Macromolecular Crowding Regulates Assembly of mRNA Stress Granules after Osmotic Stress

NEW ROLE FOR COMPATIBLE OSMOLYTES

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Stress Granules after Osmotic Stress

Macromolecular crowding regulates stress granule assembly and, thus, the cell fate after osmotic stress.

Background: The intracellular accumulation of compatible osmolytes in hypertonic conditions reduces macromolecular crowding and ionic strength.

Results: Compatible osmolytes disassemble mRNA stress granules. Hypertonic-preconditioning and gap-junction communication favor cell survival via compatible osmolyte accumulation.

Conclusion: Macromolecular crowding regulates stress granule assembly and, thus, the cell fate after osmotic stress.

Significance: Compatible osmolytes can promote cell survival through their action on stress granules.

The massive uptake of compatible osmolytes such as betaine, taurine, and myo-inositol is a protective response shared by all eukaryotes exposed to hypertonic stress. Their accumulation results mostly from the expression of specific transporters triggered by the transcriptional factor NFAT5/TonEBP. This allows the recovery of the cell volume without increasing intracellular ionic strength. In this study we consider the assembly and dissociation of mRNA stress granules (SGs) in hypertonic-stressed cells and the role of compatible osmolytes. In agreement with in vitro results obtained on isolated mRNAs, both macromolecular crowding and a high ionic strength favor the assembly of SGs in normal rat kidney epithelial cells. However, after hours of constant hypertonicity, the slow accumulation in the cytoplasm of compatible osmolytes via specific transporters both reduces macromolecular crowding and ionic strength, thus leading to the progressive dissociation of SGs. In line with this, when cells are exposed to hypertonicity to accumulate a large amount of compatible osmolytes, the formation of SGs is severely impaired, and cells increase their chances of survival to another hypertonic episode. Altogether, these results indicate that the impact of compatible osmolytes on the mRNA-associated machineries and especially that associated with SGs may play an important role in cell resistance and adaption to hyperosmolarity in many tissues like kidney and liver.

After hypertonic exposure, the immediate response of mammalian cells relies on changes in membrane ion channel and transporter activity (1), water efflux, and remodeling of the cell cytoskeleton. Such a cascade of events leads to a decrease of cell volume and increase in intracellular ionic strength (2–5). The accumulation of ions then produces an inverse osmosis-driven water movement that allows a partial cell volume recovery. This situation is, however, temporary as cells cannot sustain a high intracellular ionic strength for long as it affects most enzymatic reactions. In mammalian cells long term protection and adaptation to hypertonicity is triggered by translocation of NFAT5 (or TonEBP) (6, 7), a transcription factor that activates genes leading to the synthesis of neutral compatible osmolytes (sorbitol (8), glycerophosphorylcholine (9)) or their active uptake from the extracellular medium (betaine (10), taurine (11), and myo-inositol (12)). As a high intracellular ionic strength enhances the NFAT5 activity (13), the consecutive accumulation of these neutral organic compounds in place of the inorganic ions allows the reduction of the intracellular ionic strength while equilibrating the extracellular/intracellular osmotic balance. Nevertheless, the time required to complete such a massive accumulation is long, about 12–24 h (14–16) whatever the nature of the mammalian cell involved. Mean- time, the intracellular environment is not optimal to keep the cellular machineries going (3, 17). For DNA-associated processes, the appearance of DNA breakages after hypertonic shock has been particularly under focus as well as the impairment of the DNA repair machineries. Surprisingly, when kidney epithelial cells become adapted to long term exposures to high salinity, DNA breakages persist even though compatible osmolytes have been accumulated (18).

Regarding mRNA-associated processes that are investigated in this study, the presence of compatible osmolytes in the cytoplasm may have a profound impact, as observed in cell-free protein synthesis systems (19). In cells, an acute hypertonic shock rapidly leads to a translational arrest (20–22) and thus to the formation of stalled preinitiation complexes composed of mRNAs and multiple protein partners like initiation factors. The subsequent assembly of these complexes finally leads to the formation of stress granules (SGs) in the cell cytoplasm (22–24). This assembly is probably promoted by the shuttling from the nucleus to the cytoplasm of some mRNA-binding proteins like TIA-1 (25), as observed for stresses mediated by arsenite.
hypoxia, and hyperthermia (26). However, there are no data regarding the role of compatible osmolyte accumulation on SG assembly/disassembly, which might be critical for the recovery and the protection of the mRNA-associated machineries under chronic hypertonic exposure.

