Hyperosmolality Causes Growth Arrest of Murine Kidney Cells

INDUCTION OF GADD45 AND GADD153 BY OSMOSENSING VIA STRESS-ACTIVATED PROTEIN KINASE 2*

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Osmotic stress severely compromises functioning of eukaryotic cells, which must maintain homeostasis of inorganic ions as a prerequisite for metabolic processes to proceed properly. When eukaryotic cells are osmotically stressed they generally respond by regulating three functionally distinct sets of proteins. First, molecular chaperones and heat shock proteins are activated to counteract destabilization and unfolding of proteins because of osmotic stress (1, 2). Second, enzymes or transporters regulating compatible osmolyte levels are activated upon hyperosmotic stress and deactivated upon hypotonic stress (3, 4). Compatible organic osmolyte concentrations are regulated such that homeostasis of inorganic ion concentrations after disturbance because of cell volume regulation is reestablished. Third, proteins that define structural and functional aspects of specialized cell types at different osmolalities and certain immediate early genes are tightly regulated by osmotic strength. This is particularly the case in epithelial cells of animals that maintain osmotic homeostasis of their extracellular body fluids (osmoregulators), e.g. mammalian kidney cells (5) or teleost gill cells (6, 7).

In this study we asked first if a fourth group of proteins, the growth arrest and DNA damage-inducible (GADD) genes, that are commonly induced by stressors such as UV radiation, chemical carcinogens, starvation, etc. are also induced by osmotic stress. These proteins have been shown to be associated with growth arrest and to be involved in DNA damage repair in a variety of cell types (8). In vitro approaches have demonstrated strong effects of osmotic stress on DNA structure and function (9–12). On the contrary, prior to this study it was unknown whether animal cells are able to arrest their growth and induce GADD expression when exposed to osmotic stress or simply undergo cell death if a certain osmotic threshold is exceeded.

Our second objective was to test whether osmosensing mitogen-activated protein kinase (MAPK) signal transduction pathways are involved in osmotic regulation of GADD expression. The MAPK family of protein kinases contains many subfamilies of which at least 5 are represented by paralogous isoforms in vertebrates (13). Two of these MAPK subfamilies (the stress-activated protein kinases SAPK1 = JNK and SAPK2 = p38) display the highest degree of homology to the osmosensing yeast MAPK high osmolality glycerol kinase 1 (HOG1) and are able to complement ΔHOG1 mutants (14, 15). However, we previously demonstrated that SAPK1 and SAPK2 are not necessary for transducing hyperosmotic stress to the adose reductase osmotic responsive enhancer element and that osmoregulation of this mammalian enzyme differs in this respect from osmolyte-producing enzymes in yeast (16). We continued to search for possible targets of MAPK-osmosensing pathways and found that GADD protein expression is at least partially activated by SAPK2 phosphorylation and inhibited by extracellular signal-regulated kinase (ERK) phosphorylation. A
working model explaining these results is presented and the implications of our findings for strategies of cellular adaptation to osmotic stress are discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Experimental Design**—Murine inner medullary collecting duct (mIMCD) cells (17) of passage numbers 12–16 were used for all experiments. Monolayers were cultured in medium consisting of 45% Hams-F12 (Life Technologies, Inc.), 45% DMEM (Life Technologies, Inc.), and 10% fetal bovine serum (Life Technologies, Inc.). The final osmolality of this medium was 300 ± 5 mosmol/kg water and was prepared by addition of NaCl to regular growth medium. Exposure of mIMCD cells to either iso- or hyperosmotic medium was initiated during all experiments by addition of one-tenth of the total volume of either regular medium or 10x hyperosmotic medium, respectively. Quick mixing with regular medium was achieved by gently swirling the dish. Final osmolality in dishes exposed to hyperosmolality was 600 ± 5 mosmol/kg water as checked using a freezing point osmometer (μOsmette, Precision Instruments). 10x hyperosmotic medium of 3000 ± 5 mosmol/kg water was prepared by addition of NaCl to regular growth medium. Exposure of mIMCD cells to either iso- or hyperosmotic medium was initiated during all experiments by addition of one-tenth of the total volume of either regular medium or 10x hyperosmotic medium, respectively. Quick mixing with regular medium was achieved by gently swirling the dish. Final osmolality in dishes exposed to hyperosmolality was 600 ± 5 mosmol/kg water as checked using a freezing point osmometer (μOsmette, Precision Instruments). mIMCD cells were incubated at 37 °C, 5% CO₂ during all experiments.

