Mannitol at Clinical Concentrations Activates Multiple Signaling Pathways and Induces Apoptosis in Endothelial Cells

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Background and Purpose—Hyperosmotic mannitol therapy is widely used in the clinical setting for acute and subacute reduction in brain edema, to decrease muscle damage in compartment syndrome, and to improve renal perfusion. Though beneficial rheological effects commonly are attributed to mannitol, its direct effects on endothelial cells are poorly understood.

Methods—We studied the effect of hypertonic and hypotonic stress on bovine aortic endothelial (BAE) cells, using mannitol, urea, and sodium chloride and medium dilution in vitro.

Results—Exposure to incremental osmolar concentrations of 300 mOsm of each osmotic agent increased apoptosis in BAE cells (mannitol > NaCl > urea). Induced programmed cell death was detected by DAPI staining of intact cell nuclei, and by TUNEL and DNA fragmentation ladder assays. Mannitol-induced apoptosis exhibited dose dependence (42% of cells compared with 1.2% of control cells) and was also observed in bovine smooth muscle cells. Mannitol-induced apoptosis was attenuated ~50% in the presence of cycloheximide or actinomycin D. Hypertonic mannitol and NaCl, but not urea, increased tyrosine phosphorylation of the focal adhesion contact-associated proteins paxillin and FAK. Hypotonic medium, which did not lead to apoptosis, increased protein tyrosine phosphorylation of FAK but not of paxillin. Addition of mannitol or NaCl also produced sustained increases in c-Jun NH2-terminal kinase (JNK) activity. In addition, hypertonic mannitol increased intracellular free [Ca2+] in a dose-dependent manner. Chelation of intracellular Ca2+ with quin2-AM (10 μmol/L) inhibited mannitol-induced apoptosis ~50%, as to a lesser extent did inhibition of tyrosine kinase activity with herbimycin (1 μmol/L).

Conclusions—We have shown that hypertonic mannitol exposure induces endothelial cell apoptosis, accompanied by activation of tyrosine and stress kinases, phosphorylation of FAK and paxillin, and elevation of intracellular free [Ca2+]. The apoptosis is attenuated by inhibition of transcription or translation, by inhibition of tyrosine kinases, or by intracellular Ca2+ buffering. These data suggest that clinical use of the osmotic diuretic mannitol may exert direct deleterious effects on vascular endothelium. (Stroke. 1998;29:2631-2640.)

Key Words: hypertonicity ■ hypotonicity ■ osmotic agents ■ protein-tyrosine kinases ■ calcium
agents and gene expression vectors to the central nervous system.\textsuperscript{11,12} Mannitol is customarily administered every 3 to 4 hours in bolus doses of up to 1 to 1.5 g/kg body weight,\textsuperscript{4} resulting in step increments in serum osmolarity of up to 320 mOsm and in sustained increments of 80 mOsm.\textsuperscript{6}

Mannitol and other hyperosmotic agents may activate inflammatory mediators\textsuperscript{13} and produce deleterious effects on certain cell types.\textsuperscript{14} Little is known about mannitol’s effects on the function of endothelial cells lining the vascular system to which it largely remains confined. Recently, hyperosmotic stress has been shown to activate a number of mitogen-activated protein kinases (MAPKS),\textsuperscript{15} specifically stress kinases such as p38 and c-Jun N-terminal kinase (JNK).\textsuperscript{16} Prolonged stress has been shown to activate a number of mitogen-activated protein kinases (MAPKs),\textsuperscript{15} specifically stress kinases.

JNK activation, whether triggered by environmental stresses or gamma or UV irradiation,\textsuperscript{12} contributes to apoptosis, or programmed cell death.\textsuperscript{17} We hypothesized that maximal clinical doses of mannitol may under certain conditions adversely affect endothelial cells. The present report investigates and contrasts the effects of mannitol and other hyperosmotic agents with those of hypotonicity on vascular endothelial cell survival and signaling.

Materials and Methods

Materials

Herbimycin was purchased from Life Technologies. All other reagents were obtained from Sigma, and except where noted were of the highest grade available.