In this study we first analyzed by atomic force microscopy (AFM) the importance of macromolecular crowding (27–29) and ionic strength. Both are theoretically expected to promote self-attraction between liked-charged surfaces (30–33) and, thus, the association of anionic mRNAs into SGs or, as the control, anionic microtubules (MTs) into bundles (34), a prediction confirmed here by the results. Indeed, using MT bundling as an indicator of exacerbated excluded volume interactions (30) in the cell cytoplasm, the results show that macromolecular crowding and ionic strength are critical for the formation of mRNA granules in hypertonic-stressed normal rat kidney cells (NRK). We then analyzed whether the uptake of compatible osmolytes like betaine, taurine, and myo-inositol, which reduces excluded volume interactions, could lead to SG disassembly. In addition, in cells preconditioned to hypertonicity, we investigated the putative impact of compatible osmolyte accumulation on SG assembly and, as a consequence, their resistance to osmotic challenge. In light of the results, we propose that the maintenance of cell homeostasis, thanks to compatible osmolytes, may be of particular importance in preventing the formation of SGs and maintaining the mRNA-associated machineries under chronic hypertonicity, with possible applications in many tissues. The kidney and especially the inner medulla are known to be exposed to varying hyperosmolarity (35), but among other examples, the liver, which responds to hydration changes by altering cell volume (36), and the cornea of dry eye (37) are also potentially exposed to varying osmolarity.

**MATERIALS AND METHODS**

**Cell Culture**—NRK (proximal tubule)-52E cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotics (penicillin and streptomycin) in a humidified 5% CO₂ atmosphere at 37 °C. The osmolality of the DMEM with 5% (v/v) FBS is considered to be 320 mosmol/kg (referred here as isotonic). The hypertonicity of the medium was adjusted by

**Atomic Force Microscopy**—mRNAs and MTs were imaged by AFM under macromolecular crowding conditions as previously described (32). Briefly, 5 μl of solutions containing mRNA molecules (2 nm) or taxol-stabilized MTs (20 μM tubulin, 5 μM taxol) in 20 mM Tris-HCl, pH 7.5, and different polyethylene glycol (PEG) and KCl concentrations were deposited onto NiCl₂ pretreated mica for 1 min. Samples were then thoroughly rinsed with 0.02% uranyl acetate. Finally, the mica was dried with a filter paper. AFM imaging was carried out using a Nanoscope IIIa AFM (Veeco Instrument) in tapping mode with silicon cantilevers AC160TS (Olympus). The scan frequency was typically 1 Hz per line, and the modulation amplitude was of about a few nanometers.

**Tubulin Preparation**—Tubulin was purified from sheep brain crude extracts. Before use, a cycle of tubulin polymerization was performed in 50 mM MES-KOH, pH 6.8, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.5 mM EDTA, 6 mM MgCl₂, 0.6 mM GTP, 30% glycerol. MTs were sedimented by centrifugation (52,000 × g, 30 min at 37 °C), at the end of which the MT pellet was resuspended in 25 mM MES-KOH, pH 6.8, 0.5 mM EGTA, 1 mM DTT, and disassembled at 4 °C for 20 min. Tubulin aggregates were finally eliminated by a further centrifugation at 4 °C (52,000 × g, 20 min). Tubulin concentration was determined by spectrophotometry using an extinction coefficient of 1.2 mg cm⁻² at 278 nm.

**Synthesis of mRNA**—Plasmid pSP72-2Luc was used as a template for synthesis by T7 polymerase of 2Luc mRNAs (3000 nucleotides). After transcription, unincorporated NTPs were removed by gel filtration through a NAP-5 column (GE Healthcare), and mRNAs were further isolated with RNAble (Eurobio) following the manufacturer’s recommendation.

**Quantification of Betaine by Solution NMR Spectroscopy**—NRK cells were grown to confluency and exposed to hypertonicity (uptake) or to isotonicity (efflux) under conditions specified in the figure captions. Cells were rapidly washed with adjusted PBS to maintain a constant osmolarity and avoid the release of organic osmolytes. Cells were then scraped off in 200 μl of PBS and analyzed by one-dimensional ¹H NMR spectroscopy without further treatment. A 10-μl aliquot was used to quantify by hemacytometry the number of cells analyzed by NMR. NMR spectra were acquired at 20 °C on a Bruker Avance 600 spectrometer. All experiments were performed on 60-μl samples with a MATCH system (Cortecnnet, Paris, France). The water signal was suppressed using the Watergate method. Each spectrum was obtained after 64 scans. Spectra were processed with Topspin 2.0 (Bruker). To have statistically relevant results, three different samples were used for each condition. 2,2-Dimethyl-2-silapentane-5-sulfonic acid was added as an external reference in pure D₂O. The area of betaine at 3.25 ppm peak was calculated relative to this normalized area and then divided by the number of cells (arbitrary units (a.u.)); 1 a.u. corresponds to 72 fmol of betaine per NRK cell for all figures.

