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Letter to the Editor

An Unexpected Link Between Energy Metabolism, Calcium, Chromatin Condensation and Cell Cycle

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NOTE

Supplemental material can be found at:
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Energy metabolism and calcium homeostasis play a central role in cell cycle progression and other cellular functions making them important targets for high throughput drug screenings. In the course of our studies on molecule dynamics and chromatin throughout the cell cycle we uncovered an unexpected link between energy metabolism, calcium release and chromatin condensation. To differentiate cellular processes driven by molecule diffusion versus active transport, cellular energy depletion experiments are commonly performed.[1-4] One of the chemicals often used is sodium azide (NaN_3), which inhibits the cytochrome oxidase and thus the mitochondrial respiratory chain resulting in ATP depletion.[5]

During our experiments on the live-cell dynamics of chromatin labeled with fluorescent histones throughout the cell cycle we made the observation that 10 min NaN_3 incubation causes a concentration dependent condensation of nuclear chromatin and a correspondingly enlarged interchromatin space (Fig. 1A).[2] In NaN_3 treated Hela cells the chromatin volume decreased down to 64 % of its size in untreated cells (Fig. 1A). In images of untreated cells, the diffuse borders of the chromatin result from decondensed chromatin fibers too small to be resolved by conventional microscopy. After NaN_3 incubation, this type of chromatin structure disappears in the images and only condensed chromatin is visible (Fig. 1). A similar effect was recently reported when living cells were subjected to hyperosmolar media. We, therefore, tested whether the NaN_3 induced chromatin condensation could be the result of intracellular changes of ion levels.[6]

First we tested the direct effect of different cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) on chromatin in cells. We permeabilized the plasma membrane briefly with digitonin[7] and incubated the cells with hypertonic salt solutions (Fig. 1B). Fluorescently labeled dextrans were added to the media to monitor the permeabilization since the plasma membrane of untreated cells excludes dextrans (Fig. S1). In agreement with previous results[6] we could observe chromatin condensation in intact cells incubated with any of the four cations tested (Fig. S1). However, in permeabilized cells only Ca^{2+} ions caused chromatin condensation, while even at high concentrations the other ions had no effect (Fig. 1B and Fig. S1). Calcium as a bivalent cation binds to DNA and was shown to be associated with chromatin during mitosis.[8] We measured the change in chromatin volume by following cells progressing from mitosis into interphase and treating them with NaN_3. In this manner we overcome the problem of high variability within the cell population (Fig. 1C). While NaN_3 treatment caused a decrease of the chromatin volume to 74%, in mitosis this was further reduced to 41 % compared to untreated interphase cells. These data indicate that the NaN_3 causes chromatin condensation within a physiological range, which is lower than mitotic chromosomal condensation.

Next we tested whether intracellular calcium level changes in living cells during NaN_3 incubation using a fluorescent sensor molecule Fluo 3.[9] The cell permeable Fluo 3 AM was loaded for 30 min into living cells and the Fluo 3 fluorescence emission measured before and during NaN_3 incubation. The data indeed revealed a 1.5 fold increase of the calcium levels following the NaN_3 incubation (Fig. 2A). The drop of the Fluo 3 fluorescence over time is likely due to the depletion of free calcium from the cytosol and nucleoplasm by binding to chromatin. The dynamic range of Fluo 3 fluorescence was tested by adding 5 μM ionomycin to the medium and reached a maximum of five-fold increase leading concomitantly to chromatin condensation (Fig. S2). The fact that NaN_3 induced chromatin condensation also in calcium free PBS with 10 mM EGTA (Fig. S3) indicates that the calcium increase is caused by release from intracellular stores and not by uptake from the extracellular milieu. Beside the endoplasmatic reticulum, mitochondria are potent buffering sites for calcium and NaN_3 impairs mitochondrial functions.[5] The effect of

