detected under the NAMPT-blocked condition by 1 nM FK866 treatment with or without supplementation of NA. As a result, FK866 treatment decreased cellular NAD^+ levels by half, then it was recovered to the unblocked level by the treatment with NA (Fig. 1E).

Nicotinic acid supplementation ablates rotenone-induced mitochondrial ROS in normal human epidermal keratinocytes

Because NAD^+ is a transporter of electrons to mitochondria, NAD^+ up-regulation is thought to affect mitochondria. We monitored mitochondrial superoxide dismutase (SOD2) as a marker of effect against mitochondria. As a result, the SOD2 protein level was up-regulated by NA supplementation (Figs. 4A, and C). Contrary, the subcellular SOD enzyme (SOD1) was not significant and not dose-dependent, although a slight increase was observed (Figs. 4A, and B). NAD supplementation also reduced rotenone-induced mitochondrial ROS production (Figs. 4D and E). These results indicate that NAD^+ production can be resistant to mitochondrial damage.

Discussion

Vitamin B3, including NA and NAM, is a pivotal nutrition as precursors of NAD^+. If this vitamin is lacking in the diet, it causes a significant skin disorder causes, called pellagra [7]. NAD^+ was first discovered in 1906 by Arthur Harden, as a required small molecule for alcoholic fermentation, and now, we know NAD^+ is one of the key regulators of various cellular processes [14]. Conrad Elvehjem discovered Vitamin B3 (NA and NAM) as a cure for pellagra, which is a severe skin disease in an era of lacking nutrition science [9]. Simultaneously, Otto Warburg and Hans von Euler connected the Vitamin B3s to its bioactive form, NAD^+. Then, NAD^+-metabolism has been recognized as an important field in health research. After the second half of the century, another vitamin B3, NR, was discovered by Charles Brenner [3, 16]. Recently, NAD^+ supplementation therapy has improved various impairments of our bodies caused by aging. Especially, NMN and NR get our attention to accomplish rejuvenation. Many preclinical experiments and clinical trials have been conducted and successfully improved our body function [7].

Outside the liver, most cells do not express the full array of enzymes necessary to convert TRYP to NAD^+ by *the de novo* pathway [1]. Many types of cells properly select the pathways in each environment to guarantee the pivotal currency of energy i.e., neurons use a part of the *de novo* and the NRK pathways,

adipocytes only use the salvage pathway, immune cells use a part of the *de novo* and the salvage pathway, and intestinal epithelium uses the Preiss-Handler and the salvage pathways [3]. In the same context, an appropriate selection of NAD^+-precursors for upregulating cellular NAD^+ levels on keratinocytes should be considered. Gensler et al. revealed that oral administration of NA at levels that increase skin NAD^+ content inhibits UV-induced carcinogenesis and photoimmune suppression in an animal model [11]. However, the ingredients that are necessary and sufficient for topical applications, such as lotion, cream, and ointment, are poorly understood. In this study, the NAD^+ level was up-regulated by the supplementation of NA and NAMN, which indicates that the Preiss-Handler pathway is active in NHEK cells.

According to the supplier, NAM in the cultured medium is 0.3 μM. To sum up these data and information, 0.3-μM NAM is sufficient for the maintenance of cellular physiological conditions, partly because of its effective recycling system of NAD^+. However, the Preiss-Handler pathway is active but not used in the normal conditions of culture because no NA derivatives are supplemented in the culture medium. Compared to the upper limit of NAM (0.3 μM), the upper limit of NA is about 10–30 μM (Fig. 2A). These results suggest that NA is suitable for the NAD^+ up-regulation therapy for human keratinocytes as topical supplementation compared with NAM. Figure 2A also shows a slight decrease in NAD^+ level in 6 h after treatment with NAM. NAM is known as a negative regulator of SIRT1, which is the key component for the circadian clock [15, 21]. The negative feedback loop may be related to our observation that NAM supplementation causes a slight decrease in cellular NAD^+ level to some extent.

The *in vivo* studies of NMN treatment are administered mainly by oral gavage and intraperitoneal injection. Zhou et al. demonstrated that both types of administration of NMN protect against UVB-induced skin damage in mice [23]. Contrary, NMN supplementation in the medium did not up-regulate NAD^+ levels directly in our study. To sum up, NMN may protect against skin damage indirectly. In the future, *in vivo* topical application studies of UVB-damaged skin should be compared to confirm the results of the present study.

