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N-methyl-N'-nitro-N-nitrosoguanidine Reduces the Intracellular Calcium Level Through NAD Depletion in NIH3T3 Cells

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Abstract: The effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on the intracellular Ca^{2+} level was studied in NIH3T3 fibroblast cells. A reduction of the intracellular Ca^{2+} level was observed after exposure to 300 μM MNNG. However, the intracellular level of IP_3, a well-known regulator of Ca^{2+} release from internal storage, was not changed by MNNG treatment. Instead, a reduction of the intracellular NAD level was observed. NAD as well as IP_3 stimulated intracellular Ca^{2+} release from permeabilized cells. The treatment of 3-aminobenzamide, which inhibited the MNNG-induced reduction of the NAD level, also prevented the MNNG-induced decrease of the Ca^{2+} level. Our data suggest that MNNG reduces the intracellular Ca^{2+} level by NAD depletion in NIH3T3 cells.

Key words: 3-aminobenzamide, calcium, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), NAD, poly(ADP-ribose)polymerase.

It is well established that many DNA damaging agents inhibit replicative DNA synthesis (Painter, 1978; Mak *et al.*, 1979; Aujard and Trincal, 1983; Slamenova *et al.*, 1990; Suciu, 1991). Many reports suggested that poly(ADP-ribose)polymerase (PARP) plays an important role in the inhibition of replicative DNA synthesis at damaged DNA sites. PARP is a nuclear enzyme which is activated by DNA strand breaks (Ueda and Hayaishi, 1985). Once activated, PARP modifies many enzymes involved in cellular DNA metabolism. Topoisomerase (Ferro and Olivera, 1984) and DNA polymerase α, β (Yoshihara *et al.*, 1985) were reported to be inhibited by polyADP-ribosylation, which may in turn induce an emergency halt of chromatin functions, including replication at damaged DNA sites.

However, it is well known that the replication is also regulated by intracellular second messengers, among which Ca^{2+} plays a very important role. The intracellular Ca^{2+} level is known to be regulated by another second messenger, IP_3, but recently it was reported that NAD can also regulate the intracellular Ca^{2+} level. Clapper *et al.* (1987) reported that a NAD metabolite can induce Ca^{2+} release from the internal Ca^{2+} store in a manner that is independent of IP_3. NAD is the substrate of PARP and it was reported that the cellular NAD level was reduced when PARP was activated by DNA damaging agents (Jacobson *et al.*, 1980; Rankin *et al.*, 1980). Therefore, there is some possibility that DNA damaging agents may cause a change in the intracellular Ca^{2+} level by the reduction of the intracellular NAD level, which is the result of PARP activation.

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Materials and Methods

Materials

Fluo-3/AM, Fura-2, 3-aminobenzamide (3-AB), d-*myo*-inositol-1,4,5-triphosphate (potassium salt) (IP_3) and β-nicotinamide adenine dinucleotide (NAD) were purchased from Sigma (St. Louis, USA). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was from Aldrich (Milwakee, USA). l-3-Phosphatidyl [2-^{3}H] Inositol (18.2 Ci/mmol) was from Amersham (Amersham, UK.) All other chemicals were of analytical grade.

Measurement of the individual Ca^{2+} level

NIH3T3 cells were cultured in Dulbecco's Modified Eagle's Media containing 10% fetal bovine serum and were loaded with Fluo-3/AM (10 μM final) for 1 h. Media were then replaced with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). After incubation for 2 min in Ca^{2+} and Mg^{2+}-free PBS, MNNG was added to 300 μM. Changes in the individual intracellular Ca^{2+} level was measured using a laser scanning microscope (5W argon laser) equipped with an inverted fluorescence microscope and 80386 computer system (ACAS

Model 570, Meridian). The excitation wavelength was 488 nm and emissions above 515 nm were collected as described by Nathanson *et al.* (1992).