**Immunofluorescence**—NRK cells grown on plastic dishes were fixed with 4% paraformaldehyde in PBS, 200 mM sucrose for 20 min at 37 °C. After fixation, cells were then washed and incubated for 1 h with mouse monoclonal anti-tubulin antibody E7 (1:2000 dilution), a mouse anti-HuR antibody (Molecular Probes, 10 μg/ml), and a rabbit anti-YB-1 (produced as described in Davydova et al. (38)). Cells were washed extensively in PBS and incubated for 1 h with fluorochrome (Alexa Fluor®488 and -555)-coupled secondary antibodies (Invitrogen) in blocking solution. For statistics, the mean granule area was measured using ImageJ over at least 500 granules coming from at least 10 different representative cells.

**Immunoblotting**—Cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, and protease inhibitor mixture (Roche Applied Science). Lysates were

JANUARY 20, 2012 • VOLUME 287 • NUMBER 4
centrifuged at 14,000 × g for 15 min at 4 °C, and supernatants were collected. Proteins were separated on 12% SDS-PAGE gels and transferred onto a PVDF membrane (Invitrogen). The membranes were blocked in 5% (w/v) nonfat dried milk, PBS for 30 min at room temperature (20 °C) and incubated for 1 h at room temperature with primary antibodies (anti-phospho-eIF2α antibodies (Cell Signaling, 1:2000 dilution) and anti-GAPDH (Abcam, 1:5000). Bound antibodies were detected and quantified using anti-rabbit-IRDye 800 or anti-mouse-IRDye 680 secondary antibodies (Odyssey, 1:5000 dilution) with an Odyssey imaging system (LI-COR Biosciences).

**PLASMID CONSTRUCTION AND TRANSFECTION**—The cDNA encoding the full-length YB-1 was amplified by PCR and cloned into the Xhol and BamH1 sites of the pEGFP-C3 vector (Clontech). PCR-amplified products were then sequenced. NRK cells were transfected with plasmid DNA by using the Nucleofector™ technology.

**RESULTS**

**EXCLUDED VOLUME INTERACTIONS MEDIATED BY MACROMOLECULAR CROWDING AND HIGH IONIC STRENGTH TRIGGER mRNA AND MT SELF-ASSOCIATION**

Theoretical Prerequisites—All macromolecular complexes in the cell cytoplasm are *a priori* sensitive to exacerbated excluded volume interactions (39), which is an important aspect of macromolecular crowding. However, in the case of hypertonicity, charged macromolecules are likely to be more sensitive due to the uptake of inorganic ions (K⁺, Na⁺, Cl⁻), which neutralize charged surfaces and screen electrostatic repulsion. Consequently, in contrast with oppositely charged biomacromolecules, excluded volume interactions between two like-charged biomacromolecules are promoted by high ionic strength (31). Among these, mRNA and MTs may be particularly sensitive because of their highly negatively charged surface. The

**FIGURE 1.** High resolution AFM imaging reveals mRNA assembly into granules and MT bundling under macromolecular crowding environment.

A, at moderate ionic strength (20 mM Tris-HCl, pH 7.4, 50 mM KCl), 1% PEG 35K (w/v), a crowding agent, triggers the assembly of MTs into thick bundles, and 20% PEG 35K triggers that of 2Luc mRNA into granules. In contrast, PEG 1K was unable to induce mRNA self-assembly, as expected for excluded volume interactions because of its small size. Incubation time, 30 min. Scale bars, 2.5 μm (AFM images of MTs) and 1 μm (AFM images of mRNAs). B, with 0.5 and 15% PEG 35K at moderate ionic strength, no MT bundles or mRNA aggregates were detected, respectively. However, increasing KCl concentration led to the progressive appearance of MT bundles or mRNA granules. When betaine, a neutral osmolyte, was used instead of KCl to increase the osmolality without increasing the ionic strength, no mRNA granules or MT bundles were observed. Incubation time, 30 min. Scale bars, 2.5 μm (AFM images of MTs) and 1 μm (AFM image of mRNAs).
expected results of a self-attraction mediated by excluded volume interactions are the formation of mRNA granules and MT bundles, respectively.

High Ionic Strength and Macromolecular Crowding Promote mRNA Granule Formation and MT Bundling in Vitro—To illustrate these theoretical predictions, we analyzed by AFM in vitro formation of MT bundles and mRNA granules under macromolecular crowding condition using PEG, a neutral polymer, to mimic macromolecular crowding (Fig. 1A). We found that high molecular weight PEG $M_r$ 35,000 (PEG 35K) induced mRNA aggregation and MT bundling, whereas PEG $M_r$ 1000, which is not large enough to trigger macromolecular crowding-induced attraction, was inefficient. Macromolecular crowding can then induce MT bundling and mRNA granule formation via excluded volume interactions. In addition, as stated above, ionic strength is also an important parameter that promotes self-attraction of like-charged macromolecules. Accordingly, we observed that at PEG 35K concentrations below the threshold values for mRNA granule assembly and MT bundling, respectively, increasing KCl concentration leads to the appearance of mRNA granules and MT bundles (Fig. 1B). In contrast, the addition of betaine to obtain a similar osmolality without increasing the ionic strength fails to induce mRNA aggregation and MT bundling.