In the present data, the supplementation of NA up-regulated SOD2 protein, which indicates mitochondrial activation (Figs. 4A and C). During aging processes, NAD^+-consuming enzymes increase, which leads to mitochondrial ablation and cellular senescence. Additionally, the deterioration of mitochondrial metabolism in T cells imbalances macrophage types toward a pro-inflammatory M1 state. This process is called inflammaging, and it should be evaded [7]. The up-regulation of SOD2 protein level might have removed mitochondrial superoxide (Figs. 4D and E), resulting in inhibiting these silent changes in mitochondria in the keratinocytes. As reviewed in the ref [4], the appropriate sources of NAD^+-precursor differ by the cell types. Generally, NAM is used for topical applications for removing wrinkles and moisturizing [10]. Conversely, in this study, NA was revealed to act against the epidermal keratinocyte to increase NAD^+ levels, which potentially leads to skin-barrier functions of anti-aging mechanisms. We should combine effective methods to maintain our healthy skin conditions according to our purpose.

Note that NA administration has a risk of niacin flushing, of which cutaneous vasodilation with a sensation of tingling and burning [8, 17]. Recently, NA receptor GPR109A was found in adipocytes and skin Langerhans cells as the cause of niacin flushing [2]. Hanson et al. revealed that keratinocytes also express GPR109A and it was involved in NA and monomethyl fumarate-induced flushing and COX2-dependent prostanoid formation in mice [12]. In our result in Fig. 2A, at least 30 μM NA supplementation conversely decreased the NAD^+ level compared with 10 μM application. This might be a phenomenon related to GPR109A-induced flushing. Regardless of this concern, NA has already been used as 0.01% – 0.1% (ca. 8–80 mM eq) in several cosmetic formulations in USA [6]. The toxicity report analyses by the Cosmetic Ingredient Review Expert Panel revealed that Niacinamide and Niacin are safe in the current practices of use and concentration in cosmetic products [6]. In the future, whether the effect of NA in a safe dose window can affect cellular NAD *in vivo* needs to be estimated.

Conclusion

This study showed that the Preiss-Handler Pathway is the most efficient NAD^+ production system in normal human epidermal keratinocytes. In conclusion, NA is essential nutrition for the maintenance of physiological conditions of skin keratinocytes. This knowledge suggests that NA is a useful ingredient for supplements and cosmetics, especially for topical applications.

Abbreviations

CD, cluster of differentiation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NA, nicotinic acid; NAD^+, nicotinamide adenine dinucleotide; NADS, NAD synthetase; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAR, nicotinic acid riboside; NHEK, normal human epidermal keratinocyte; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyl transferase; NR, nicotinamide riboside; NRK, nicotinamide riboside kinase; PARP, poly(ADP-ribose) polymerase; QPRT, quinolinate phosphoribosyltransferase; SIRT, sirtuin; SOD, superoxide dismutase; TRYP, tryptophan

Declarations

CRediT author statement

Takahiro Oyama: Conceptualization, Validation, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project administration, Data Curation **Takumi Yamamoto**: Investigation, Writing – Review & Editing **Takeshi Kameda**: Investigation, Project administration **Hideaki Abe**: Supervision **Takanori Kamiya**: Writing – Review & Editing, Investigation, Supervision, **Takehiko Abe**: Supervision, and **Sei-ichi Tanuma**: Supervision, Funding acquisition: The authors declare that all data were generated in-house and that no paper mill was used.

Conflicts of interest

The authors declare no conflict of interest

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Compliance with Ethical Standards