Measurement of IP_3

Cells were harvested by centrifuging at 700×*g* for 3 min, the supernatants were aspirated, the cell pellets were suspended in 4% $HClO_4$ and placed on ice for 20 min. Cell samples were then centrifuged at 2,000 ×*g* for 15 min, supernatants were collected and neutralized with 5 N KOH and kept on ice for 15 min. After removing $KClO_4$ precipitations by centrifugation at 2,000×*g* for 15 min, the IP_3 content in supernatants were measured using Amersham's IP_3 assay kit (Amersham code No. TRK 1000).

Measurement of phospholipase C (PLC) activity

NIH3T3 cells were treated with various concentrations of MNNG for 30 min. PLC activities in the plasma membrane of NIH3T3 cells were assessed by measuring the ability to convert [^{3}H] labeled phosphatidyl inositol (PI) into aqueous inositol phosphates as described by Hofmann and Majerius (1982) with some modifications. Briefly, the cells were suspended in 1 ml of lysis buffer containing 100 mM KCl, 50 mM HEPES, 5 mM NaCl, 0.5 mM EGTA and 3.5 mM $MgCl_2$ (pH 7.3). After sonication for 90s, nuclei and unbroken cells were removed by centrifugation at 500×*g* for 7 min. The remaining cell samples were centrifuged at 20,000×*g* for 30 min, supernatants were discarded and the pellets were resuspended in 0.1 ml of reaction buffer containing 0.3 μCi/ml of [^{3}H] PI, 150 μM of PI, 0.1% sodium deoxycholate, 3 mM $CaCl_2$, 1 mM EGTA and 50 mM HEPES (pH 7.0). After incubation for 30 min at 37℃, the reaction was stopped by adding 1 ml of chloroform/methanol (1 : 2). To achieve phase separation, 0.5 ml of chloroform plus 0.5 ml of 0.25 N HCl was added to each sample preparation. Samples were vortex-mixed and centrifuged at 9,000×*g* for 1 min. 1 ml aliquots of the aqueous phase were radiometrically assessed in a liquid scintillation counter (Beckman LS 3801).

Measurement of Ca^{2+} release

Intracellular Ca^{2+} release by IP_3 or NAD from permeabilized cells were measured using Fura-2 as a fluorescent Ca^{2+} indicator. Briefly, cells were detached from culture dishes using Trypsin and EDTA, and washed twice with PBS. Cells were then permeabilized by suspending the cell pellets in the permeabilizing buffer containing 110 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 5 mg/ml BSA, 5 mM EGTA and saponine (10 μg per μl of cell pellet). Saponine was reported to selectively permeabilize plasma membrane without affecting endoplasmic reticulum (ER) membrane (Joseph *et al.*, 1984; Prentki *et al.*, 1984). 1 mM MgATP, 20 mM phosphocreatin, 8 U/ml creatin kinase and 1 μM Fura-2 were added to the permeabilized cell suspension, which was then transferred into a quartz cuvette chamber and was continuously stirred. Fura-2-Ca^{2+} fluorescence was measured by monitoring the fluorescence emission intensities at 540 nm with excitation at 400 nm in Perkin Elmer LS-3B fluorescence spectrophotometer (Clapper *et al.*, 1987). Calibration of Fura-2-Ca^{2+} signals was made by adding known amounts of $CaCl_2$ to the permeabilizing buffer containing 1 μM of Fura-2.

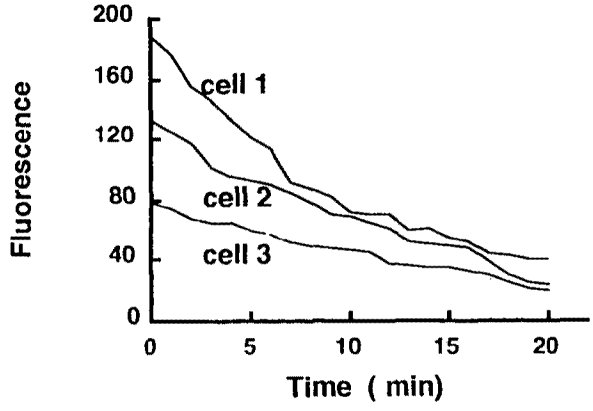

Fig. 1. Effect of MNNG on intracellular Ca^{2+} level. Semiconfluent cells were loaded with 10 μM of Fluo-3/AM for 1 h prior to the treatment of MNNG (300 μM final concentration). The changes of intracellular Ca^{2+} levels in three individual cells were monitored using a laser scanning microscope.

