Three GADD45 isoforms contribute to the hypertonic stress phenotype of murine renal inner medullary cells

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Abstract

Mammalian renal inner medullary (IM) cells routinely face and resist hypertonic stress. Such stress causes DNA damage, to which IM cells respond with cell cycle arrest. We report that three Growth Arrest and DNA Damage inducible 45 isoforms (GADD45α, GADD45β, GADD45γ) are induced by acute hypertonicity in murine IM cells. Maximum induction occurs 16 - 18 h after the onset of hypertonicity. GADD45γ is induced stronger (7-fold) than GADD45β (3-fold) and GADD45α (2-fold). GADD45α and GADD45β protein induction is more pronounced and stable compared to the corresponding transcripts. Hypertonicity of various forms (NaCl, KCl, sorbitol, mannitol) always induces GADD45 transcripts, whereas non-hypertonic hyperosmolality (urea) has no effect. Actinomycin D does not prevent hypertonic GADD45 induction, indicating that mRNA stabilization is the mechanism. GADD45 induction patterns in IM cells exposed to ten different stresses suggest isoform-specificity, yet similar functions of individual isoforms during hypertonicity, heat shock, and heavy metal stress, when GADD45γ induction is strongest (17-fold). These data associate all known GADD45 isoforms with the hypertonicity phenotype of renal IM cells.

Key words: GADD45, cell cycle, hypertonicity, nephrotoxins, kidney inner medulla.
Introduction

Cells of the mammalian renal inner medulla are routinely subjected to a wide range of osmolality as part of their function in renal urinary concentration. They express a phenotype that allows them to counteract the threat posed by hypertonicity and other stresses prevalent in the renal inner medulla. Because hypertonic stress represents such an immense threat to most human cells, it is critical to understand the molecular basis of the phenomenon and to study the cellular mechanisms by which cells minimize its consequences. Hypertonic stress damages proteins leading to their unfolding and malfunction (35). This is compensated by compatible and counteracting organic osmolytes, which are accumulated during hypertonic stress in many cell types, including renal IM cells (reviewed in (4, 14, 31). DNA is also threatened by hypertonic stress, which increases the amount of DNA double-strand breaks (19) and chromosomal aberrations (13).

We have previously provided evidence that hypertonicity leads to activation of a complex network of intracellular signaling pathways including mitogen-activated protein (MAP) kinase pathways (20), the p53 pathway (8) and DNA-dependent protein kinases (19). Previous evidence also indicates that the GADD 45 (Growth Arrest and DNA Damage inducible 45) family of genes is part of such networks (20, 33) but little is known about the osmotic regulation of the three mammalian GADD45 isoforms, prompting us to analyze this aspect of the hypertonic stress phenotype of IM cells.

The first GADD45 gene, later designated GADD45α, was cloned on the basis of its induction following ionizing radiation-induced DNA damage and cell cycle arrest (12). Subsequently, two other GADD45 genes have been identified and designated GADD45β and GADD45γ (2, 18, 24, 32, 33, 37). GADD45 proteins are necessary for maintaining DNA integrity and genome stability
in mammalian cells (16) and their induction is often a consequence of DNA damage. They are involved in many processes during cellular adaptation to a diverse array of cellular stresses, including apoptosis, DNA repair, chromatin regulation, and cell cycle delay (5, 28). Understanding the osmotic regulation of GADD45 should help identify the cell functions that are regulated by these proteins during hypertonic stress.
Materials and methods

**Culture of mIMCD3 cells:** Murine inner medullary collecting duct (mIMCD3) cells of passage number 15 were used for all experiments (25). Monolayers were grown to 90% confluency in isosmotic medium consisting of 45% Hams-F12, 45% D-MEM, 10% fetal bovine serum, and 1% of a saline solution containing 10 mU/ml penicillin and 10 µg/ml streptomycin (Invitrogen-Life Technologies Corp.). The final osmolality of the medium was 300 ± 5 mosmol/kg as verified using a vapor pressure osmometer (Wescor, Model 5500). Hypertonic media were prepared by addition of the appropriate solute to the isosmotic medium to achieve the desired osmolality. UV irradiation was done in a sterile tissue culture hood by epi-illumination of cell monolayers with a UV-C and UV-B probe (UVP, San Diego, CA). The distance of the lamp to the cell monolayers and the time interval of UV exposure were calibrated to achieve a dose of 500 J/m². From the start of UV irradiation cells were given a period of 18 h to respond to the UV pulse before they were analyzed to be consistent with the response period allowed for other stresses and previously published protocols for stressing cells with UV. Chemical toxicants were added to the medium in the concentrations indicated and cells exposed to these concentrations for a period of 18 h. Cells were incubated at 37°C, 5% CO₂ during all experiments except during heat shock when they were incubated at 42°C.