Cell Culture

Bovine aortic endothelial (BAE) and bovine smooth muscle (BSM) cells (passage 6 to 15) were harvested from descending thoracic aortas obtained from the local abattoir by collagenase digestion, as previously described.\textsuperscript{18} More than 98% of endothelial cells so isolated displayed uptake of Ac-LDL (Biomedical Technologies). Cells were cultured at 37°C, 5% CO\textsubscript{2} in a humidified incubator in serum-free medium 24 hours before addition of the experimental stimulus. Loss of cell viability as assessed by intact cell uptake of propidium iodide (Molecular Probes) using a FACS analyzer (Becton Dickinson) was assessed uptake of Ac-LDL (Biomedical Technologies). Cells were cultured at 37°C, 5% CO\textsubscript{2} in a humidified incubator in serum-free medium 24 hours before addition of the experimental stimulus. Loss of cell viability as assessed by intact cell uptake of propidium iodide (Molecular Probes) using a FACS analyzer (Becton Dickinson) was assessed.

Western Blotting

Cell monolayers were lysed in boiling hot 2X SDS-Laemmli lysis buffer (1X = 125 mmol/L Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 5% glyceral, 0.003% bromophenol blue, 1 mmol/L sodium orthovanadate, and 1% β-mercaptoethanol) and incubated at 100°C for an additional 5 minutes. Samples were centrifuged for 5 minutes (12 000g) to remove SDS-insoluble material, and the clarified supernatants were assayed for protein concentration (BCA Assay). Sample aliquots (10 μg) were applied to a 0.75-mm thick SDS-polyacrylamide gel (10%) and transferred to nitrocellulose (Schleicher and Schuell) using a semidy transfer apparatus (Owl Scientific) in transfer buffer (1X = 25 mmol/L Tris, 190 mmol/L glycine, 20% methanol). The filters were rinsed briefly in wash buffer (1X = 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20) and incubated in blocking buffer (wash buffer containing 3% nonfat dry milk) for 1 hour at room temperature. Filters were then incubated in blocking buffer containing either mouse monoclonal anti-phosphotyrosine (1 μg/mL of clone 4G10, Upstate Biotechnology Inc), mouse monoclonal anti-paxillin (1:10 000 dilution of clone 349, Transduction Laboratories), or rabbit polyclonal anti-human FAK (1 μg/mL, Upstate Biotechnol-

gy) with constant agitation for 1 hour at room temperature, washed 6×5 minutes in wash buffer, then incubated with anti-mouse or anti-rabbit IgG:horseradish peroxidase conjugate (Jackson Immunoresearch Laboratories) in blocking buffer. Filters were then washed for 30 minutes and developed with an enhanced chemiluminescence kit (ECL) using Kodak X-Omat radiographic film.

Immunoprecipitation

Cells monolayers were rinsed with ice-cold PBS twice for 1 minute, then lysed in cell lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L PMSF, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mmol/L sodium orthovanadate, and 1 mmol/L NaF) at 4°C for 15 minutes on a rotating platform. The lysed cells were centrifuged, washed using a 26-gauge needle and centrifuged at 4°C for 15 minutes to remove insoluble material. Cleared cell lysates were diluted to 1 mg/mL protein. Four micrograms of anti-human FAK antibody or 4 μg anti-paxillin antibody were added to 500 μg cell lysate protein and incubated overnight with gentle rocking. Secondary rabbit anti-mouse antibody was then added in the case of paxillin for 1 additional hour. Fifty microliters of 10% Protein A-Sepharose (Pharmacia Biotech Inc) was then added with vortexing and incubation with agitation for 60 minutes at 4°C. The beads were then centrifuged, rinsed several times with immuno precipitation buffer, and subjected to immunoblot analysis.

DNA Ladder Analysis

BAE Cells (∼2×10\textsuperscript{5}) were collected by centrifugation at 100g for 5 minutes. The cell pellet was resuspended in 500 μL TNE (100 mmol/L NaCl, 10 mmol/L Tris pH 8.0, 1 mmol/L EDTA). Ten percent SDS (25 μL) was added with 5 μL proteinase K (20 mg/mL) and 5 μL RNase A (200 μg/mL). The mixture was incubated for 2 hours at 37°C, then extracted twice with Tris-EDTA buffered phenol/chloroform and once with chloroform. The genomic DNA was precipitated with ethanol and the pellet dissolved in 50 μL TE buffer. DNA (10 μg) was then electrophoresed through a 1% agarose gel and visualized with ethidium bromide under ultraviolet light.

TUNEL Assay and DAPI Staining

A terminal deoxynucleotide transferase (TdT)-mediated X-dUTP nick-end labeling (TUNEL) kit was used according to the manufacturer’s instructions (Boehringer Mannheim). BAE cells were fixed with paraformaldehyde, triton permeabilized, then incubated with a TUNEL reaction mixture containing fluorescein-dUTP and TdT to catalyze attachment of fluorescein-dUTP to the free 3 OH ends of DNA strand breaks. The fixed cells were stained with 4,6-diamidino-2-phenyindole dihydrochloride (DAPI, Molecular Probes) to visualize total nuclear DNA. Cells were then counted and nuclear morphology assessed with an epifluorescence microscope (Olympus BH-2), with use of selective band-pass filters.