Determination of NAD

Cells were treated with test chemicals for stated periods of time prior to the NAD assay. After incubation, the cells were harvested and the media were discarded. Pyridine nucleotides were extracted with ice-cold 0.5 M $HClO_4$ as described by Jacobson and Jacobson (1976) and the intracellular NAD level was measured using an enzymatic cycling assay (Bernofsky and Swan, 1973).

Results

To elucidate the relationship between DNA damage and intracellular second messenger level, the intracellular Ca^{2+} level was measured after treatment with MNNG, a typical DNA damaging agent (Fig. 1). MNNG induced rapid decrease of the intracellular Ca^{2+} level in 3 individual cells. The difference of the initial intracellular Ca^{2+} level may reflect the heterogeneous state of the cell population, such as cell cycle stage and so

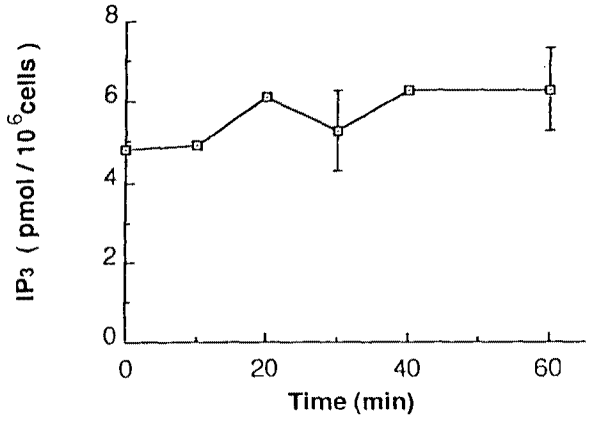

Fig. 2. The time course of intracellular IP_3 levels after MNNG treatment. Semiconfluent cells were treated with 300 μM MNNG and harvested at indicated time points after the treatment. Cellular IP_3 was extracted with 4% $HClO_4$, and measured using an IP_3 asay kit (Amersham code No. TRK 1000). Each value is expressed as the mean and standard deviation of three experiments.

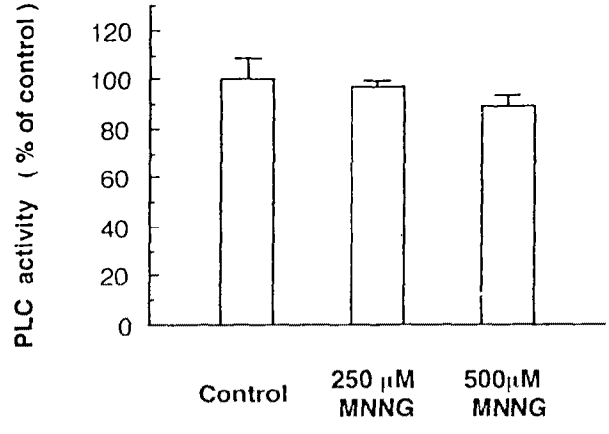

Fig. 3. Effect of MNNG on PLC activity in NIH3T3 cells. Semiconfluent cells were treated with MNNG for 30 min. Membrane fractions from cell samples were used for the measurement of PLC activity using [^{3}H]PI as a substrate. Radioactivity transfer from organic phase to aquous phase was determined as a measure of PLC activity.