**Determination of Growth Characteristics**—mIMCD cells were seeded at a density of 7.6 × 10⁴ cells in 10-cm dishes and allowed to attach for 6 h. At this time half of the dishes were exposed to hyperosmotic medium while the other half served as isosmotic controls (see above). Cells grown in either of these two different osmotic environments were harvested at the following times after seeding: 6, 30, 54, 78, 102, 126, and 150 h. Cell harvest was achieved by trypsinization (18). 2 aliquots were used for counting cells in a Neubauer hemocytometer, and the remainder was centrifuged at 500 × g for 2 min. The resulting cell pellets were stored frozen at −80 °C. Cell pellets were analyzed for total nucleic acid, DNA, and RNA contents using an assay based on binding of a fluorescent dye to cellular nucleic acids (CyQuant®, Molecular Probes). All assays were performed according to the manufacturer's instructions (Molecular Probes), and fluorescence intensity was measured with an SLT Fluorstar microplate fluorometer (Tecan). The excitation wavelength was set at 485 nm, and fluorescence intensity was recorded at an emission wavelength of 538 nm.

**Inhibitor Experiments**—To prevent targets of SAPK2 and ERK pathways from being activated by hyperosmolality we utilized highly specific inhibitors (19, 20) of SAPK2 (SB 203580, Calbiochem) and MEK (FD98059, Calbiochem). Both compounds were administered 2 h prior to osmolality shift of hyperosmotic or isosmotic conditions to allow for sufficient time for diffusion into cells and complete inhibition. Inhibitors were dissolved in ethanol in the dark to yield 25 mM stock solutions (PD98059, Calbiochem). For each blot an exposure time was chosen that gave good intensity of bands without exceeding the sensitivity of the film. Intensity of individual bands was quantified from an identical area by calculating the densitometric volume of this area and are given as densitometric volume units (DVU). Background correction was set to local median. All values were normalized to a reference band from the same sample that was set 100% on all films. Statistical data analysis was done using StatMost® software. Time series effects were evaluated by analysis of variance and differences between values within a single series by Student-Newman-Keuls test. Differences between pairs of data (hyper- versus isosmotic) at the same time point were tested for significance by F-test followed by either paired t test or Mann-Whitney test. All experiments were replicated between 4 and 6 times.

**RESULTS**

mIMCD cells arrest their growth after medium osmolality is increased from 300 to 600 mosmol/kg water by addition of NaCl. Cell numbers double during the first day after seeding in isosmotic medium (Fig. 1A). This initial growth is suppressed by hyperosmolality (Fig. 1A). Starting from day 5 the difference in cell numbers diminishes because cell monolayers start to reach confluence by this time (Fig. 1A). The duration of growth arrest was approximately 18 h as illustrated by the magnitude of shift of the growth curve after hyperosmotic exposure. Total nucleic acid content showed the same pattern compared with cell numbers and also illustrated growth arrest after hyperosmotic shock (Fig. 1B). Interestingly, the difference in DNA content between iso- and hyperosmotic samples is very small (Fig. 1C), whereas it is very large for RNA content (Fig. 1D). These data suggest that growth of mIMCD cells is arrested in G₁ rather than G₂ phase. Presumably, DNA replication rate is not strongly affected by hyperosmolality, whereas mitosis is delayed. This is reflected by low abundance of cellular RNA versus DNA (Fig. 1, C and D). In addition, data illustrating a strong decrease in RNA content (Fig. 1D) may reflect an inhibition of transcription by hyperosmolality.

Because we were able to demonstrate that mIMCD cells undergo a period of 18 h of growth arrest after hyperosmotic shock we tested if GADD proteins are induced by hyperosmolality. Indeed, we found that two GADD proteins, GADD45 (Fig. 2A) and GADD153 (Fig. 2B), are strongly induced by hyperosmolality. Time courses of induction are depicted for GADD45 (Fig. 2C) and GADD153 (Fig. 2D). The levels of GADD45 and GADD153 expression are very low in isosmotic controls and during the first hours following hyperosmotic exposure, but they increase dramatically 24–48 h after onset of...
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Our data on RNA and DNA contents in mIMCD cells indicate that hyperosmolality causes G2 arrest. The minor effect of hyperosmolality on cellular DNA content is consistent with relatively undisturbed DNA replication. On the contrary, transcription may be inhibited by hyperosmotic stress as indicated by the large difference in RNA content between populations of cells grown in iso- versus hyperosmotic medium. These findings, taken together with the differences observed in growth rates based on cell numbers, indicate that mIMCD cells proceed through G1 and S phase but are arrested in G2 before starting to divide. Most DNA-damaging agents are known to cause growth arrest in G2 suggesting potential DNA damage by hyperosmolality (8). G2 arrest may have a selective advantage to cells insofar as transcription and overall metabolism are slowed down such that possible osmotic damage may be kept at minimum.