JUN Kinase Assay

Confluent BAE cells exposed to control or experimental treatments were washed 3 times in ice-cold PBS and lysed in 1 mL of a 1% Triton X-100/PBS solution containing the following inhibitors: 100 mg/mL aprotinin, 100 mg/mL leupeptin, 1 mmol/L peptatin, 1 mmol/L phenylmethylsulfonyl chloride, 100 mmol/L Na\textsubscript{2}VO\textsubscript{4}, 1 mmol/L benzamidine, and 50 mmol/L NaF. The cell lysate was incubated for 20 minutes on ice and centrifuged 5 min at 10 000g to remove cellular debris. The clarified lysate was then incubated (3 hours at 4°C) with 20 μL GST-c-Jun conjugated to glutathione beads (approximately 2 μg of c-Jun (5–89); kind gift of Dr. James Woodgett, Princess Margaret Hospital and University of Toronto, Toronto, Canada), then washed at least 4 times in PBS containing the above protease inhibitors. Washed beads were resuspended in 20 μL kinase buffer (50 mmol/L Tris-Cl, 1 mmol/L EGTA, 10 mmol/L MgCl\textsubscript{2}, 4 mmol/L K-ATP and 2 nCi of [γ-\textsuperscript{32}P]- ATP, pH 7.5) and incubated for 30 minutes at 30°C. After the reaction was stopped by addition of 20 μL of 2X Laemmli’s sample buffer, samples were separated by SDS-PAGE (10%), stained with Cooomassie blue,
destained, and dried. Dried gels were then exposed to phosphor plates and quantified by Phosphorimager analysis (Molecular Dynamics).

**Determination of Cytoplasmic Free Ca\(^{2+}\) Ion Concentration ([Ca\(^{2+}\)\(_{i}\)]\(_{\text{free}}\) Using Fura-2 Ratio Imaging**

The fluorescence ratio imaging of intracellular free calcium concentrations using the indicator fura-2 AM was as previously described. Briefly, BAE monolayers cultured at subconfluent density on 25 mm coverslips were incubated in growth media containing 2 \(\mu\)mol/L fura-2-acetoxyethyl ester (fura-2 AM, Molecular Probes) for 30 to 40 minutes at 37°C in humidified 5% CO\(_2\). The coverslips were then washed in a Ca\(^{2+}\) measurement buffer (modified Hanks’ buffered saline solution) and mounted in a modified Leiden chamber in which the coverslip constituted the bottom. One milliliter of buffer was added to the chamber. [Ca\(^{2+}\)]\(_{i}\) was measured in room air and at room temperature by fura-2 ratio imaging using an Image-1 digital ratio imaging system (Universal Imaging) equipped with an Olympus IMT-2 inverted microscope, a Dage-MTI CCD7 camera, a Genesys image intensifier, a Pinnacle REO-650 optical disk drive, and color video printer. Fura-2 fluorescence was monitored and images acquired at 340 and 380 nm; 340/380 ratio images of individual cells calculated on a pixel-by-pixel basis were recorded to optical discs for data processing. Fura-2 ratio values were calibrated in vitro to free Ca\(^{2+}\) concentrations ranging from 0 nmol/L to 39.8 \(\mu\)mol/L (Calcium Calibration Buffer Kit #2, Molecular Probes) using the same imaging parameters. \(K_d\) was determined by fitting the experimental R value at various free [Ca\(^{2+}\)]\(_{i}\) using the equation:

\[
\frac{[\text{Ca}^{2+}]_{\text{free}}}{K_d} = \frac{S_A}{S_A - S_B} \left(\frac{R - R_{\text{min}}}{R_{\text{max}} - R}\right),
\]

where the factor \(S_A/S_B\) corrects for fura-2 ion sensitivity at 380 nm. A similar procedure was used for in situ calibration, in which 2 \(\mu\)mol/L of the nonfluorescent Ca\(^{2+}\) ionophore, A23187 (Molecular Probes), was used to collapse Ca\(^{2+}\) gradients during 15 min incubation of fura-2-loaded BAECs in a series of Ca\(^{2+}\) and EGTA buffers with free [Ca\(^{2+}\)]\(_{i}\) ranging from 36 to 1270 nmol/L. \(K_d\) in these conditions was 224 nmol/L. Although calculated values of resting [Ca\(^{2+}\)]\(_{i}\), determined by in vitro and in situ calibration curve differed only slightly, [Ca\(^{2+}\)]\(_{i}\), was calculated from the in situ calibration curve. Extracellular medium contained 1.27 mmol/L CaCl\(_2\).