**Passage 2 mouse IM epithelial (p2mIME) cells:** p2mIME cells were prepared and maintained as described previously (9, 38). Briefly, male mice (4-8 weeks old, B6SJL) were sacrificed by cervical dislocation, shaved, and cleaned with an antiseptic solution containing 70% ethanol and Betadine. After dissecting the kidneys using aseptic technique they were transferred into a sterile plastic tube containing phosphate buffered saline (PBS). The inner medulla was dissected from both kidneys using aseptic technique and minced into 1-2 mm cubes. These tissue pieces were
transferred to an Erlenmeyer flask containing a sterile solution of 500 ml of D-MEM/F12 (Gibco BRL cat. number 21041-025), 80 mM urea, 130 mM NaCl, 50 mg/25 ml of collagenase B (Roche, cat number 1088 807), and 18 mg/25 ml of hyaluronidase (Worthington Chemical Corporation, cat number 2592). The final osmolality of this solution was approximately 615 mOsmol/kg. The tissue pieces were incubated in this solution under constant agitation (300 rpm) for 90 minutes at 37 °C, humidified air, and 5% CO2. Every 20 minutes the cell suspension was mixed by repeated aspiration into a 10 ml pipette. At the end of the enzymatic cell disaggregation the cell suspension was centrifuged at 160 g for 1 minute, and washed 3 times at 37°C. Cells were suspended in 5 ml medium containing 45% DMEM (low glucose, Cellgro 99-688-CV), 45% Coon’s improved F-12 (Cellgro 99-687-CV), 10% Fetal Bovine Serum, 10 mM HEPES (pKa 7.5, pH 7.5), 5 mg/L T3, 5 pM transferrin, 10 nM selenium, 50 nM hydrocortisone, 2 mM L-glutamine, 2.5 ml/L Penicillin G/Streptomycin sulfate (10,000 U/ml, Irvine 936620120), 80 mM urea, and 130 mM NaCl. The final osmolality of this medium was 640 mOsmol/kg. Cells were seeded into a 6 cm dish (Corning). When confluent, they were split at a 1:4 ratio and grown to confluence. Then, prior to the experimental manipulation, the medium was switched for 48 hours to an otherwise identical one, lacking serum and urea, and with reduced NaCl, yielding a final osmolality of 300 mosmol/kg. Based on our previous studies with p2mIME cells, 48 hours are sufficient for them to recover from the hypotonic stress, considering that their subsequent response to hypertonicity is little affected (38). Thus, we were able to use a hypertonic stress protocol for p2mIME cells that is comparable to the protocol used for mIMCD3 cells.

**RNA and protein extraction:** For total RNA extraction cells were lysed in 800 µl per 10 cm dish of denaturing solution (4M guanidine isothiocyanate, 0.02M sodium citrate, 0.5% sarcosyl,
and 0.7% β-mercaptoethanol. Total RNA was isolated by phenol-chloroform extraction (6). The RNA pellet was quickly resuspended in 50 – 100 µl of DEPC-treated water. A 1µl aliquot was mixed with 119µl of Tris-EDTA buffer (pH 8.0) and used for spectrophotometric quantification of RNA yield. The A260/A280 ratios were typically ≥ 2.0.

For protein extraction cells were lysed by addition of 500 µl ice-cold cell lysis buffer to each 10 cm tissue culture dish followed by a 10 min at 4°C. The cell lysis buffer contained 50 mM Tris-HCl (pH 7.4), 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 tablet mini-complete protease inhibitor cocktail (Roche), 1 mM activated Na₃VO₄, and 1 mM NaF. Samples were centrifuged at 20,000 g for 10 min, 4°C and the supernatants saved into a fresh Eppendorf tube. A 5 µl aliquot was used for assaying protein concentration and the remainder divided into 50µl aliquots and stored at –80°C. Protein concentration was measured using BCA reagents (Pierce) and a Spectromax Plus microplate reader (Molecular Devices).

**GADD45 cloning and Riboprobe generation:** PCR primers for cloning full-length GADD45 cDNAs were designed based on Genbank accession numbers NM_007836 (murine GADD45α), NM_008655 (murine GADD45β), and NM_011817 (murine GADD45γ) using Vector-NTI 5.5. The following primer pairs were used for amplification of the GADD45 CDS from mIMCD3 cell RNA by RT-PCR: GADD45α: primer 1 = ACTTTGGAGGAATTCTCGGCT, primer 2 = AATCACGGGCACCCACTGATCCA; GADD45β: primer 1 = ATGACCCTGGAAGAGCTGGT, primer 2 = CCAGGAGGCAGTGCAGGTCT; GADD45γ: primer 1 = TCTGGAAGAAGTCCGTGGCCA, primer 2 = GATGCTGGGCACCCAGTCGT. The three GADD45 cDNAs were then cloned into pGEM-T vector (Promega) before sub-cloning them into pCDNA5/FRT vector (Invitrogen-Life Technologies Corp.) in reverse orientation such that *in vitro* transcription of the insert with T7 polymerase yields antisense mRNA. Plasmid
minipreps were made using Qiagen kits as described by the manufacturer followed by linearizing the plasmids. The linear DNA template was purified and used for \textit{in vitro} transcription using T7 MAXIscript (Ambion). The resulting riboprobes were labeled with $^{32}$P-CTP (Perkin Elmer, NEG508X) and purified using AquaSelect-D G-25 columns (Eppendorf-5 Prime) to remove free nucleotides. Purified riboprobes were recovered in water for subsequent use in Northern hybridization.