In addition to these results, we can remark that MT bundling occurs at very low PEG 35K concentrations (~1% w/v) compared with that required for mRNA granule formation (~20%). This could be due to the large interacting surface between MTs in bundles (30) compared with that of mRNAs and the heterogeneous charge distribution of MTs with some positive charge on the MT body (40) and a highly negatively charged C-terminal tail that facilitates short-ranged electrostatic attraction.

We may then wonder why MTs do not naturally form bundles in the cell cytoplasm, which is naturally crowded with macromolecules (27–29). In fact, MT diffusion in the cell cytoplasm is hindered by obstacles like actin filaments (41), which can slow down the bundling rate (42), and MTs are highly dynamical polymers, which reduces their chance to form bundles because of their limited lifetime. In line with this, taxol, an agent that stabilizes MTs, promotes the formation of MT bundles in cells (43). Interestingly, hypertonicity also favors the formation of MT bundles in taxol-treated cells, whereas hypotonicity inhibits their formation (supplemental Fig. S1).

Regarding the high PEG 35K concentration required to form mRNA granules in vitro, we should note that multiple mRNA protein partners are associated with mRNAs in SGs, like the prion-like protein TIA-1, which may act in combination with macromolecular crowding to favor the formation of SGs in cells. For example, when YB-1, a major mRNA-binding protein (44), is mixed with mRNA to form ribonucleoprotein particles, the PEG concentration required to trigger in vitro the compaction is lower than that needed for naked mRNAs (supplemental Fig. S2).

Hypertonicity Promotes SG Assembly and MT Bundling in NRK Cells

Given the AFM results, we explored how hypertonicity leads to the formation of SGs in NRK cells and whether MT bundling occurs simultaneously. To detect the formation of SGs, we used YB-1, which is an interesting marker to track the formation of SGs because of its cytoplasmic location and its binding to mRNA before and after stress (45). In control experiments (supplemental Fig. S3) we confirmed that YB1 is indeed present in SGs and co-localizes with other SG markers like HuR (46). SGs were detected for NaCl- and sucrose-mediated hypertonic shocks but not with molecules that cross the cell membrane like urea (Fig. 2), thus indicating that these granules were truly of osmotic origin. The formation of YB-1-containing SGs was observed above 520 mosmol/kg (Fig. 3A). These granules shared the typical properties of other SGs related to oxidative stress or hyperthermia (47). Cycloheximide, a polysome stabilizer, prevents their formation (supplemental Fig. S4), whereas puromycin, which induces premature chain termination during translation, has no effect on the assembly mechanism (data not shown). The extracellular osmolality modulates the size of SGs, which significantly increases from 520 to 720 mosmol/kg (Fig. 3B). A similar pattern was obtained for MT

Compatible Osmolytes Induce Stress Granule Disassembly
bundling, which starts to be significant above 620 mosmol/kg. This suggests that a similar mechanism may be involved in these two processes, i.e. excluded volume interactions. To investigate whether the appearance of SGs could provide clues to the cell fate, we measured the rate of cell survival after 6 h of hypertonicity and found a sharp decrease of cell viability at osmolalities higher than 520 mosmol/kg (Fig. 3C). In addition, we noticed that a significant phosphorylation of eIF2α, which was reported to promote apoptosis upon hypertonic stress (22), also occurs at a critical osmolality of 620 mosmol/kg (Fig. 3D).