The [Ca\(^{2+}\)]\(_{i}\) responses to hypertonic media were monitored over 15- to 20-minute periods by ratio image acquisition at programmed intervals. In response to mannitol addition, 405 to 60% (average, 50%) of the cells responded with increases in cytoplasmic calcium. Images were stored to optical disks for subsequent replay and data sampling from 8 to 12 individual responsive cells for each coverslip. The average mean value of [Ca\(^{2+}\)]\(_{i}\) for each coverslip was then plotted versus time with Image-1 software.

**Immunostaining and Photomicroscopy**

BAE monolayers were washed 3 times with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde in PBS for 30 minutes, permeabilized in PBS containing 0.01% triton X-100 for 15 minutes, then washed in PBS. Fixed, permeabilized cells were incubated with DAPI or with appropriate antibodies as described above. Cells were visualized on an inverted microscope (Olympus Model IMT-2) using phase contrast or Hoffman phase-modulation optics and were photographed with T-Max 400 film (Kodak).

Photomicrographs were optically scanned and saved as Adobe Photoshop files. Areas of DAPI-stained non-apoptotic nuclei were measured using the public domain NIH Image program, version 1.62 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on an Apple Macintosh computer.

**Statistics**

Data were expressed as mean±SEM. Statistical analysis was performed by analysis of variance (ANOVA) combined with a Tukey-Kramer HSD correction test for all comparison pairs. Comparisons with values of \(P<0.05\) were interpreted to represent statistically significant differences except where otherwise stated.

**Results**

**Hyperosmotic Agents Induce Detachment and Cell Loss in Endothelial Monolayers**

To determine the response of vascular endothelial cells to osmotic stress, confluent monolayers of BAE cells were exposed to step additions of 100 and 300 mmol/L mannitol (corresponding to final osmolarities of 400 or 600 mOsm, respectively). Within 6 hours after addition of mannitol, some cells began to round up and lose adhesion from the monolayer (compare Figures 1B and 1C with Figure 1A). The cell loss phenomenon was evident at 100 mmol/L mannitol but was more dramatic at 300 mmol/L mannitol, resulting by 6 hours in partial denudation of the previously confluent monolayer (Figure 1C). Both NaCl (Figures 1D and 1E) and urea (Figures 1F and 1G) added at of 100 and 300 mOsm induced the same cell loss phenomenon as mannitol, with a definite dose-dependence. However, the more permeant urea appeared to cause less cell detachment than did impermeant mannitol and NaCl.

**Hyperosmotic Treatment With Mannitol, Urea, and Sodium Chloride Induce Apoptosis in Endothelial Cells**

To determine whether the endothelial cell loss is the result of necrosis or apoptosis, we used DAPI to stain nuclear chro-
matin in endothelial cells fixed 3 hours after treatment with mannitol (Figure 2A). The proportion of cells exhibiting nuclear chromatin condensation increased in mannitol-treated cells in a dose-dependent manner. The nuclei of apoptotic cells showed condensed chromatin that was brightly and uniformly stained by DAPI and ranged in shape from a single uniform sphere to a collection of multiple chromatin dots typical of fragmented apoptotic nuclei. Nuclear fragmentation and condensation was observed not only in response to mannitol but also in cells exposed to urea and sodium chloride (Figure 2A). A closer observation of the DAPI staining pattern of nonapoptotic nuclei demonstrates that nuclei of cells treated with 300 mOsm of the permeable solute urea retained the same shape and projected area (102±1.9%, n=43) as those of control cells (100±1.6%, n=55), in contrast to the DNA contraction and irregular shrunken appearance of nonapoptotic nuclei in cells treated with impermeant mannitol (84.8±2.0%, n=42, P<0.0001) or NaCl (85±3.1%, n=33, P<0.0001) (Figure 2B).

To ascertain that the cells with condensed chromatin had undergone DNA fragmentation, an in situ TUNEL assay was performed on fixed endothelial cells to label the 3'-OH ends of DNA strand breaks. Cell nuclei were also labeled with DAPI. The double staining revealed that positive staining with the TUNEL assay was coincident with DAPI-visualized nuclear condensates. DNA fragmentation ladder of bovine aortic endothelial cells treated for 6 hours with 100 or 300 mOsm mannitol, sodium chloride, or urea reveals a significant increase in low-molecular-weight fragmentation of DNA.