Growth arrest has been shown to strongly induce GADD gene products in a variety of cell types (26, 27). In this study we provide evidence that this paradigm holds true for hyperosmotically stressed mIMCD cells. However, only GADD45 and GADD153 are induced by osmotic stress in mIMCD cells, whereas GADD34 remains unchanged at very low levels (data not shown). Therefore, GADD45/GADD153 and GADD34 are not cooperatively regulated by osmotic stress in mIMCD cells. At present it is unknown at which level (transcriptional, posttranscriptional, or translational) GADD45 and GADD153 are

FIG. 1. Hyperosmotic induction of growth arrest in mIMCD cells. Cells grown in isosmotic medium (300 mosmol/kg water) are symbolized by open squares, and cells grown in hyperosmotic medium (600 mosmol/kg water) by filled circles. A, cell numbers per 10-cm dish illustrating 18 h growth arrest that results in a shift of the growth curve to the right; B, total nucleic acid content per 10-cm dish; C, DNA content per 10-cm dish; D, RNA content per 10-cm dish. Data represent means ± S.E., n = 4. Asterisks symbolize significant effects of hyperosmolality (p < 0.005).

To assess whether the changes in phosphorylation of ERK and SAPK2 account for the observed induction of GADD proteins, we inhibited ERK and SAPK2 pathways using highly specific pharmacological inhibitors of MEK (specific ERK activator) and SAPK2. Inhibition of SAPK2 resulted in a 37.5% decrease in hyperosmotic GADD45 induction (Fig. 6A) and 19.6% decrease in hyperosmotic GADD153 induction (Fig. 6B). On the contrary, inhibition of the ERK pathway resulted in further potentiation of hyperosmotic GADD45 induction by 65.2% (Fig. 6A) and of GADD153 induction by 28.7% (Fig. 6B). These results suggest that hyperosmotic induction of GADD45, and to a lesser degree of GADD153, is positively regulated via SAPK2 and negatively regulated via ERK osmosensing pathways. However, because SB203580 only partially prevented the increase in GADD45 and GADD153 expression, it is clear that additional, as yet unidentified elements, are also involved in hyperosmotic GADD induction.

DISCUSSION

Hyperosmolality did not affect the expression of any of the MAPKs analyzed within a period of 2 days (Fig. 4). Therefore changes in the abundance of these kinases can be excluded from being responsible for hyperosmotic induction of GADD45 and GADD153 expression. Interestingly, SAPK2 phosphorylation had an inhibitory effect on ERK phosphorylation even though activation of both of these MAPKs is hyperosmotically induced (Fig. 5). This cross-talk between SAPK2 and ERK pathways may explain the relatively high variability of phospho-ERK levels following hyperosmotic stress (Fig. 3D).

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primarily induced in response to osmotic stress. The elements involved in this regulation remain to be elucidated.

Currently, the function of GADD proteins is also unknown although GADD153 has been shown to dimerize with and alter the binding capacity of the CCAAT/enhancer-binding protein transcription factors (28, 29). Time courses of GADD45 and GADD153 induction are somewhat stressor-specific, being very rapid (maximum at 1 h, returning to baseline levels within 24 h) in response to DNA-damaging carcinogens and UV radiation (26, 27). In these cases it has been suggested that growth arrest, often in G1 phase, is a result of GADD induction (30, 31). Our data indicate that osmotic stress leads to a much slower but more persistent induction of GADD45 and GADD153 resembling the kinetics observed by medium depletion (32). By comparing time courses of growth arrest and GADD45/153 induction in mIMCD cells it is evident that induction of GADD proteins does not precede G2 arrest in mIMCD cells. Rather, hyperosmotic GADD45/153 induction seems to be a conse-
sequence of growth arrest or occur in parallel to growth cessation. The tumor suppressor p53 has also been reported to either induce growth arrest (33) or to be induced as a result of growth arrest (32) depending on the nature of the stress and cell type.