**Extracellular Osmolarity Modulates Assembly of SGs Triggered by Oxidative Stress**

Albeit there is a concomitant appearance of MT bundles and SGs, this does not signify that macromolecular crowding and high ionic strength drive the forces behind SG assembly. To further address this issue, NRK cells were incubated in media of osmolality ranging from pronounced hypotonicity (170 mosmol/kg) to high hypertonicity (620 mosmol/kg) for 45 min. The cells were then exposed to varying concentrations of arsenite for 45 min to trigger the formation of SGs via oxidative stress (25). Arsenite, which is probably taken up in NRK cells via aquaglyceroporins (48), induces the rapid formation of SGs under isotonic conditions (45) at lethal doses (LD₅₀ about 100–300 μM for 1–2 h exposition in hepatoma cells and mouse fibroblasts (49, 50)). As shown in Fig. 4A, increasing the extracellular osmolality promotes the formation of arsenite-mediated SGs. On the other hand, hypotonicity inhibits their assembly. We may, however, wonder if hypotonic conditions impair the formation of SGs through other effects than decreasing macromolecular crowding and lowering ionic strength, for instance, by preventing the shuttling of critical proteins from cell nucleus to cytoplasm. To explore this idea, we first treated cells with arsenite to trigger the shuttling of the critical proteins and the formation of SGs and then exposed cells to varying osmolalities for 45 min before observation. Here again hypotonicity promotes SG dissociation, whereas hypertonicity preserves SG assembly (Fig. 4B). In addition, for cells first exposed to arsenite under hypotonic conditions, which does not lead to SG assembly, increasing the extracellular osmolality leads to their formation even in the absence of arsenite. Then the extracellular osmolality modulates the ability of oxidative stress agents to form SGs, most probably via its influence on both macromolecular crowding and the intracellular ionic strength. To finish, SGs and MT bundles formed under acute hypertonicity disappeared within minutes upon return to isotonicity (Fig. 4C), thus indicating that excluded volume interactions are mandatory to preserve SG assembly.
The Accumulation of Compatible Osmolytes Is Required for Dissociation of SGs

When cells grown in isotonic conditions are exposed to hypertonicity, the first SGs appear after about 15 min of hypertonicity (supplemental Fig. S5). Such a time lapse is required for critical proteins like TIA-1 to shuttle from the cell nucleus to the cytoplasm and promote SG assembly (25). In addition, the aggregation of mRNA-containing particles into large SGs occurs at a slow rate due to hindered diffusion in the cytoplasm (45). For hypertonic stress, the SGs are rather homogeneously distributed in the cytoplasm, and in contrast with arsenite-mediated SGs (45), their size and localization weakly depended on the presence of MTs (supplemental Fig. S6), probably a consequence of MT bundling.

In contrast with SG assembly, SG dissociation under constant hypertonicity is long and occurs between 9 and 24 h in the absence of extracellular osmolytes (Fig. 5A). Such a time lapse is required for critical proteins like TIA-1 to shuttle from the cell nucleus to the cytoplasm and promote SG assembly (25). In addition, the aggregation of mRNA-containing particles into large SGs occurs at a slow rate due to hindered diffusion in the cytoplasm (45). For hypertonic stress, the SGs are rather homogeneously distributed in the cytoplasm, and in contrast with arsenite-mediated SGs (45), their size and localization weakly depended on the presence of MTs (supplemental Fig. S6), probably a consequence of MT bundling.

In parallel, we measured the intracellular content of betaine to estimate its accumulation in NRK cells. We found that the betaine uptake may be accelerated in the presence of a high extracellular concentration of betaine, as detected by NMR after 9 h (Fig. 5, B and C). Indeed there are both high affinity ($K_m = 0.12$ mm) and low affinity ($K_m = 5.6$ mm) betaine transport sites (52); the latter may then explain the accelerated accumulation. In this case the disassembly of SGs indeed occurs significantly earlier (Fig. 5, D and E). Here again, MT bundling follows the same trend, as thick MT bundles were observed in the absence of betaine after 9 h of hypertonicity, whereas these bundles were already dissociated with 10 mM betaine. Altogether, these results indicate that compatible osmolytes play a key role in the disassembly of SGs and MT bundles. Interestingly, a high extracellular betaine concentration (in the millimolar range) was already shown to have a protective effect to hypertonicity (53, 54).

Cell Preconditioning to Hypertonicity and Inhibition of SG Assembly

As compatible osmolyte accumulation leads to the dissociation of SGs, the idea is to accumulate compatible osmolytes in cells and to analyze whether this accumulation inhibits SG assembly under such condition. To that end, NRK cells were first preconditioned under moderate hypertonic conditions for 24 h to accumulate compatible osmolytes in the presence of betaine and then exposed to an additional hypertonic shock of large amplitude, +400 mosmol/kg, whatever the osmolality of the pretreatment. The results clearly show that cells preconditioned to hypertonicities higher than 370 mosmol/kg displayed significantly smaller granules than those grown under isotonic condition (Fig. 6A). In fact, the mean granule size decreases with the intracellular accumulation of betaine, which