**Northern analysis:** 7µg of total RNA from each sample were premixed with 5µl of formaldehyde-formamide loading dye (Ambion) containing ethidium bromide (50µg per 1ml of loading dye). Samples were heated to 65°C for 15min, quick-chilled on ice, and centrifuged at maximum speed for 10sec before loading them into the wells of 1% agarose-formaldehyde gel. Gels were electrophoresed in MOPS buffer and visualized by UV-transillumination in a Fluor-S Multiimager (Bio-Rad). RNA was transferred overnight from the gels to positively charged Biodyne B nylon membrane (Pall) by upward capillary transfer using an alkaline transfer buffer (0.01N NaOH /3M NaCl). RNA transfer was followed by cross-linking the RNA to the membrane as described by Sambrook and Russell (26). The membrane was pre-hybridized in UltraHyb buffer (0.1 ml/ cm$^2$ Ambion) at 65°C in a rotisserie-type hybridization oven for 3 h. Following pre-hybridization, radiolabeled riboprobe was added (1/1000, v/v) and allowed to hybridize overnight at 65°C. The membrane was washed twice with low stringency wash solution (2x SSC, Ambion #8673) for 5 min each, followed by two washes with high stringency buffer (0.1x SSC Ambion #8674) for 15 min each and imaged using a phosphorimager (Molecular Dynamics). Quantification was done with ImageQuant and QuantityOne software and values normalized for 28s rRNA.
Ribonuclease protection assay: RPA analysis was carried out as described previously (8) using the RPA III Kit (Ambion). Briefly, antisense, biotin-labeled RNA probes encoding the murine GADD45 mRNAs were synthesized using T7 polymerase (MAXIscript, Ambion) with purified RT-PCR products as templates. Antisense probes were then hybridized with 5 μg of RNA, and nonhybridized RNA was digested using ribonuclease. Protected fragments were separated on 6% TBE/urea polyacrylamide gels (NOVEX) at 23 mA constant current followed by transfer of RNA from gels to BrightStar nylon membrane (Ambion) at 200 mA constant current for 60 min. RNA immobilization to the nylon membrane was achieved by UV cross-linking with a Stratalinker 1800 (Stratagene). A non-isotopic detection kit from Ambion (Bright-Star BioDetect) was used for visualization of biotinylated probes hybridized to GADD45 on the membrane.

SDS-PAGE and Western immunodetection: Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described previously (20). Equal amounts of protein (20 μg) were loaded in each lane of 8% Tris/glycine polyacrylamide gels. The first lane of each gel was loaded with prestained molecular weight standards (Kaleidoskope, Bio-Rad). Samples were electrophoresed at 125 V constant voltage, the gels were briefly rinsed in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol), and proteins were blotted onto Immunobilon P membrane (Millipore) at 1 mA/cm² constant current for 90 min using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were blocked for 30 min at room temperature in a solution containing 137 mmol/ liter NaCl, 20 mmol/liter Tris, pH 7.6, with HCl, 0.1% v/v Tween-20, 5% w/v nonfat dry milk, and 0.02% w/v thimerosal. Then they were incubated for 3 h in blocking buffer containing either GADD45α (Santa Cruz Biotechnology sc-792, 1:100) or GADD45β antibody (Santa Cruz Biotechnology sc-7775, 1:100). After three washes in blocking buffer the blots were
incubated for 1 h in blocking buffer containing secondary antibody coupled to horseradish peroxidase (1:2000). Following more washes, blots were developed with SuperSignal Femto (Pierce) for 5 min and imaged using a Fluor-S MultiImager (Bio-Rad). Quantification of GADD45 bands was performed using Quantity-One software (Bio-Rad).

**Statistics:** Data analysis was carried out with STATMOST32 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 for the same time point were analyzed by F test followed by either paired t test or Mann–Whitney test. The significance threshold was set at P<0.05 and values represent means of at least three experiments for all data shown.
Results

All three GADD45 transcripts are induced in response to hypertonic stress in mIMCD3 cells. The expression of GADD45 mRNAs correlates with the adaptation of mammalian cells to a wide spectrum of genotoxic stresses (15). In addition, we have previously provided evidence that acute hypertonicity is genotoxic to renal cells (19) and previous reports have indicated that GADD45α protein and GADD45β transcript are induced during hypertonicity (20, 33). Therefore, we decided to investigate systematically the regulation of all three GADD45 transcripts in mIMCD3 cells after exposing these cells to hypertonicity, ranging from 400 to 675 mosmol/kg, for 18 h. These experiments show that the abundance of all three GADD45 mRNAs increases during hypertonicity. The increase in GADD45 mRNA abundance is statistically significant for all three GADD45 transcripts and highest when the hypertonic medium has an osmolality of 600 mosmol/kg (Fig. 1). GADD45α mRNA is induced 2.2-fold, GADD45β mRNA is induced ca. 3.3-fold, and GADD45γ mRNA is induced 7.1-fold. Interestingly, we did not observe any significant induction of either GADD45 transcript during less severe hypertonicity (400 and 500 mosmol/kg) and the level of induction was also less at 675 mosmol/kg compared to 600 mosmol/kg. Thus, the maximum level of induction of all three GADD45 transcripts coincides with the highest degree of acute, non-lethal hypertonicity that mIMCD3 cells can tolerate.