Growth arrest and GADD protein induction were shown to be triggered by a wide variety of DNA-damaging agents, including chemical carcinogens, starvation, hypoxia, UV radiation, and other stresses (8). Moreover, GADD153 and GADD45

Fig. 4. Hyperosmolality does not affect MAPK expression in mIMCD cells. One example of time courses of expression in isosmotic and hyperosmotic medium is depicted for the following MAPKs: ERK (p44, p42) (A), SAPK1 (JNK) (B), and SAPK2 (p38) (C). Results of quantitative densitometric analysis are depicted for ERK (D), SAPK1 (E), and SAPK2 (F). Squares with S.E. at bottom symbolize samples exposed to isosmotic medium, and circles with S.E. on top symbolize samples exposed to hyperosmotic medium. ERK1 (p44) (D) and p46 SAPK (E) are depicted as closed symbols connected by continuous lines, whereas ERK2 (p42) (D) and p54 SAPK (E) are plotted as open symbols connected by dotted lines. Data are given as DVU (see “Experimental Procedures”) and represent means ± S.E., n = 4. Asterisks symbolize significant effects of hyperosmolality (p < 0.05). Note that there is an axis break and change of units of time after 60 min.

Fig. 5. Hyperosmotic induction of ERK is increased by inhibition of SAPK2. Panel A depicts data obtained for ERK1 (p44) phosphorylation and panel B for ERK2 (p42) phosphorylation at 30 min following onset of hyperosmotic exposure. The SAPK2-specific inhibitor SB203580 does not affect basal levels of phospho-ERK in isosmotic medium but leads to significantly higher phospho-ERK levels in hyperosmotic medium. Data are given as DVU (see “Experimental Procedures”) and represent means ± S.E., n = 6. Asterisks symbolize significant effects of SB203580 (p < 0.05).

Fig. 6. Hyperosmotic induction of GADD expression is positively regulated via SAPK2 phosphorylation and counteracted by ERK phosphorylation. Panel A depicts data obtained for GADD45 expression and panel B for GADD153 expression at 24 h following onset of hyperosmotic exposure. SB203580 specifically inhibits SAPK2, and PD98059 specifically inhibits the ERK activator MEK. Neither inhibitor has an effect on basal levels of GADD45 or GADD153 in isosmotic medium. SB203580 partially suppresses and PD98059 further elevates the induction of expression of both GADD45 and GADD153 in hyperosmotic medium. Data are given as DVU (see “Experimental Procedures”) and represent means ± S.E., n = 6. Asterisks symbolize significant inhibitor effects (p < 0.05).
are specifically involved in DNA damage repair and activated in this capacity via the tumor suppressor p53 (34). Thus, it is tempting to speculate that osmotic stress leads to DNA damage that in turn triggers growth arrest and GADD45/153 induction as part of a DNA repair mechanism. The time courses of GADD45 and GADD153 induction seem to contradict this notion. These proteins are induced strongest only after mIMCD cells have resumed to grow again (at 24 h). Thus, the need for DNA repair may not be immediate anymore by the time GADD45 and GADD153 are present at highest levels. Alternatively, GADD45/153 may be involved in DNA stabilization at high osmotic strength and/or required for lifting the G2 growth arrest.

A variety of in vitro experiments employing different methodologies have convincingly demonstrated strong effects of osmotic strength on DNA structure (10, 12) and transcriptional activity (5, 9, 11). In general, transcription is severely inhibited by deviations from intracellular osmotic homeostasis that is caused by noncompatible osmolytes (9). In fact, optimization of transcriptional activity for specific (homeostatic) osmotic conditions may well represent one of the major forces leading to the extraordinary wide phylogenetic conservatism of intracellular inorganic ion concentrations. This scenario is supported by our data demonstrating a large difference in RNA content between populations of cells grown in isosmotic versus hyperosmotic medium. However, despite this overall negative effect of hyperosmolality on RNA content, certain genes of particular adaptive value are induced instead of repressed under these conditions. Examples include genes for osmolyte-producing enzymes, e.g. aldose reductase, or osmolyte transporters, e.g. betaine transporter (4). In some of these genes, specific osmotically or toxicity-responsive enhancer elements have been identified in the 5'-untranslated region, and progress is being made in elucidating the molecular pathways involved in the regulation of these elements (35, 36). The adaptive value of these genes lies in the restoration of ionic homeostasis after a disturbance by replacing excessive inorganic ions with compatible osmolytes. The adaptive advantage of GADD45 and GADD153 gene expression may lie in enhanced repair of damaged DNA caused by osmotic fluctuations, but this remains to be confirmed. Alternative functions of GADD proteins may include lifting of G2 growth arrest or stabilization of DNA in unfavorable situations, e.g. during hyperosmotic stress. Similarly, it has been shown that hyperosmotic stress induces molecular chaperones that protect proteins from unfolding and degradation (1). Presently, it is not known whether osmolyte-producing enzymes and transporters, molecular chaperones, and GADD proteins are all regulated by a similar mechanism operating coordinately in situations of hyperosmotic stress or by multiple strategies. A key question remains how these genes and proteins of adaptive value are induced, whereas the majority of genes and metabolic activity seems to be repressed by osmotic stress.