Figure 4. The extracellular osmolality regulates the assembly of SGs in arsenite-stressed cells. A, NRK cells were pretreated at the indicated osmolalities for 45 min before the addition of arsenite for another 45 min. SGs were detected via anti-YB1 labeling. We observed that hypertonicity favors SG assembly in contrast to hypotonic conditions. Scale bar, 15 μm. Statistical analysis clearly indicates a positive correlation between SG formation and extracellular osmolality. B, NRK cells were first pretreated with arsenite for 45 min in iso- or hypotonic conditions and then exposed to various osmolalities in the absence of arsenite. Hypotonic exposure dissociates SGs, in contrast with hypertonicity, which preserves preformed SGs or can make them appear (pretreatment under hypotonic environment). Insets, NRK cells fixed at the end of the 45 min arsenite pretreatment. We observed the presence of SGs in iso- but not hypotonic environment. SGs were detected via anti YB1-labeling. Scale bar, 15 μm. C, NRK cells displayed a homogenous distribution of MTs and YB1 protein under isotonic conditions. SGs and MT bundles appeared after 45 min of hypertonic treatment, and interestingly, upon return to isotonicity for 5 min, both SGs and MT bundles were dissociated. SGs and MTs were observed via anti-YB1 and anti-tubulin staining, respectively. Scale bar, 15 μm.
is monitored by the extracellular osmolality of the pretreatment medium (Fig. 6B). The impaired SG assembly in cells preconditioned to hypertonicity is not specific to betaine but is shared by the other compatible osmolytes like taurine and myo-inositol (Fig. 6A). We also noticed that cell preconditioning inhibits the formation of MT bundles, which as usual indicates that MT bundling and SG assembly follow the same pattern of association and dissociation.

The presence of SGs may have a negative impact on cell survival due to their reported proapoptotic influence (22) and the necessity to recover mRNA translation for cell adaptation to chronic hypertonicity. In this context the disassembly of SGs mediated by compatible osmolytes may have a positive impact on cell survival. In agreement with this, cell preconditioning inhibits the formation of MT bundles, which as usual indicates that MT bundling and SG assembly follow the same pattern of association and dissociation.

Osmylate Efflux Induces Formation of SGs in Preconditioned Cells

To further explore the role of compatible osmolytes in impaired SG assembly after hypertonic preconditioning, we analyzed SG formation during the return to isotonic conditions and its relationship with compatible osmylate efflux that occurs under such conditions (55). To this end, after cell preconditioning for 24 h in hypertonic medium in the presence of betaine, cells were returned to isotonicity to allow the efflux of accumulated osmolytes for varying times and were again exposed to hypertonicity (400 mosmol/kg). The results clearly indicate that the osmolyte efflux promotes the formation of SGs (Fig. 7A), and the size of the granules increases with the magnitude of the efflux (Fig. 7B and C). We note, however, that even after 24 h of efflux, SGs were smaller than for non-preconditioned cells, which may be due to the partial osmolyte removal that persists for days (55). We also note that the osmolyte efflux promotes MT bundling, thus indicating exacerbated excluded volume interactions in the cell cytoplasm (Fig. 7D).

Cell-Cell Interactions Allow Rapid Preconditioning of Epithelial Cells

Compatible osmolytes are small neutral molecules that can be easily exchanged between cells communicating via gap junctions in NRK cells (56). We then explored the idea that cell preconditioning, if monitored by compatible osmolytes, can be transmitted from cell to cell. For this purpose, non-precondi-

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**FIGURE 5.** The accumulation of compatible osmolytes after hypertonic exposure promotes SG disassembly. A, shown is time-lapse immunolabeling of NRK cells labeled with anti-YB1 and anti-tubulin after hypertonic shock. Up to 9 h of hypertonic exposure, SG size grew with time, and MT bundles were thicker. Between 9 and 24 h, SGs and MT bundles dissociated in the few cells that survived. Scale bar, 15 μm. B, we analyzed by NMR the intracellular betaine accumulation in NRK cells after a hypertonic shock with various extracellular concentrations of betaine for 9 h. The results show that betaine transport in NRK cells under hypertonicity is accelerated in the presence of extracellular betaine. Results are the means ± S.D., and 1 a.u. corresponds to 72 fmol of betaine per NRK cell (see "Materials and Methods"). C, two typical NMR spectra were obtained as described in B. In the presence of increasing betaine concentration, the area of cellular betaine peaks (see the asterisks) significantly increases. D, NRK cells were exposed for 9 h to constant hypertonicity in the presence of varying concentrations of betaine. Betaine, above 2.5 mM, promotes the disassembly of SGs and MT bundles. SGs and MTs were detected with anti-YB1 and anti-tubulin immunostaining, respectively. Scale bar, 15 μm. E, shown are statistical measurements of the mean SG area obtained from the analysis of NRK immunostained with anti-YB1 after the indicated treatment. Although SG size increased between 6 and 9 h for hypertonic-stressed cells in the absence of betaine, a net decrease in SG size was detected in the presence of 10 mM betaine. Results are the means ± S.D.
tioned NRK cells were transfected with GFP-YB1 and then deposited on a layer of NRK cells that had been preconditioned to hypertonic stress or not. After 4 h, to allow the incorporation of transfected cells in the cell monolayer and the formation of gap junctions (57), samples were exposed to acute hypertonic shock to eventually form SGs. The cell transfection and the expression of GFP-YB1 by themselves did not inhibit the formation of SGs, and tagged YB-1 also co-localized with SGs (Fig. 8A). As a control, the incorporation of transfected cells into the non-preconditioned monolayer of NRK cells did not change the SG appearance, with both transfected and non-transfected cells displaying large granules. On the other hand, their incorporation into a monolayer of preconditioned cells led to a significant inhibition of SG assembly. In addition, when transfected cells