The hypertonic induction of GADD45 transcripts is transient but GADD45 protein induction is more stable. The kinetics of induction of GADD45 was determined for all three transcripts and for GADD45α and GADD45β also at the protein level to further our insight into their osmotic regulation and correlate GADD45 induction with other adaptive mechanisms utilized by mIMCD3 cells during hypertonicity. Exposure of mIMCD3 cells to hypertonicity of 600
mosmol/kg (NaCl↑) causes a rapid and transient increase of all three GADD45 transcripts as determined by Northern blot analysis (Fig. 2a). This increase was maximal after 18 h exposure to hypertonicity. As seen also in Fig. 1, the degree of hypertonic induction of the three GADD45 transcripts in response to hypertonicity differed considerably, in particular for GADD45γ, which was induced much more strongly than GADD45α and GADD45β. The kinetics of hypertonic GADD45 induction in p2mIME cells is very similar to that in mIMCD3 cells even though it was analyzed by ribonuclease protection assay and not Northern blots (Fig.2b). Maximum levels of induction were observed at 16 h for all three GADD45 transcripts. The only notable difference between the two cell types concerns the lesser degree of GADD45β induction in p2mIME cells compared with mIMCD3 cells. We performed Western blot experiments for GADD45α and GADD45β to complement the kinetic data on hypertonic GADD45 mRNA induction at the protein level. Western blot analysis of these two GADD45 isoforms in mIMCD3 cells revealed that the kinetics of hypertonic induction of GADD45 protein lags behind that of the mRNA induction (Fig. 2c). Even though this is expected, it was unexpected to see the levels of GADD45α and GADD45β protein still elevated at 36 h after the onset of hypertonicity, because the corresponding mRNA levels had already declined to near baseline values within 24 h (Fig. 2d). In addition, the maximal hypertonic induction of GADD45α and GADD45β protein is 4.3-fold and 4.1-fold, respectively, which is almost twice that observed at the level of mRNA abundance. Thus, the increase in the abundance of these two GADD45 proteins appears to be more stable and not exclusively mediated by increased transcription but by additional mechanisms as well. We did not succeed in Western blot analysis of GADD45γ because no suitable antibodies were identified.
**Osmotic regulation of GADD45 transcripts is specific to hypertonic stress and not seen with urea-imposed hyperosmotic stress.** The next question we addressed was whether the hypertonic induction of GADD45 transcripts were solute-specific. We tested this by comparing mRNA levels of GADD45 in mIMCD3 cells after dosing these cells for 18 h in 600 mosmol/ kg hyperosmotic medium, made by addition of either NaCl, KCl, sorbitol, mannitol, or urea. Hypertonicity in either form (NaCl↑, KCl↑, sorbitol↑, or mannitol↑) results in an induction of all three GADD45 mRNA transcripts, although the degree of induction is variable to some extent (Fig. 3a). Sorbitol has the most pronounced effect on the abundance of all three GADD45 transcripts. In contrast, non-hypertonic hyperosmolality resulting from urea addition to the medium does not induce any GADD45 transcript significantly (Fig. 3b). Surprisingly, the hypertonic induction of GADD45β and, to an even greater extent, GADD45γ mRNA is not as strong during hypertonicity in the form of KCl compared to hypertonicity in the form of the other solutes tested (Fig. 3). The cause underlying this effect is unknown. Nevertheless, these data clearly demonstrate that hypertonicity (i.e. a change in cell volume or intracellular ionic strength) and not just hyperosmolality *per se* is the trigger for the induction of all three GADD45 transcripts in mIMCD3 cells.

**Induction of GADD45 results from mRNA stabilization and not from nascent mRNA synthesis.** The up-regulation of GADD45 transcripts in response to hypertonicity could either be mediated by increased transcription or by mRNA stabilization. To differentiate between these two possible mechanisms we added actinomycin D to the cell culture medium during hypertonic stress experiments. Actinomycin D is a commonly used blocker of transcription that inhibits RNA synthesis. Addition of 10 µg/ ml actinomycin D to mIMCD3 cells kept in isosmotic medium strongly inhibits nascent mRNA synthesis of all three GADD45 transcripts. Using
Northern blot analysis we demonstrate that the reduction of mRNA abundance is fourfold for GADD45α (Fig. 4a), and nearly tenfold for GADD45β (Fig. 4b) and GADD45γ (Fig. 4c). For GADD45β and GADD45γ virtually no mRNA is detectable after growing mIMCD3 cells for 18 h in isosmotic medium in the presence of actinomycin D. In contrast to the dramatic inhibition of GADD45 mRNA synthesis in mIMCD3 cells grown in isosmotic medium, none of the three GADD45 transcripts shows diminished hypertonic induction after dosing mIMCD3 cells in 600 mosmol/kg medium (NaCl↑) in the presence of actinomycin D (Fig. 4). In fact, GADD45α and GADD45β transcripts even significantly increase in the presence of actinomycin D (Figs. 4a, b). These data clearly indicate that the induction of all three mammalian GADD45 transcripts in response to hypertonicity is not a result of increased nascent mRNA synthesis, but instead due to increased mRNA stabilization. Because such stabilization of GADD45 transcripts is absent in isosmotic medium, the mechanisms involved must be activated by hypertonic stress.