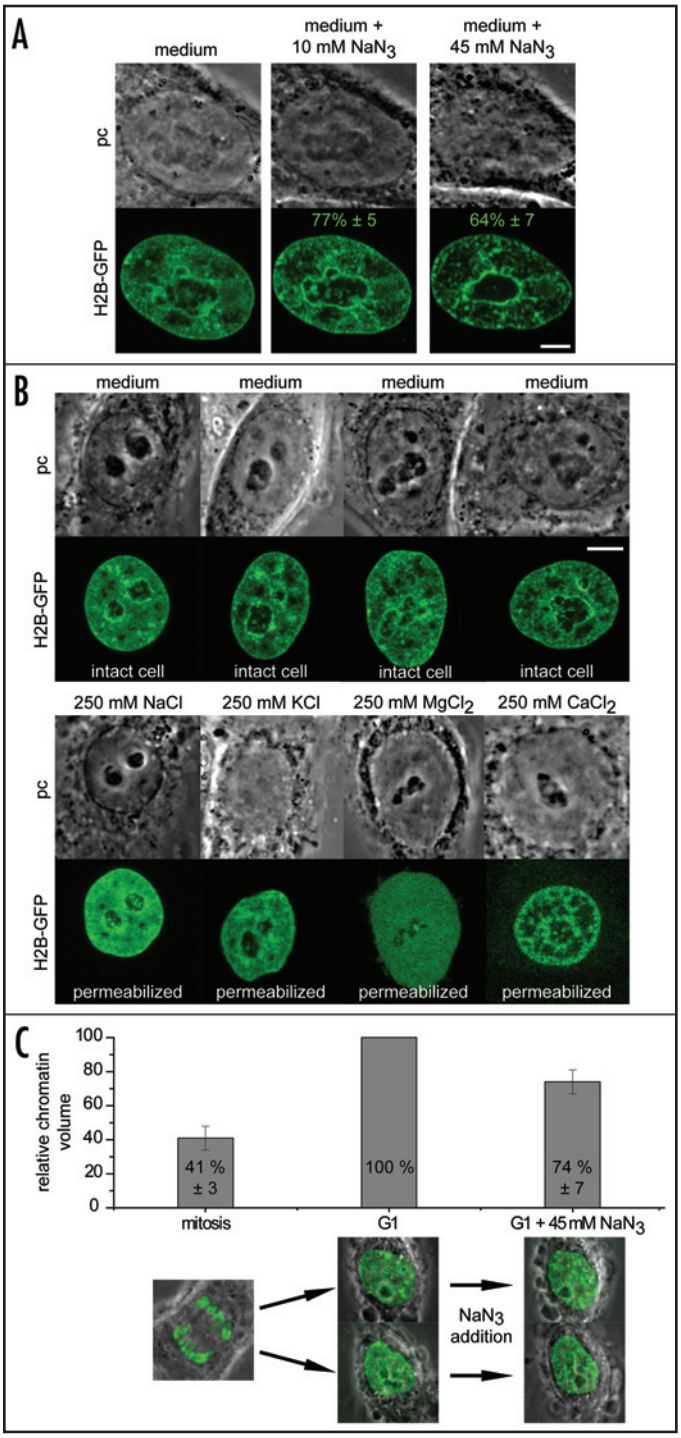

Figure 1. Chromatin condensation in living cells induced by NaN_3. (A) NaN_3 (Calbiochem) induces within 10 min a condensation of the nuclear chromatin in living HeLa cells expressing a histone H2B-GFP chromatin label. The chromatin volume is reduced progressively with increasing amounts of NaN_3 and the interchromatin space extends. The quantitative evaluation of the chromatin volume relative to the untreated cells was performed using ImageJ software and is given in the lower panels ± standard deviation (n = 10 cells). The analysis was performed by determining and subtracting the background, followed by Gauss filtering and applying a fluorescence intensity threshold that included all chromatin. Finally, the thresholded chromatin volume for whole 3D image series was calculated using the voxel counter tool. (B) The effect of different salt ions on the chromatin condensation level. The plasma membranes of the living cells were mildly permeabilized by digitonin to test for direct effects of ions on chromatin. The top images show the intact cells in medium and the bottom images the same cells after digitonin treatment in the different 250 mM salt solutions. A condensation of the nuclear chromatin occurs only as a result of incubation in Ca^{2+} solution. The other ions Na^+, K^+ and Mg^{2+} do not induce condensation. The addition of Mg^{2+} caused a rather homogeneous distribution of chromatin, which probably is the result of nuclease activation by this cation. (C) The bar diagram displays the percentage of the chromatin volume of mitotic and NaN_3 treated G_1 cells compared to the chromatin volume in the same G_1 cell before treatment (see scheme below). For the comparison, the combined chromatin volume of the two G_1 daughter cells was taken to keep the total amount of chromatin constant relative to the mitotic cell. The chromatin is most condensed in mitosis with only about 41% of the interphase chromatin volume, while in cells treated with 45 mM NaN_3 the chromatin volume decreases to 74 % of the interphase volume. Scale bars, 5 μm. pc, phase contrast.