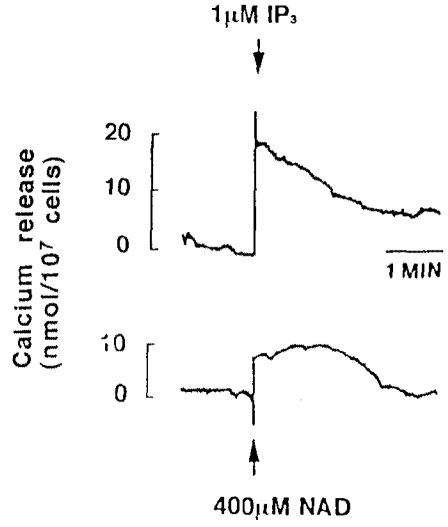

Fig. 4. Intracellular Ca^{2+} release mediated by IP_3 and NAD. NIH 3T3 cells (10^7) were harvested and permeabilized. After Fura-2 was added to 1 μM, the permeabilized cells were transfered into a quartz cuvette. Fura-2-Ca^{2+} fluorescence was measured using fluorescence spectrophotometer.

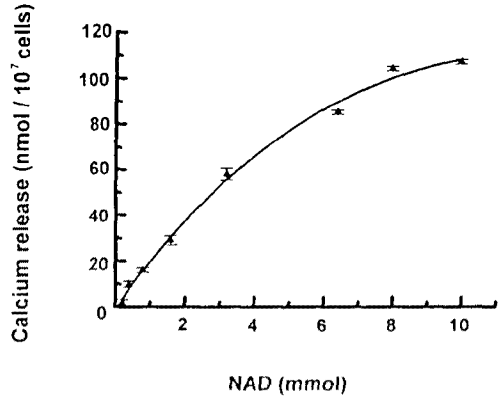

Fig. 5. Dosage response of NAD on the intracellular Ca^{2+} release in permeabilized cells. Cells (10^7) were harvested and permeabilized. After Fura-2 was added to 1 μM, the permeabilized cells were transfered into a quartz cuvette. Fura-2-Ca^{2+} fluorescence was measured using fluorescence spectrophotometer. Calibration of Fura-2-Ca^{2+} fluorescence was performed by adding known amounts of $CaCl_2$ to the permeabilizing buffer containing 1 μM Fura-2.

on. This phenomenon could not be the result of cytotoxicity of MNNG, because 300 μM MNNG treatment up to 1 h had no effect on cell viability, which was tested by trypane blue staining (data not shown).

The IP_3 has thus far been known to be the regulator of intracellular Ca^{2+} (Majerius *et al.*, 1986). We thought there could be some possibility that the reduction of intracellular Ca^{2+} level by MNNG treatment had been the result of reduced IP_3 contents. To test this hypothesis, we measured the intracellular IP_3 level at various time periods of MNNG treatment. However, data in Fig. 2 show that MNNG treatment did not change the intracellular IP_3 level. We also examined the effect of MNNG on the activity of PLC, an enzyme which produces IP_3 by the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), but there was no change (Fig. 3). These data suggest a different mechanism of intracellular Ca^{2+} regulation by MNNG.

In recent years, many observations have implied that a NAD metabolite is involved in the regulation of intracellular Ca^{2+}. We compared the mobilization of intracellular Ca^{2+} by IP_3 and NAD using Fura-2 as a fluorescent Ca^{2+} indicator (Fig. 4). To minimize the fluores-

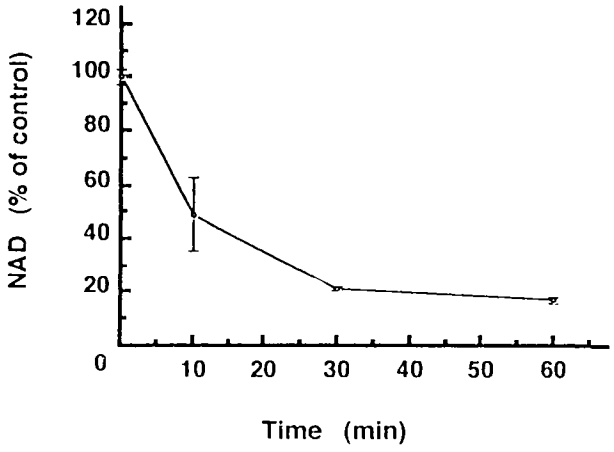

Fig. 6. NAD level following treatment of NIH3T3 cells with 300 μM MNNG. Semiconfluent cells were treated with 300 μM MNNG for the indicated time periods. Cellular NAD was extracted with 0.5 M $HClO_4$ and the changes of intracellular NAD levels were measured using an enzymatic cycling assay. Levels of NAD are expressed as the percent of initial NAD level. Each value represents the mean and standard deviation of three experiments.