**GADD45 isoforms show distinct profiles of induction in response to exposure of mIMCD3 cells to various other stresses.** Because we have shown that all three GADD45 isoforms are induced in response to hypertonic stress and because this response was found to be solute-specific (urea↑ did not elicit an increase in any GADD45 mRNA), we asked whether there are discernable differences among the three isoforms regarding the pattern of GADD45 induction in response to a whole array of cellular stresses. Thus, we carried out a comprehensive experiment, in which mIMCD3 cells were exposed for 18 h to ten different stresses that are either known to have effects on cells similar to those of osmotic stress or have previously been shown to be genotoxic. We show that, despite the uniform hypertonic induction of all three GADD45 transcripts, there are clear differences with regard to the stressor-specificity of induction for each GADD45 isoform (Fig. 6). This is particularly evident during the cellular response to altered
extracellular pH. In response to acid stress (pH 5.5), GADD45β is strongly induced (3.5-fold),
while GADD45α is not much affected. In contrast, alkaline stress (pH 9.0), induces GADD45α
significantly (2-fold) while GADD45β is unaffected. The mRNA abundance of GADD45γ does
not increase in response to any pH change. mIMCD3 cells subjected to heat-shock (42°C)
respond by significant induction of all three GADD45 isoforms but the degree of induction of
GADD45α transcript is higher (4-fold) compared to GADD45β (1.5-fold) and GADD45γ (2.6-
fold). A cold-shock (2°C) only marginally induces GADD45α transcript (1.5-fold) while the
other two GADD45 transcripts do not increase significantly. The response to ultraviolet
irradiation (UV-C and UV-A/B) is not very pronounced and comparable to that during cold
shock. For UV treatment cells were irradiated for 40 seconds at a dose of 500 J/m² and allowed
to recover for 18 h before they were analyzed. Hydrogen peroxide has a more marked effect than
UV radiation, leading to the induction of all three GADD45 transcripts, although the degree of
induction is strongest for GADD45α (2.5-fold), and less for GADD45γ (1.6-fold) and
GADD45β (1.4-fold). In response to heavy metal toxicity, GADD45γ is induced far more
potently than the other two isoforms (Hg²⁺ stress: 17-fold, Cd²⁺ stress: 11-fold), although such
stress also results in significant induction of GADD45α (Hg²⁺ stress: 4-fold, Cd²⁺ stress: 2.8-
fold) and GADD45β (Hg²⁺ stress: 2-fold, Cd²⁺ stress: 1.5-fold). Thus, GADD45α is mostly
induced during hypertonic, alkaline, heat, hydrogen peroxide, and heavy metal stress, whereas
GADD45β is only induced appreciably during hypertonic and acid stress, and to some extent
also during heat and heavy metal stress (Fig. 6). GADD45γ is the most strongly induced isoform
during hypertonic and heavy metal stress, and is also induced by heat and H₂O₂ stress. Clearly,
there is overlap with regard to stressor-specificity of particular GADD45 isoforms but it is also
evident from these data that the induction profile of GADD45 transcripts is far from identical
when comparing cellular responses to various stresses. These data should facilitate the discovery of biological functions of individual GADD45 isoforms.
Discussion

The ability of renal inner medullary (IM) cells to adapt to a severely hyperosmotic milieu and wide osmotic fluctuations is essential for the renal concentrating mechanism. Therefore, it is important to understand the nature of the osmotic threat that these cells face routinely and to investigate the hypertonic stress phenotype that serves to alleviate such threat. We have previously reported that mIMCD3 cells suffer DNA damage during acute hypertonicity (19). This observation may explain the activation of cell cycle checkpoints and the p53 pathway in mIMCD3 cells exposed to hypertonicity (8, 19). In addition, hypertonicity causes induction of GADD45α protein in mIMCD3 cells (20) and GADD45β mRNA in ML-1 myeloid leukemia cells (33). GADD45 proteins have been implicated in multiple aspects of cellular stress adaptation that could be of critical importance during hypertonicity, including DNA repair (29, 30), regulation of cell growth (15), modulation of chromatin compactness (5), and apoptosis (28). Despite these potentially critical functions of GADD45 proteins in hypertonically stressed IM cells it was not known prior to the current study which GADD45 proteins contribute most to the hypertonic stress phenotype of renal IM cells and how the GADD45 isoforms are regulated by hypertonicity.