To address this question we analyzed three osmosensing signal-transduction pathways for their potential to confer osmotic regulation of GADD45 and GADD153. The three pathways analyzed are all phosphorylation cascades arranged around a particular MAPK. We chose to evaluate the ERK pathway, the SAPK1 (JNK) pathway, and the SAPK2 (p38) pathway for their potential to signal GADD45 and/or GADD153 induction. Our results demonstrate that all three pathways are hyperosmotically activated in mIMCD cells, which is consistent with an independent study published recently (37). We find that differences in the kinetics of hyperosmotic activation of ERK, SAPK1, and SAPK2 exist and that activation of all three MAPK pathways was exclusively because of phosphorylation without a change in the number of kinases present. Activation kinetics was very fast for SAPK2 and ERK, and slower for SAPK1. In contrast to SAPK2s, constitutive levels of phospho-ERK are significant and do not increase much after exposing mIMCD cells to hyperosmotic medium. A negative feedback mechanism might explain the early maximum and rather large fluctuations in phospho-ERK levels observed after hyperosmotic shock. Indeed, it has been demonstrated that SAPK2 activation leads to activation of a MAPK phosphatase (MKP1), which specifically dephosphorylates ERK (38).

Recent evidence suggests that the betaine transporter gene is a target of SAPK2 in Madin-Darby canine kidney cells (39). However, we previously demonstrated that SAPK-osmosensing pathways in rabbit PAP-HT25 cells do not target the osmotic responsive enhancer element of the aldose reductase gene (16). In addition, it has been demonstrated that osmolyte transporter genes are not regulated via the ERK pathway (40) or the SAPK1 pathway (41). We were therefore interested in other potential targets of MAPK-osmosensing pathways in mammalian kidney cells and tested if GADD45 and GADD153 are osmotically regulated via SAPK2 or ERK phosphorylation cascades. We find that GADD45/153 expression is positively regulated by the SAPK2 and negatively regulated by the ERK pathways (Fig. 6). The physiological significance of negative feedback regulation of phospho-ERK levels may lie in conferring signal specificity and/or to reinforce the positive effect of SAPK2 phosphorylation on GADD expression by minimizing negative effects of phospho-ERK. Because inhibition of SAPK2 only partially diminishes hyperosmotic induction of GADD proteins, other pathways are likely to be involved. One candidate is the SAPK1 (JNK) pathway, which has been shown to be involved in cell cycle regulation (42), apoptosis (43), and p53 phosphorylation (44). Unfortunately, a specific pharmacological inhibitor of SAPK1 was not readily available and a negative dominant mutant of SEK1 (MKK4) could not be employed for these studies because of side effects of the transfection procedure on GADD45 and GADD153 expression in mIMCD cells. Our current working hypothesis summarizing events leading to hyperosmotic GADD45/GADD153 induction is depicted in Fig. 7. Hyperosmolality activates SAPK2 and ERK very rapidly (within minutes) and also causes mIMCD cells to arrest their growth very rapidly. This growth arrest lasts for about 18 h. Growth arrest in G2 may be either a direct consequence of hyperosmotic inactivation of molecular components required...
for mitosis, or it may be mediated by SAPK1, SAPK2, ERK, or other, as yet unknown, pathways. GADD45 and GADD153 are only expressed at highest levels at the time when cells start to divide and overcome the growth arrest (24–48 h after onset of hyperosmotic stress). Induction of GADD45 and GADD153 is at least in part mediated via the SAPK2 pathway. SAPK2 reinforces its positive effect on GADD induction by inhibiting the ERK pathway, which suppresses GADD45 and GADD153 induction after hyperosmotic stress (Fig. 7). This negative feedback may be mediated by a mitogen-activated protein kinase phosphatase, which specifically dephosphorylates ERK, e.g., via mitogen-activated protein kinase phosphatase 1. Additional pathways are involved in hyperosmotic GADD45/GADD153 induction. Potential candidates are SAPK1 and p53-dependent pathways.

In conclusion, we demonstrate that hyperosmolality induces growth arrest in murine kidney (mIMCD) cells and that this apparently triggers a late induction of GADD45 and GADD153. Hyperosmotic induction of GADD45 and GADD153 is partially conferred via the SAPK2 osmosensing pathway and negatively regulated by the ERK pathway. These results stimulate further research addressing the nature of DNA-damage resulting from osmotic stress and the integration of molecular messengers in response to hyperosmolality.

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