Figure 2. Exposure of quiescent monolayers of endothelium to hypertonic stimuli induces programmed cell death. A, Staining of endothelial nuclear DNA using DAPI of cells exposed to 300 mmol/L mannitol, sodium chloride, or urea (final osmolality, 600 mOsm) at 6 hours after addition reveals condensation and formation of dense bodies characteristic of apoptosis. Note the shrunken morphology of the nucleus in membrane impermeant mannitol and sodium chloride compared with membrane permeant urea and control. B, Quantitative morphologic analysis of nonapoptotic BAE nuclei using DAPI staining reveals 15% decrease in projected surface area in the nuclei of cells treated with mannitol and NaCl (**P<0.0001) but no significant change in cells treated with urea. C, TUNEL assay of endothelial cells exposed to 300 mmol/L mannitol addition at 6 hours reveals significant DNA fragmentation coincident with DAPI-visualized nuclear condensates. D, DNA fragmentation ladder of bovine aortic endothelial cells treated for 6 hours with 100 or 300 mOsm mannitol, sodium chloride, or urea reveals a significant increase in low-molecular-weight fragmentation of DNA.
To confirm further that hyperosmotic treatment results in apoptosis, DNA was analyzed from endothelial monolayers exposed for 4 hours to hypertonic mannitol, NaCl, or urea. Isolation of nuclear DNA and subsequent gel electrophoresis revealed the ladder pattern characteristic of regular DNA fragmentation produced by all 3 osmolar agents in a dose-dependent manner (Figure 2D). Thus, 3 independent assays, including morphological analysis of nuclear condensation, TUNEL end-labeling of DNA strand breaks, and gel electrophoretic detection of nuclear DNA fragmentation (laddering), were all consistent with an apoptotic process induced by exposure to hypertonic mannitol, NaCl, or urea at incremental osmolarities of 100 and 300 mOsm.

Permeant Urea Is a Weaker Inducer of Apoptosis Than Impermeant Mannitol and NaCl

Quantitation of condensed bodies and nuclear fragmentation in DAPI-stained live cells revealed a baseline rate in serum-deprived BAE cells of 1.2±0.4%, which increased to 3.4±0.5% at 100 mOsm mannitol, 3.6±0.4% at 100 mOsm NaCl, and 2.2±0.7% at 100 mOsm urea (Figure 3). None of these values reached statistical significance. In contrast, apoptosis was increased significantly (*P<0.001) to 41.9±4.0% by 300 mOsm mannitol, to 42.3±6.6% by 300 mOsm NaCl, and to 30.5±3.2% by 300 mOsm urea. Urea exposure led to significantly fewer apoptotic cells than did either mannitol or NaCl.

Hypoosmotic Stress Is Much Weaker Than Hyperosmotic Stress as an Inducer of Apoptosis

To determine the effect of hypotonic exposure on the endothelial cell, we performed step decreases in medium osmolarity to 240, 200, and 150 mOsm, the latter corresponding to halving of the initial osmolarity. DAPI analysis of nuclear chromatin condensation (1.43±0.22% in isotonic medium) showed no significant change in apoptosis at 240 mOsm (0.88±0.21%) or at 200 mOsm 1.36±0.36%). At 150 mOsm, the rate of nuclear condensation increased to 4.67±0.26% (*P<0.05, Figure 4B). This was, however, significantly lower than the apoptosis rate elicited by doubling osmolarity with mannitol, 44.6±1.85% (*P<0.001). The weaker induction of apoptosis by hypotonic stimulation was also confirmed using DNA fragmentation ladder analysis. No visible increase in low-molecular-weight DNA fragments was evident at 240 and 200 mOsm. Only at 150 mOsm were faint low molecular weight bands observed (Figure 4A), consistent with the TUNEL and DAPI staining results.

Figure 3. Apoptotic index in response to mannitol, sodium chloride and urea. Analysis of nuclear fragmentation and condensation using DAPI staining at 3 hours following addition of hyperosmolar agent in 5 to 6 representative fields of 130 to 150 cells each. *P<0.05.