Our results demonstrate that all three GADD45 transcripts are induced during hypertonicity in mIMCD3 cells and in p2mlME cells. We report different degrees of induction of individual GADD45 isoforms (GADD45γ > GADD45β > GADD45α), which could be reflective of varying significance during hypertonic stress adaptation. GADD45γ, which was not previously known to be osmoregulated, is induced most potently by hypertonicity (7-fold) and may be most important. The kinetics of GADD45 induction is similar for all three isoforms and suggests that these proteins are involved in adaptive events taking place during later stages, i.e. 18 – 24 h after
the onset of hypertonicity. At this time, most of the increase in mRNA and protein levels of all three GADD45 isoforms is observed. Such kinetics does not support a role of either GADD45 in the onset of cell cycle arrest. Conversely, the kinetics of hypertonic GADD45 induction supports a role of these proteins in DNA repair following hypertonicity, which does not have a significant impact until 24 h after the onset of hypertonicity (19). In this regard, it is interesting that the amount of all GADD45 transcripts increases up to 600 mosmol/ kg in mIMCD3 cells but starts to decline if these cells are exposed to higher osmolalities. We have previously shown that the same pattern of osmolality dependence applies for the tumor suppressor protein p53 and for cell survival in mIMCD3 cells (8). Apoptosis is much more prevalent in mIMCD3 cells when the acute hypertonicity exceeds 600 mosmol/ kg (22, 27). In addition, high urea does not cause DNA double-strand breaks and fails to induce GADD45 or p53. Any increase of extracellular osmolality that is mediated by non-permeable solutes causes hypertonicity (cell shrinkage and increased intracellular ionic strength) and triggers DNA damage, and the induction of GADD45 and p53. Collectively, these observations suggest that all three GADD45 proteins may be involved in DNA repair or chromatin modulation rather than having anti-proliferative effects or being pro-apoptotic during hypertonicity. Abcouwer and co-workers (1) suggest that GADD45 could be functionally analogous to traditional stress response genes such as molecular chaperones, which protect cells from stress-induced damage and aid in the recovery of cell function following stress.

Hypertonic induction of all three mammalian GADD45 transcripts apparently is not dependent on nascent mRNA synthesis, but is mediated via mRNA stabilization, evidenced by lack of prevention by the universal transcription inhibitor actinomycin D. Thus, the mechanism of osmotic regulation of GADD45 transcripts differs from that of other hypertonically regulated
genes, most of which are transcriptionally induced (4). For some of these osmoregulated genes, including *BGT1*, and *aldose reductase*, it is known that their hypertonic induction is mediated by tonicity-responsive enhancer elements in the 5’ flanking region, named TonEs (34) or OREs (11). Our finding that actinomycin D blocks nascent GADD45 synthesis in isotonic medium is not surprising and verifies the well-documented property of actinomycin D to be a universal blocker of transcription in mIMCD3 cells. However, we find that actinomycin D does not elicit any decrease in GADD45 mRNA levels in hypertonic medium. This observation is consistent with increased mRNA stability of all three GADD45 transcripts in response to hypertonic stress. Stabilization of GADD45α mRNA is also seen in CHO cells exposed to methyl methane sulfonate (MMS) and UV irradiation (17) and in human breast cell lines deprived of glutamine (1). Interestingly, hypertonicity and actinomycin D have a synergistic effect on the abundance of GADD45α and GADD45β transcripts in mIMCD3 cells. This apparent paradox may have its roots in slight interference of actinomycin D with the RNA degradation machinery (10). Such interference could be potentiated during hypertonicity and limit the production of labile regulatory factors, resulting in enhanced mRNA stability. However, it is unlikely that such interference is dominant because, unlike for GADD45 genes, actinomycin D potently inhibits the hypertonic induction of many other genes, including *ATA2*, *BGT1*, *Cox-2*, and the α and β subunits of the Na⁺/K⁺-ATPase, (3, 7, 23, 36). In addition, our data of actinomycin D effects on overall poly-A+ mRNA content and an osmoregulated control transcript (c-abl tyrosine kinase) illustrate that hypertonicity does not lead to non-specific mRNA stabilization but that GADD45 transcripts are stabilized selectively in hypertonic medium while most other mRNAs are reduced under these conditions (Fig. 5). Of great interest, increases in GADD45α and GADD45β protein levels may result in part from protein stabilization that parallels the mRNA stabilization. Two
arguments support this speculation. First, increases in GADD45α and GADD45β protein abundance (ca. 4-fold) exceed the increases in their mRNAs (ca. 2- to 3-fold). Second, and perhaps more importantly, both GADD45 proteins continue to increase in abundance 36 h after the onset of hypertonicity when the corresponding transcript levels have already returned to baseline. An increase in GADD45 stability during hypertonicity is unusual because most proteins are de-stabilized by hypertonicity (31). Nevertheless, aquaporin 1 protein, whose hypertonic induction is physiologically relevant, is also stabilized during hypertonicity (21). In this case, stabilization results from a phosphorylation-dependent decrease in aquaporin 1 ubiquitination during hypertonicity.

We have previously provided evidence that GADD45α protein induction during hypertonicity depends in part on the p38 MAP kinase pathway (20). Furthermore, inhibition of p38 MAP kinase by a specific inhibitor (25µM SB203580) results in partial (50%) suppression of the hypertonic induction of GADD45β and GADD45γ transcripts (data not shown). Thus, proteins involved in GADD45 mRNA stabilization may be hypertonically induced via the p38 MAP kinase cascade.