NaN_3 on mitochondria in living cells can be directly tested with dyes (e.g., MitoTrackerRed) that are taken up into the organelle and accumulated via the membrane potential. We incubated living cells with MitoTracker for 30 min followed by change to media without dye for 45 min. Cells were imaged before and after adding NaN_3 to monitor possible changes in the mitochondria. Within a few minutes of NaN_3 incubation the filamentous mitochondrial population were fragmented into small sphere like remains (Fig. 2B). Furthermore the decrease of the dye signal from the mitochondria indicates a lowering or loss of the membrane potential. Altogether, these results show that NaN_3 disrupts the mitochondrial membrane potential inducing an increase in the calcium level in the nucleus. Since extracellular calcium was not required for chromatin condensation, the intracellular calcium rise could be due to release from mitochondria or from the endoplasmatic reticulum. The lowering of the ATP production by NaN_3 should concomitantly prevent calcium buffering by inhibiting the transport of free calcium by ATP dependent ion pumps into the mitochondria, endoplasmatic reticulum as well as to the outside of the cell.[10]

These findings have important consequences for the interpretation of experiments using NaN_3 for ATP depletion and the analysis of molecule dynamics within the nucleus. Although the diffusive mobility of nuclear proteins is not ATP dependent,[11] the enlarged interchromatin space on the one hand and the higher condensation of chromatin on the other hand likely affects local protein concentration, mobility and access. Most importantly, our results show that external stimuli, manipulations and drugs directly or indirectly changing cellular calcium levels, by either energy depletion or direct interference with calcium homeostasis, have an effect on chromatin condensation and arrest cell cycle progression. These unexpected links between energy metabolism, calcium homeostasis, chromatin condensation and cell cycle should be taken in account for the design of drug screenings and the interpretation of cellular responses. Even small changes of free intracellular calcium levels may affect chromatin condensation and gene expression with still unknown consequences.