Figure 4. Hypoosmotic stress is much weaker than hyperosmotic stress at inducing apoptosis. A, DNA fragmentation ladder of bovine aortic endothelial cells treated for 6 hours to hypoosmotic medium of 240, 200, and 150 mOsm reveals significant DNA fragmentation only at the most extreme value. B, Analysis of nuclear fragmentation and condensation in response to hypoosmotic stress.
Smooth Muscle Cells Are Less Vulnerable Than Endothelial Cells to Mannitol-Induced Apoptosis

To assess the cell specificity of mannitol-induced apoptosis, we exposed confluent monolayers of bovine smooth muscle cells to the same experimental regimen and quantitated apoptotic bodies by DAPI staining. BSMC cells underwent similar changes in nuclear morphology and in TUNEL staining (data not shown). The isotonic apoptosis rate of 1.58 ± 0.35% was unchanged at 100 mOsm (2.06 ± 0.63%, NS) but increased at 300 mOsm to 8.4 ± 1.09% (P < 0.05). Thus, BSMC exhibited an approximately 8-fold lower increase in the rate of mannitol-induced apoptosis than observed in endothelial cells (Figure 5).

Mannitol Addition Induces Increased Tyrosine Phosphorylation of Multiple Proteins in Endothelial Cells

Addition to BAE cells of 300 mOsm mannitol increased tyrosine phosphate content of at least 4 polypeptides of Mr 300, 220, 125, and 90 kDa (Figure 6A). This increase was maximal at 30 minutes and was sustained at decreasing levels out to 180 minutes. Although both NaCl and urea at equivalent osmolarities induced the same pattern of p-tyr increase, both the magnitude and duration of the response to the permeant urea were less (Figure 6A). In contrast, a step-hypotonic stimulus via medium dilution to 200 mOsm produced no detectable change in polypeptide phosphotyrosine content (Figure 6A).

Effects of Hypertonic and Hypotonic Stimulation on Tyrosine Phosphorylation of Focal Contact-Associated Proteins

To initiate the process of identifying the proteins undergoing increased tyrosine phosphorylation in response to hyperosmotic stimulus, we hypothesized that the cell shrinkage accompanying hyperosmotic stress may be transmitted to focal adhesion contacts anchoring the endothelial cell to its substratum. Such a transmitted force might then modulate the tyrosine phosphorylation and/or activity of FAK or paxillin. Anti-p-Tyr immunoblot analysis of FAK immunoprecipitates from BAE cells exposed either to hypertonic addition of 300 mOsm mannitol or to hypotonic medium (final osmolarity, 150 mM/L) for 30 minutes showed significantly increased tyrosine phosphate content in FAK produced in both conditions (Figure 6B). Anti-p-Tyr immunoblot analysis of paxillin immunoprecipitates from mannitol-treated BAE cells revealed a smaller increase in paxillin phosphotyrosine content than that noted in FAK. Hypotonic stimulation produced no change in paxillin phosphotyrosine content, in contrast to the increase noted in FAK.

Mannitol Addition Activates JNK in Endothelial Cells

To determine the possible contribution of stress kinases in the endothelial cell response to mannitol, we measured the activity of JNK activity at timed intervals following exposure...
to mannitol. Addition of 300 mmol/L mannitol to confluent BAE cells led within 5 minutes to a time-dependent increase in JNK activity measured by incorporation of γ-32-P into GST-c-Jun. BAE cell JNK activity measured 1 hour after mannitol exposure revealed a biphasic dose-response relationship, peaking at 300 mOsm and decreasing at higher osmolarities (Figures 7A and 7B). Both the nonpermeable osmolytes mannitol and NaCl activated JNK activity to almost twice the extent (6- to 7-fold) achieved by the permeable osmolyte urea (4-fold; Figures 7C and 7D). JNK activity elicited by all 3 hyperosmotic agents remained elevated 6 hours after initial exposure, though the effect of urea decreased beyond 3 hours. In contrast, hypotonic treatment of BAE cells transiently decreased JNK activity.

**Mannitol Addition Elicits Intracellular Calcium Transients**

To test additional second messenger systems that might be involved in the endothelial cell response to hyperosmotic stress, we measured cytoplasmic free calcium concentration ([Ca²⁺]ᵢ) by ratiometric imaging of fura-2. Only ≈50% of subconfluent BAE cells on coverslips responded to addition of mannitol by a rapid increase in [Ca²⁺]ᵢ, peaking at 15 seconds and remaining tonically elevated at 12 minutes (Figure 8A). Peak [Ca²⁺]ᵢ in mannitol-responsive BAE cells increased in a dose-dependent manner (Figure 8B).