We have compared the degree of hypertonic induction for all three GADD45 transcripts with that in response to other stresses to obtain information about the relative significance of the three GADD45 isoforms during exposure of mIMCD3 cells to various insults. These experiments suggest that, although all three GADD45 isoforms are induced during hypertonic, heat, and heavy metal stress, the degree of induction differs greatly. In addition, individual GADD45 isoforms show discernable patterns of specificity with regard to additional types of stress. GADD45α is involved in adaptation to alkali, cold-shock, UV-irradiation, and hydrogen peroxide stress. In contrast, only GADD45β is induced significantly in response to acid stress.
The pattern of stressor-specificity of GADD45γ induction is dominated by its strong responsiveness to hypertonic and heavy metal stress. In fact, our study shows that mercury-induced GADD45γ induction represents the highest degree of induction of any GADD45 transcript under any of the conditions tested (17-fold). Overall, the hypertonic induction profile of GADD45 isoforms is most similar to that seen in response to heat shock and heavy metal stress. This may indicate that induction of all three GADD45 proteins is critical for cell functions that are common during adaptation to triple-H stress (hypertonicity, heat, and heavy metal stress). These cell functions remain to be determined. On the other hand, the relative ineffectiveness of UV radiation for inducing the GADD45 transcripts may point to the possible involvement of GADD45 in cell functions that are poorly or not at all affected by UV radiation stress in mIMCD3 cells. Alternatively, higher doses of UV radiation may be required to induce an adaptive response. We view the latter possibility as unlikely because mIMCD3 cells showed massively signs of apoptotic cell death in response to the doses of UV radiation that were used in our study (unpublished observations). UV radiation leads to a special form of DNA damage, the formation of pyrimidine dimers suggesting that GADD45 may not be involved in the recognition or repair of this form of DNA damage in kidney cells. Nevertheless, the GADD45 transcripts are induced by UV radiation in skin fibroblasts, which, in contrast to kidney cells, are exposed to UV radiation under physiological conditions. Thus, the contingencies involved in GADD45 induction and maintenance of genomic integrity may be cell type-specific and reflect the physiological relevance of a particular stressor.

In summary, we have shown that all three GADD45 isoforms are part of the hypertonic stress phenotype of renal IM cells. Their induction kinetics supports a possible involvement in DNA repair and/ or chromatin regulation. The induction of GADD45 is dependent on hypertonicity,
not seen during isovolumetric hyperosmolality, and based on mRNA stabilization of all three transcripts. Because the pattern of hypertonic GADD45 induction is most closely resembled by that seen in response to heat and heavy metal stress it is likely that these proteins are involved in cell functions that are common for adaptation to these three types of stress. In contrast, individual GADD45 isoforms may be involved in other aspects of cell adaptation during pH, oxidative, and ionizing radiation stress.

Acknowledgements
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References


Figure Legends

**Fig. 1:** All three GADD45 transcripts are maximally induced in mIMCD3 cells after raising medium osmolality from 300 to 600 mosmol/ kg by addition of NaCl. Cells were kept for 18 h in osmolalities ranging from 300 (isosmotic) to 675 mosmol/ kg followed by total RNA isolation. Northern blots of GADD45 transcripts in cells exposed to this osmolality range are shown (A = GADD45α; B=GADD45β; C=GADD45γ). The upper image in each panel (dark bands on light background) depicts one representative Northern blot. The image in the center of each panel depicts the corresponding RNA gel with ethidium bromide-stained 28S and 18S ribosomal RNA. The 28s rRNA served as a control for even loading and for normalization of the Northern blots data. The histogram in the bottom of each panel depicts the mean mRNA abundance of GADD45α, GADD45β, and GADD45γ ± SEM of three independent experiments.

**Fig. 2:** Hypertonic GADD45 induction is transient at the mRNA level and sustained at the protein level. Time course studies on mIMCD3 cells and p2mIME cells show that the increase in mRNA abundance following hypertonic stress peaks between 16 and 18 h. **A:** Abundance of GADD45 mRNAs in mIMCD3 cells in response to 600 mosmol/kg (NaCl↑) hypertonicity as determined by Northern blot analysis. **B:** Abundance of GADD45 mRNAs in p2mIME cells in response to 700 mosmol/kg (NaCl↑) hypertonicity as determined by RNAse protection assay. **C:** Representative Western blots of GADD45α and GADD45β from mIMCD3 cells exposed to 600 mosmol/kg (NaCl↑) hypertonicity for various times. Shown below each blot is the corresponding upper part of the gel that was stained with Coomassie blue to confirm even loading of total protein. **D:** Time course of GADD45α and GADD45β protein abundance in
mIMCD3 cells exposed to 600 mosmol/kg (NaCl↑) hypertonicity, as in C. Each reading represents the mean of three independent experimental observations (± SEM).

**Fig 3:** All solutes causing hypertonicity induce GADD45 but comparable hyperosmolality due to elevated urea fails to induce GADD45. The abundance of all three GADD45 transcripts is significantly increased in mIMCD3 cells in response to hypertonic stress by elevating medium osmolality from 300 to 600 mosmol by addition of NaCl, KCl, sorbitol, and mannitol. The increase in GADD45 mRNA abundance was measured by Northern blot analysis and is absent when cells are exposed to hyperosmolality in the form of elevated urea (300 -> 600 mosmol/kg).