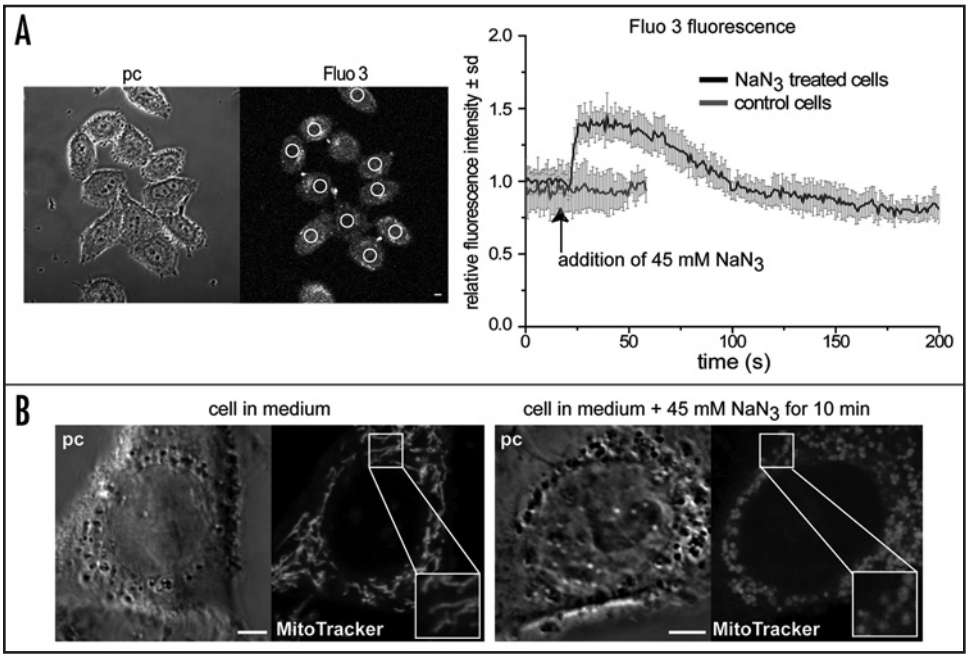

Figure 2. NaN_3 triggers cellular calcium rise and fragmentation of mitochondria. (A) Microscopic fluorescence imaging of the calcium sensor Fluo 3 (Molecular Probes) in living cells. The fluorescence image shows Fluo 3 loaded into HeLa cells in normal growth medium (corresponding to 10 s in the curve). The areas of measurement (white circles) were choosen according to the position of the nuclei identified in the phase contrast image. The curve describes the mean nuclear Fluo 3 fluorescence over time (n =10 cells ± standard deviation), showing a rise of fluorescence shortly after addition of NaN_3 to the culture medium, indicated by the arrow. The Fluo 3 fluorescence increases up to 1.5 fold over the normal level before NaN_3 addition and in control cells (gray curve) which corresponds to a calcium rise of about two-fold according to the fluorescence calibration curve given by the manufacturer. (B) The mitochondria of living HeLa cells expressing histone H2B-GFP were imaged using MitoTrackerRed (Molecular Probes). The images display confocal optical sections of control cells (left) and 10 min after NaN_3 incubation (right). The tubular and filamentous mitochondrial structure in the left image and inset collapse into small dot-like remains and indicate a loss of mitochondrial fission and fusion events. Scale bar, 5 μm. pc, phase contrast.

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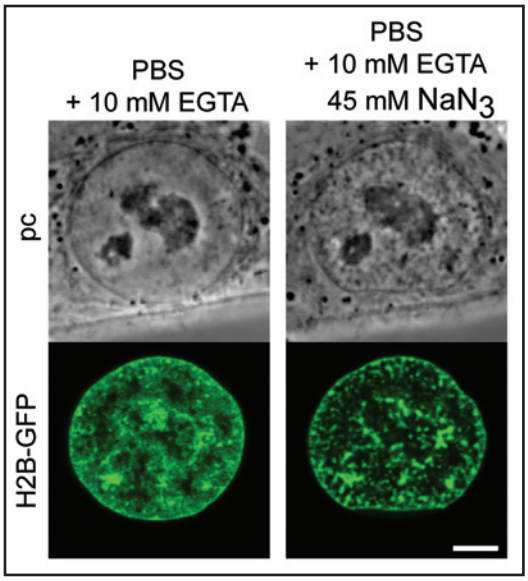

Figure S3. NaN_3 induces chromatin condensation independent of extracellular calcium. The condensation of chromatin using NaN_3 is also achieved in a calcium free buffer, which excludes extracellular calcium as a possible source for the intracellular calcium rise. The cells were incubated in PBS supplemented with 10 mM EGTA for 15 min to deplete extracellular calcium and subsequently 45 mM NaN_3 was added. Scalebar 5 μm. pc = phase contrast.