Not Applicable

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Figures

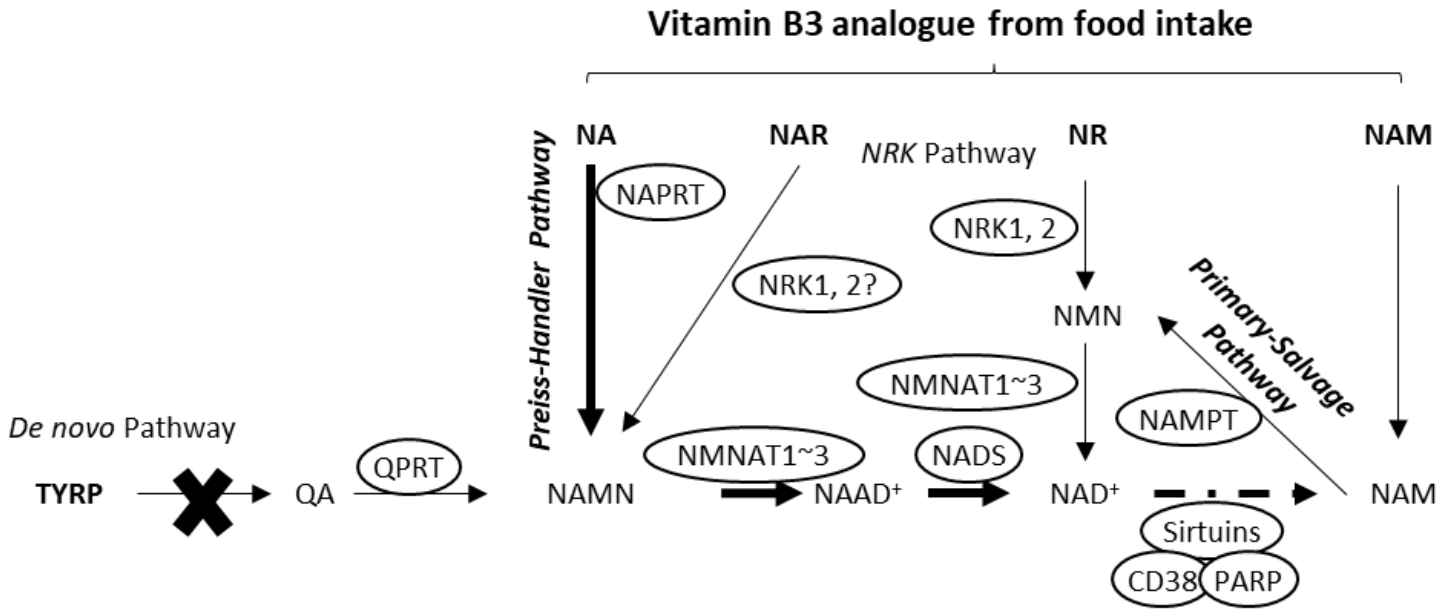

Figure 1

Nicotinic acid activates NAD^+ production in cultured keratinocytes.

(A) The scheme of the central part of NAD^+-metabolic pathways. The enzymes are depicted as circles. Arrows illustrated the synthetic route of NAD^+. The dotted arrow illustrates the catabolic route of NAD^+. The big × mean the metabolic pathway is disturbed by lacking a part of enzyme in that pathway. CD, cluster of differentiation; NA, nicotinic acid; NAD^+, nicotinamide adenine dinucleotide; NADS, NAD synthetase; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyl transferase; NR, nicotinamide riboside; NRK, nicotinamide riboside kinase; PARP, poly(ADP-ribose) polymerase; QPRT, quinolinate phosphoribosyltransferase; SIRT, sirtuin; TRYP, tryptophan