*Fig. 3:* Representative Northern blots showing the solute-specificity of mRNA abundance of all three GADD45 transcripts in mIMCD-3 cells (top = GADD45α; center =GADD45β; bottom = GADD45γ). The following solutes were tested: Lane 1 = isosmotic control; Lane 2 = NaCl; Lane 3 = KCl; Lane 4 = Sorbitol; Lane 5 = Mannitol, and Lane 6 = Urea. mIMCD3 cells were dosed by substituting isosmotic medium with the respective hyperosmotic medium and incubated for 18h. The upper dark bands on light background represent the abundance of the mRNA transcript in each panel. The lower bright bands on dark background represent the amounts of 28S and 18S ribosomal RNA on the corresponding gels. *B:* Histogram depicting the average amounts of GADD45 mRNA depending on the solute used to impose hyperosmotic stress on mIMCD3 cells. Data shown are means of three independent experimental observations (± SEM).

**Fig. 4:** All three GADD45 transcripts are hypertonically induced via mRNA stabilization. Actinomycin D (10µg/ml; Biovision Inc.) reduces the transcript abundance of GADD45α (*A*), GADD45β (*B*), and GADD45γ (*C*) in mIMCD3 cells grown in isosmotic medium. In contrast,
actinomycin D does not prevent the hypertonic induction of any GADD45 transcript when cells are treated for 18 h in hypertonic medium (600 m osmol/ kg, NaCl↑) indicating that the increase is due to mRNA stabilization. Data shown are means of three independent experimental observations (± SEM). The inset in each panel depicts one representative Northern blot for each GADD45 transcript (top) and the corresponding 28s and 18s ribosomal RNAs (bottom). Samples were loaded in the following order: Lane 1 = 300 mosmol/ kg, Lane 2 = 300 mosmol/ kg plus 10µg/ ml actinomycin D, Lane 3 = 600 mosmol/ kg (NaCl↑), Lane 4 = 600 mosmol/ kg (NaCl↑) plus 10µg/ ml actinomycin D.

**Fig. 5:** Actinomycin D reduces overall and c-abl transcript levels. Actinomycin D (10µg/ml; Biovision Inc.) reduces the abundance of overall poly-A+ mRNA (A), and c-abl tyrosine kinase transcript (B) in mIMCD3 cells grown in isosmotic (300 m osmol/ kg) and hypertonic (600 m mosmol/ kg, NaCl↑) media. These data show that the actinomycin D effect on GADD45 transcripts is unique and not a result of interference of hypertonicity with the action of actinomycin D as a universal transcriptional inhibitor. Data shown are means of three independent experimental observations (± SEM). The inset depicts one representative Northern blot for c-abl transcript (top) and the corresponding 28s and 18s ribosomal RNAs (bottom). Samples were loaded in the following order: Lane 1 = 300 mosmol/ kg, Lane 2 = 300 mosmol/ kg plus 10µg/ ml actinomycin D, Lane 3 = 600 mosmol/ kg (NaCl↑), Lane 4 = 600 mosmol/ kg (NaCl↑) plus 10µg/ ml actinomycin D.

**Fig. 6:** Stressor-specificity of GADD45 induction in mIMCD3 cells. The effect of eleven different stressful conditions on the mRNA abundance of GADD45 was analyzed by Northern blots in cells treated for 18 h (A = GADD45α, B = GADD45β, and C = GADD45γ). All gels are
loaded in the following order: Lane 1 = isosmotic control; Lane 2 = hypertonic medium (600 mosmol/ kg, NaCl↑); Lane 3 = pH 5.5; Lane 4 = pH 9.0; Lane 5 = Heat shock (42 °C); Lane 6 = Cold shock (2 °C); Lane 7 = UV-C; Lane 9 = UV-A/B; Lane 9 = 4 mM H₂O₂; Lane 10 = 30 µM HgCl₂; Lane 11 = 35 µM CdCl₂. pH 5.5 was generated by substituting HEPES in D-MEM/F12 medium with 15 mM MES (Sigma). pH 9.0 was generated by substituting HEPES in D-MEM/F12 with 15mM AMPSO (Sigma). UV treatment (UV-C = 250-300nm; UV-B = 300-350nm, UV-A = 350-380nm) was achieved by irradiating cells with a UV-lamp (Model UVGL-58, M/s UVP-Inc. USA) from a distance of 8.5 cm for 37 s – 42 s. The upper dark bands on light background in panels A-C represent GADD45 mRNA abundance and the lower bright bands on dark background represent 28S and 18S ribosomal RNA. **D:** Average effects of hyperosmolality caused by different solutes on GADD45 mRNA abundance in mIMCD3 cells. Data shown are means of three independent experimental observations (± SEM).
Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 5:
Figure 6:

A
GADD45 α
28s rRNA
18s rRNA

B
GADD45 β
28s rRNA
18s rRNA

C
GADD45 γ
28s rRNA
18s rRNA

D

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