**Roles of RNA Transcription, Protein Synthesis, Intracellular Ca²⁺, and Tyrosine Kinase Activity in Mannitol-Induced Apoptosis**

We used a pharmacological approach to initiate identification of candidate signaling pathways by which mannitol induces apoptosis in BAE cells. Both inhibition of transcription by actinomycin D and inhibition of translation by cycloheximide significantly attenuated the mannitol-induced 8.2-fold increase in apoptosis rate to 4.0-fold and 4.9-fold, respectively (both \(P<0.001\); Figure 9).

The observation that hypertonic mannitol exposure activated tyrosine kinase activity led us to test the effects on mannitol-induced apoptosis of tyrosine kinase inhibition by herbimycin. In the presence of herbimycin, the mannitol-induced 8.2-fold increase in BAE cell apoptosis rate was moderately decreased to 6.6-fold \(P<0.05\); Figure 9). The observation that hypertonic mannitol elicited increased \([Ca^{2+}]_i\) led us to test the effects of intracellular \[Ca^{2+}\] chelation by quin2-AM. In quin2-AM-loaded BAE cells, the mannitol-induced increase in apoptosis rate was almost halved to 4.4-fold \(P<0.001\); Figure 9).
Discussion

This report demonstrates that mannitol and other hypertonic agents induce programmed cell death in both endothelial and smooth muscle cells. Hypersmolality induced programmed cell death in a dose-dependent manner and was more pronounced for impermeant mannitol and NaCl than for permeant urea. Hypoosmotic stress proved to be a much less potent inducer of apoptosis than was hyposmotic stress. Hypertonicity-induced apoptosis was partially inhibited by pharmacological inhibition of RNA transcription, of protein synthesis, of tyrosine kinase activity, and by chelation of intracellular $[Ca^{2+}]$.

The 300-mOsm increment used in the present study corresponded to the maximal mannitol concentrations achieved transiently in clinical use. For a 1- to 1.5-g/kg bolus every 3 to 4 hours in a 70-kg individual with 5.8 L intravascular volume, this corresponds to a peak incremental serum osmolality of 216 to 322 mOsm, assuming equilibration throughout the vasculature, and to much higher transient local concentrations in the time following administration.

Cell shrinkage is a hallmark of apoptosis induced by all known signaling pathways and contrasts with the cell-swelling characteristic of necrotic cell death. In S49-Neo thymocytes that lack regulatory volume increase (RVI) mechanisms, hypertonic cell shrinkage itself sufficed to induce apoptosis in the presence of serum. In contrast, hypertonic serum-containing medium produced no apoptosis in other cell types (COS, HeLa, GH3) that possess RVI activity. BAE cells exhibited acute regulatory volume increase mediated by bumetanide-sensitive $Na^{+}/K^{+}/2Cl$-cotransport (Reference 23 and Jiang and Alper, unpublished data, 1998). Sustained activation of $Na^{+}/K^{+}/2Cl$-cotransport also would result in maintenance of intracellular $[K^{+}]$, itself considered protective against apoptosis. Alternatively, it may act through other pathways to antagonize or interrupt apoptotic signaling.

Cell shrinkage can force adherent cells to rearrange their focal adhesion contacts and the cytoskeletal attachments to those contacts. Recent work has shown that cell shape and interaction of integrins with the ECM plays a critical role in determining cell survival or inducing programmed cell death in adherent cells. Disruption of matrix-ECM contacts has been shown to induce anoikis, a form of apoptosis induced by cell detachment from substratum. Constitutively activated FAK can rescue epithelial cells from anoikis and induce anchorage-dependent growth in MDCK cells. In that report, cell exposure to either hyperosmotic or hypoosmotic stress increased the protein-tyrosine phosphate content of FAK, though increased FAK phosphorylation by anisosmotic perturbation did not suffice to prevent mannitol-induced apoptosis.

Cell spreading itself has been shown to protect from apoptosis regardless of the cell-ECM contact area. JNK has been shown to be activated by cell rounding, a process thought to be crucial in promoting apoptosis. However, recent findings argue against a direct contribution from either JNK or p38 in cell-detachment induced apoptosis.

![Figure 8](image1.png)

**Figure 8.** Mannitol addition induces an increase in cytoplasmic calcium. A, Fura-2–loaded endothelial cells were exposed to mannitol (240 mOsm) and cytoplasmic calcium determined as described in methods at 15-second intervals. B, Fold increase in peak cytoplasmic calcium with respect to resting value in BAE cells exposed to mannitol at 120 and 240 mOsm.