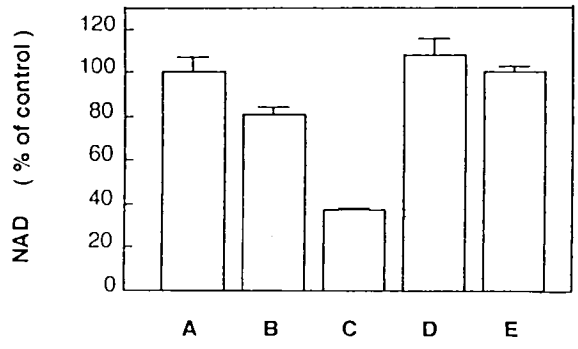

Fig. 7. Effect of 3-AB on intracellular NAD levels in NIH3T3 cells. The levels of NAD are expressed as the percent of NAD level in the control cells. A: control; B: cells treated with 30 μM MNNG; C: cells treated with 300 μM MNNG; D: cells treated with 5 mM 3-AB; E: cells treated with 5 mM 3-AB plus 300 μM MNNG. Each value represents the mean and standard deviation of three experiments.

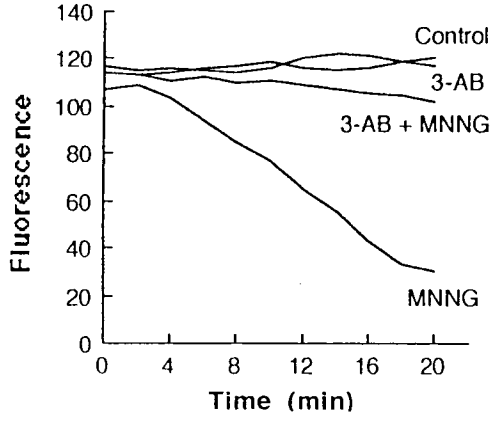

Fig. 8. Effect of 3-AB on intracellular Ca^{2+} levels in MNNG-treated cells. Semiconfluent cells were loaded with 10 μM Fluo-3/AM for 1 h followed by the addition of 300 μM MNNG, 5 mM 3-AB, or 300 μM MNNG plus 5 μM 3-AB. Fluorescence intensities were measured using a laser scanning microscope with excitation at 488 nm and emissions above 515 nm were collected.

cent interference potentially caused by reduced pyridine nucleotides, fluorescence was measured at 400 nm excitation and 540 nm emmision as reported by Clapper *et al.* (1987). It was shown that the addition of IP_3 to permeabilized cells elicited a rapid Ca^{2+} release. The IP_3-sensitive intracellular Ca^{2+} storage site of a permeabilized cell was reported to be ER, and released Ca^{2+} was resequestrated into ER by the action of the Ca^{2+} pump, which was energized by the ATP regenerating system composed of ATP, phosphocreatin and creatin kinase (Joseph *et al.* 1984). Similar to IP_3, NAD also stimulated Ca^{2+} release. The addition of NAD to the assay buffer without cells had no effect on fluorescence, showing that data in Fig. 4 are not artifacts originating from the fluorescence of NAD itself or contaminating Ca^{2+} (data not shown). Data in Fig. 5 show that NAD in a range of 100 μM to 10 mM elicits significant Ca^{2+} release. The maximal release of Ca^{2+} was observed at 8 mM with the mid-point at 2.5 mM. These concentrations of NAD are unphysiological and at physiological NAD concentration, which is 100 μM to 1 mM (Hayaishi and Ueda, 1977), Ca^{2+} release was proportional to the NAD concentration, suggesting the possibility that change in the intracellular NAD level may lead to a change in the intracellular Ca^{2+} level.