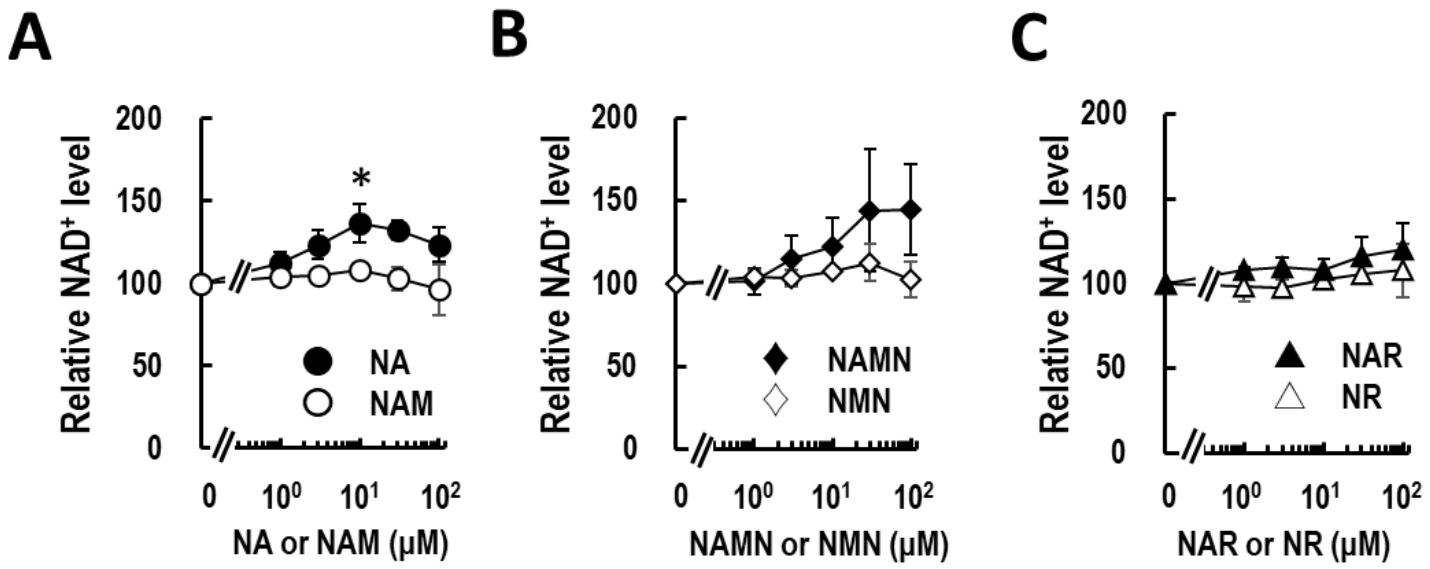

Figure 2

Nicotinic acid activates NAD^+ production in cultured keratinocytes.

The dose-dependent effects of the NA and NAM (A), NAMN and NMN (B), and NAR and NR (C) supplementation on NAD^+ in NHEK cells. The concentrations of the compounds were 1, 3, 10, 30, and 100 μM. The statistical analyses were performed by one-way ANOVA followed by Dunnet's test. All data were expressed as mean ±SE from at least three independent experiments. NA, nicotinic acid; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside;

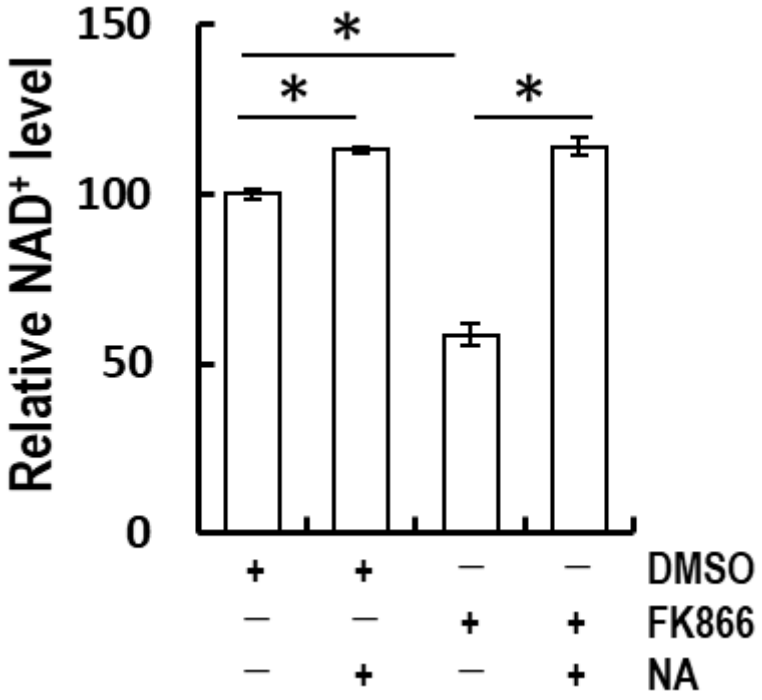

Figure 3

Nicotinic acid recovered FK866-induced NAD^+ depletion in cultured keratinocytes.

Recovery of the depletion of NAD^+ level by the pre-treatment with 1 nM FK866 for 24 h in NA-supplemented medium. After FK866 treatment, 10 μM NA was treated for 6 h. The statistical analyses were performed by Student's t-test. Data was expressed as mean ±SE from at least three independent experiments.