![Figure 9](image2.png)

**Figure 9.** Effects of inhibitors of protein synthesis, transcription, tyrosine kinase activity and of intracellular calcium chelation on mannitol-induced apoptosis. Effect of pretreatment for 1 hour with cycloheximide (CHX, 10 μg/mL), actinomycin D (ActD, 10 μg/mL), quin2-AM (10 μmol/L), and herbimycin (1 μmol/L) to inhibit on the fold induction of apoptotic index in response to 300 mOsm mannitol in BAE cells (*$P<0.05$, **$P<0.001$).
theless, the sustained activation of JNK in BAE for 6 hours, beyond the onset of apoptosis (Figure 7A), is consistent with the hypothesized dual contribution of JNK in controlling cell fate in response to external stimuli. In this dual role, transient activation of JNK is noted during a proliferative response, whereas sustained JNK activation accompanies apoptosis in response to ultraviolet-C and gamma irradiation. Rosette and Karin have shown that both hyperosmotic shock and TNF addition result in receptor clustering, a crucial step in JNK activation and subsequent apoptosis. The ability of the permeant osmolyte, urea, to induce apoptosis in the absence of nuclear (and likely also cellular) shrinkage is consistent with such a primary effect on receptor clustering or some other membrane signaling event.

The present data suggest a contributing role for protein tyrosine kinases in mannitol-induced apoptosis in BAE cells. It is unclear whether differential tyrosine phosphorylation of paxillin and FAK by hypoosmotic and hyperosmotic stress might explain their different abilities to induce subsequent apoptosis. However, inhibition of tyrosine kinase activity likely acts via a mechanism other than enhancing RVI, since isoosmotic shrinkage of human neutrophils activates NHE1 in concert with tyrosine phosphorylation of p59fgr and p56

isoosmotic shrinkage of human neutrophils activates NHE1 likely acts via a mechanism other than enhancing RVI, since isoosmotic shrinkage of human neutrophils activates NHE1 in concert with tyrosine phosphorylation of p59fgr and p56/59hck. In this case, cell shrinkage itself rather than changes in osmolarity or ionic strength proved to be the critical activating stimulus. Our demonstration of JNK activation and increased tyrosine phosphorylation of FAK by hyperosmotic stress does not, however, imply the causal involvement of these processes in inducing apoptosis; further experiments are needed to address this question.

Our data also suggest that intracellular calcium plays another contributing role in mediating apoptosis in BAE cells exposed to hypertonic media. Chelation of intracellular calcium using quin2-AM significantly decreased mannitol-induced apoptosis. Intracellular calcium has been previously implicated in oxidized LDL-mediated apoptosis in human endothelial cells. Treatment of S49 cells with the Ca

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-store releasing agents, thapsigargin or cyclopiazonic acid, leads to apoptosis. Calcium may contribute to apoptosis at multiple levels in different cell types (cf 32 for review), including activation of proteases, endonucleases, transglutaminases, increased phosphotidylserine transfer from the inner to outer surface of the plasma membrane, direct alteration of gene transcription, or activation of signal transduction intermediates, as exemplified by Ca

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-store depletion-mediated activation of lipoxigenase. Interestingly, the ability of inhibitors of transcription and translation to attenuate hypertonicity-induced apoptosis in BAE cells distinguishes it from hypertonicity-induced apoptosis in S49 cells but resembles the slower dexamethasone-induced apoptosis in the same cell type.

In conclusion, we have shown that both the impermeant hyperosmotic agents mannitol and sodium chloride and the permeant urea induce apoptosis in vascular endothelial cells. The concentration of mannitol used represented sustained exposure to a dose to which patients are repeatedly but transiently exposed. The apoptosis induced by hypertonicity exhibits a partial requirement for intact RNA transcription and protein translation, and is partially inhibited by chelation of intracellular calcium and by inhibition of tyrosine kinase activity. Several additional signaling pathways, including the Fas pathway, and a growing number of mitochondrial proteins, have been implicated in the apoptotic process in nonendothelial cell types. Future studies will examine their possible involvement in endothelial cell apoptosis induced by hypertonicity.

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References


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**Editorial Comment**

Endothelial cells, the interface between organs and blood, serve to maintain the integrity of vascular function. Endothelial cell death by apoptosis has been increasingly recognized under different pathological conditions. In the preceding article, Dr Malek and associates report that mannitol at concentrations which may be achieved in clinical conditions under different pathological conditions. In the preceding article, Dr Malek and associates report that mannitol at concentrations which may be achieved in clinical conditions.

**References**


Mannitol at Clinical Concentrations Activates Multiple Signaling Pathways and Induces Apoptosis in Endothelial Cells

Adel M. Malek, Greg G. Goss, Lianwei Jiang, Seigo Izumo and Seth L. Alper

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