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**FIGURE 6.** Hypertonic preconditioning inhibits SG assembly and promotes cell survival. A, NRK cells were pretreated for 24 h as indicated. Then, NaCl osmotic shock was applied for 45 min by increasing the osmolality by 400 mosmol/kg with respect to the osmolality of the pretreatment medium. In the first column, we observed that increasing the osmolality of the pretreatment in the presence of betaine leads to the progressive impairment of SG assembly. In the second column, we observed that not only betaine but also myo-inositol and taurine can also impair SG assembly. These same remarks are also valid for the impairment of MT bundling. SGs and MTs were detected with anti-YB1 and anti-tubulin immunostaining. Scale bar, 10 μm. B, statistical analysis of the SG areas obtained from A and NMR measurement of the betaine accumulation in cells after 24 h of hypertonicity is shown. As expected, the magnitude of the intracellular betaine accumulation is dictated by the extracellular osmolality, and there is a negative correlation between the SG size and the amount of intracellular betaine. Results are the mean ± S.D. (1 a.u. corresponds to 72 fmol of betaine per NRK cell). C, Western blotting of cells extracts from preconditioned NRK cells exposed to an increase of osmolality (+400 mosmol/kg) for 45 min is shown. The phosphorylation of the initiation factor eIF2α is significantly reduced for cells preconditioned in the presence of betaine (10 mM). Control, NRK cells under isotonic condition with 10 mM betaine without hypertonic shock. GAPDH was used as a loading control. D, shown is the rate of cell death after hypertonic shock (+400 mosmol/kg for 6 h) applied to preconditioned NRK cells as determined via trypan blue exclusion. Cell preconditioning under hypertonic environment in the presence of betaine (300 μM) promotes cell resistance to hypertonic aggression. E, shown is phase contrast optical microscopy of NRK cells preconditioned as indicated and exposed to a hypertonic shock (+400 mosmol/kg for 6 h). We observed that without hypertonic preconditioning the integrity of the epithelia was affected, and many cells were detached, in contrast to preconditioned epithelia, which were protected from these damages. Together, these results confirm that hypertonic preconditioning in the presence of betaine promotes cell resistance to hyperosmolarity. See also supplemental Fig. S7 for the effects of other extracellular compatible osmolytes (taurine and myo-inositol).
were not yet fully incorporated in the cell monolayer or in the presence of oleamide, an inhibitor of gap junction communications (58, 59), larger SGs were observed (Fig. 8, A and C). Altogether, these results indicate that cell-cell interactions, at least partly via gap junction communications, allow preconditioned cells to share with non-preconditioned cells their aptitude to limit the formation of SGs, presumably through the transit of compatible osmolytes.

**DISCUSSION**

**Biophysics of SG Assembly after Hypertonic Shock**—We show in this study that SG assembly is triggered by hypertonic shock provided that the variation of osmolality is larger than 200 mosmol/kg (Fig. 3). For such acute hypertonic shocks, cell volume can shrink within minutes to 30–40% of its initial value or even more depending of the amplitude of the hypertonic shock (60), which increases the intracellular macromolecular concentration (61), noted below as $c$. The ensuing increase of macromolecular crowding may then promote the attraction between macroscopic bodies like mRNA-containing particles or MTs (30, 62). We can estimate the impact of cell shrinkage on excluded-volume attractions by considering the benefit of interparticle interactions by reducing the electrostatic repulsion between macromolecules (63) or surfaces (32) like that of mRNA and MTs. As the recovery of a normal ionic strength requires the uptake of compatible osmolytes and thus is very long (between 12 and 24 h), this explains why the dissociation of SGs takes several hours under constant hypertonicity, as observed for NRK cells. Besides cell shrinkage, a high intracellular ionic strength also promotes excluded volume interactions by reducing the electrostatic repulsion between like-charged macromolecules (63) or surfaces (32) like that of mRNA and MTs. The recovery of a normal ionic strength requires the uptake of compatible osmolytes and thus is very long (between 12 and 24 h), which explains why the dissociation of SGs takes several hours under constant hypertonicity (Fig. 5). In line with this, when cells were returned to isotonic conditions, both the cell volume and the ionic strength returned to normal values, and the SGs dissociated within minutes (Fig. 4D). We can then conclude, as observed in vitro by AFM (Fig. 1), that both macromolecular crowding and high ionic strength promote the assembly of SGs in hypertonic-stressed cells and may favor the assembly of SGs triggered by other stresses (Fig. 4).