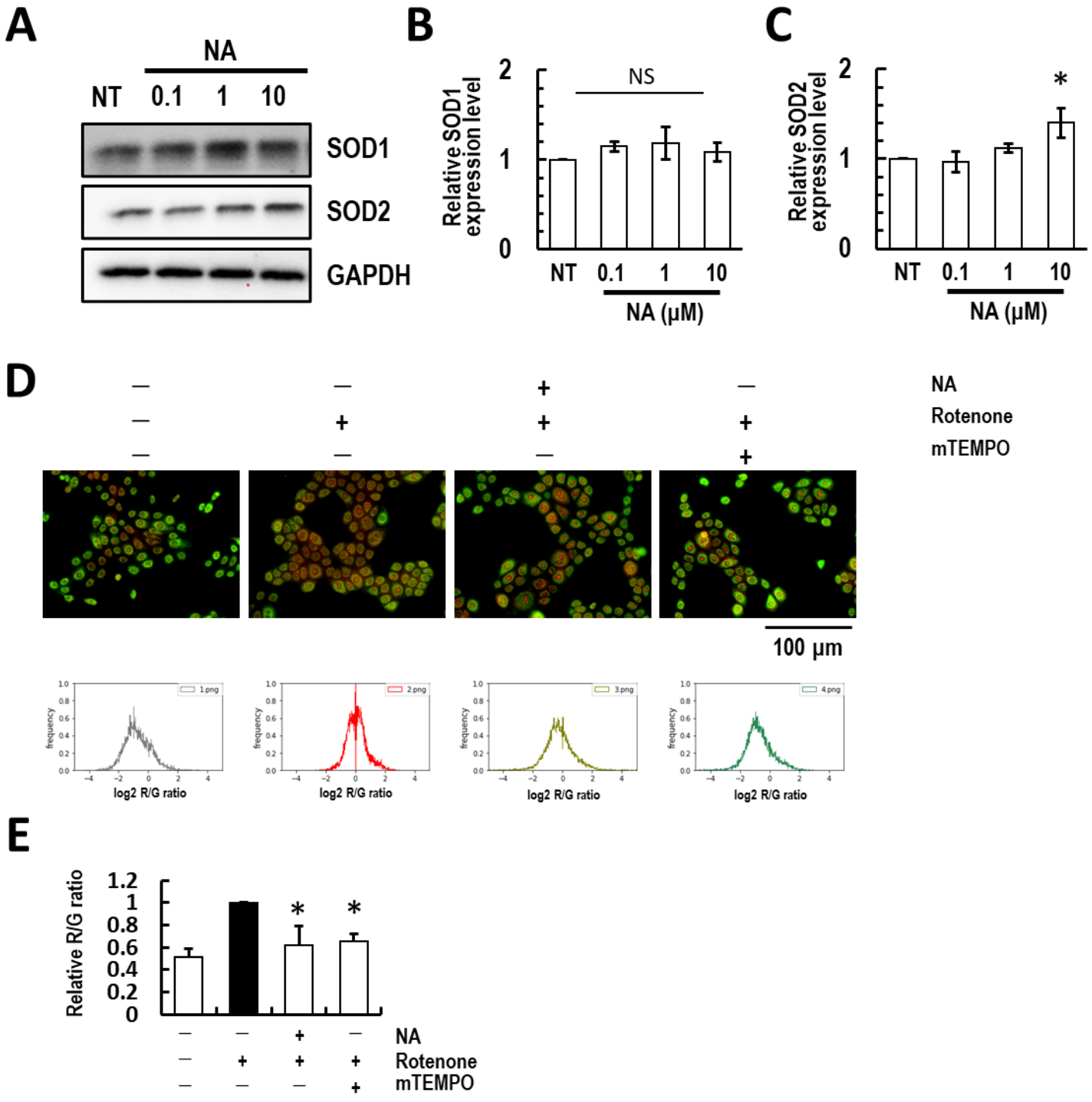

Figure 4

Ablation of mitochondrial ROS by the supplementation of nicotinic acid

(A) Representative images of the effects of NA on the mitochondrial marker, superoxide dismutase (SOD) 2, and reference cytosolic SOD1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as the loading controls. (B,C) Quantification of SOD1 (B) and SOD2 (C) immunoblot bands on (A). Data were divided by the intensity of GAPDH. (D) Representative images of the effect of NA on the mitochondrial ROS recovery activity. Mitochondria and mitochondrial ROS were stained by Green and Red fluorescent dye. The 50 μM Mito-TEMPO was used as a positive control. R: 1 μM Rotenone. Red/Green pixel ratio (R/G ratio) were depicted by histogram, and the means of the histograms were shown in (E). All data were expressed as mean ±SE from at least three independent experiments. The statistical analyses were performed by one-way ANOVA followed by Dunnet's test.