It is well known that N-alkyl-N-nitroso compounds, including MNNG, reduce the intracellular NAD level by the activation of PARP without affecting other NAD metabolism (Jacobson *et al.*, 1980; Rankin *et al.*, 1980; Smulson *et al.*, 1977). We measured the intracellular NAD level after the treatment of 300 μM MNNG. MNNG caused reduction of the NAD level as shown in Fig. 6. The MNNG-induced decrease of the NAD level was dose dependent, and was completely inhibited by 3-AB, a PARP inhibitor, showing that this reduction of the NAD pool was mediated by PARP, as reported before (Fig. 7). There was no change in the NAD level when cells were treated with 3-AB alone.

To further explore the hypothesis that the DNA damaging agent can influence intracellular Ca^{2+} level by the reduction of the intracellular NAD pool, which is mediated by the activation of PARP, we examined the effect of 3-AB on the Ca^{2+} level in MNNG-treated cells (Fig. 8). Apparently, 3-AB prevented the MNNG-induced reduction of the intracellular Ca^{2+} level, as it prevented the reduction of the intracellular NAD pool in Fig. 7. There was no significant change in the intracellular Ca^{2+} level in the control cells, and the treatment

by 3-AB alone had no effect on the intracellular Ca^{2+} level. The above results suggest that MNNG-induced intracellular Ca^{2+} decrease is mediated by the activation of PARP and subsequent reduction of the intracellular NAD level.

Discussion

Recently, many papers have reported that DNA damaging agents can influence some steps in signal transduction pathways. Neomycin was reported to inhibit the initiation of DNA synthesis by a mechanism which was suggested to involve the blockade of PIP_2 hydrolysis (Carney *et al.*, 1985), and Shin *et al.* (1993) reported that UV light reduced the intracellular concentration of IP_3 and diacylglycerol to inhibit DNA replication. It was observed that long-wave UV light inhibited the binding of epidermal growth factor to its receptor, which is a tyrosine-specific protein kinase known to be important in the regulation of cell proliferation (Laskin *et al.*, 1988), and Buscher *et al.* (1988) suggested that UV activates protein kinase to induce the expression of c-fos protooncogene.

In our study, MNNG was found to reduce the level of intracellular Ca^{2+}, an important second messnger which regulates cell division and DNA replication. It is well known that the intracellular Ca^{2+} level is regulated by another second messenger, IP_3. But there was no change in intracellular IP_3 content and PLC activity after MNNG treatment, suggesting another mechanism of Ca^{2+} regulation (Fig. 2 and 3).

In recent years, many reports suggested that NAD can also be involved in intracellular Ca^{2+} regulation. Clapper *et al.* (1987) reported that a metabolite of NAD can stimulate Ca^{2+} release from microsomes of sea urchin eggs. Subsequently, the active metabolite was purified by HPLC and its structure was determined by NMR and mass spectrometry (Lee *et al.*, 1989). This NAD metabolite was named cyclic ADP-ribose. The metabolite itself (Walseth *et al.*, 1991), its synthesizing enzyme (Rusinko and Lee, 1989; Lee and Aarhus, 1991) and its degrading enzyme (Lee and Aarhus, 1993) were reported to be present in various mammalian and invertebrate tissues, and it is thought that the regulation of intracellular Ca^{2+} by a NAD metabolite is a wide-spread phenomenon among eukaryotes. This Ca^{2+}-releasing metabolite is synthesized by a one-step reaction using NAD as the sole substrate (Lee and Aarhus, 1991; Summerhill *et al.*, 1993; Berridge, 1993) suggesting the possibility that intracellular NAD depletion may lead to the reduction of the intracellular Ca^{2+} level.

In our study, MNNG induced the reduction of both NAD and Ca^{2+} levels (Fig. 1 and 6), NAD stimulated Ca^{2+} release from the intracellular Ca^{2+} store(Fig. 4 and 5), and the cotreatment by 3-AB, a PARP inhibitor, prevented the reduction of not only the NAD level but also the Ca^{2+} level (Fig. 7 and 8). These results suggest that the MNNG-induced reduction of the intracellular Ca^{2+} level is mediated by the depletion of NAD which is the result of PARP activation.

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