**Compatible Osmolytes Promote SG Dissociation and Cell Survival**—The accumulation of compatible osmolytes in the cell cytoplasm after cell preconditioning for 24 h in hypertonic medium impairs the assembly of SGs (Fig. 6). We also show that the phosphorylation level of eIF2α is significantly reduced (Fig. 6C). eIF2α phosphorylation is considered as an important step regarding the formation of stalled preinitiation complexes and their subsequent assembly into SGs in various stressful conditions (64, 65) even if SG assembly can also occur via other path-
In the case of hypertonicity, impaired eIF2α/phosphorylation cannot explain inhibition of SG assembly, as mutants deficient in eIF2α/phosphorylation are resistant to hypertonicity but are still able to form SGs (22). The results presented here, however, indicate that SG disassembly is most probably due to weaker excluded volume interactions in the cell cytoplasm of preconditioned cells. For instance, the efflux of compatible osmolytes before osmotic shock leads to the reappearance of small SGs (Fig. 7).

Another important point of this study is that preconditioned cells acquire a resistance to hyperosmotic challenge (Fig. 6, D and E). SGs are not inert aggregates of mRNA molecules and associated proteins. They fulfill many functions like mRNA storing, sorting, and degradation (22–24) and may then influence the positive or negative outcome after stress. In the case of hypertonicity, the phosphorylation of eIF2α allows the cytoplasmic accumulation in SGs of heterogeneous nuclear ribonucleoprotein A1, which may induce cell apoptosis (22). The inhibition of eIF2α phosphorylation can thus explain the acquired resistance to hypertonic shock. However, besides specific molecular mechanisms related to SGs, the long term presence of SGs generally indicates that the translational machineries are mostly arrested, a situation that cells cannot sustain for long under chronic hyperosmolarity. Compatible osmolytes can also promote cell survival by SG-independent mechanisms. The recovery of normal cell cytoskeleton after osmotic shock (56, 67), such as the MT bundle disassembly observed after betaine accumulation, may play a role in hypertonic adaption. In addition, compatible osmolytes counteract the negative effect of ionic strength regarding protein aggregation (68, 69) and have a positive impact on many enzymatic reactions (17) and mRNA translation (19) without considering the DNA- or membrane-related processes.

Is Cell Preconditioning by Compatible Osmolytes Relevant in Vivo?—The kidney and especially the renal medulla are permanently exposed to varying hypertonicities, for example, during diuresis and antidiuresis (70). In line with this, NFAT5-null mice display a profound atrophy of the kidney medulla, thus indicating that the accumulation of compatible osmolytes triggered by the NFAT5 transcriptional response is most probably mandatory for normal renal functions (35). However, many other tissues can experience hypertonicity in pathological conditions like the brain (71) and the liver (72), for which, respectively, taurine and betaine contents are osmo-regulated (73, 74). In addition, inflammatory disorders also induce local hypertonicity like in the intestine and cornea (75).

Besides the information about the fundamental processes associated to SG assembly and disassembly in hypertonic environment, here are three examples of possible insights into in vivo osmo-protection suggested by the following results. (i) Upon decrease in environmental osmolality, compatible osmolyte concentrations in kidney cells decrease sharply due a transient and rapid efflux occurring during the first 3 h and then significantly more slowly for days due to the slow decrease of compatible osmolytes, for example, the efflux of taurine and betaine is due to a rapid and a slow component (35, 67).
Compatible Osmolytes Induce Stress Granule Disassembly

active influx (76). A partial removal of compatible osmolytes can provide a “memory,” i.e. a long term protection in case of future rise in extracellular osmolality. (ii) Interestingly, the lymphoid environments of the liver but also the spleen and thymus are moderately hypertonic (~40 mosmol/kg) (77), which could stimulate NFA/T5-related osmolyte uptake. As indicated in this study, even a moderate hypertonic pretreatment may significantly increase the cell resistance to hypertonicity, which in this case may apply to lymphocytes or other cells exposed to this environment. (iii) We also show that the beneficial effect of cell preconditioning can be transferred from cell to cell provided that they are interacting via gap junctions (Fig. 8). Indeed cells exposed to constant hypertonicity can accumulate compatible osmolytes and then share these precious osmolytes with adjacent communicating cells even if these cells do not express compatible osmolyte transporters. This way, epithelia can sense a potential osmotic aggression coming. Such a strategy, if any, may be useful in many tissues and especially in the kidney, which abundantly expresses connexins (78).

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