Hypertonic Preconditioning Inhibits Macrophage Responsiveness to Endotoxin

Joseph Cuschieri, David Gourlay, Iris Garcia, Sandra Jelacic and Ronald V. Maier

*J Immunol* 2002; 168:1389-1396; doi: 10.4049/jimmunol.168.3.1389
http://www.jimmunol.org/content/168/3/1389

References
This article cites 47 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/168/3/1389.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Hypertonic Preconditioning Inhibits Macrophage Responsiveness to Endotoxin

Joseph Cuschieri, David Gourlay, Iris Garcia, Sandra Jelacic, and Ronald V. Maier

Hypertonic saline has been shown to modulate cell shape and the response of components of the innate immune response. However, the effect of hypertonic saline on the macrophage remains unknown. We hypothesized that hypertonic preconditioning would impair subsequent inflammatory mediator signaling through a reduction in stress fiber polymerization and mitogen-activated protein kinase activity after LPS stimulation. Rabbit alveolar macrophages were stimulated with 100 ng/ml of LPS. Selected cells were preconditioned with 40–100 mM of NaCl, mannitol, or urea for 4 h and returned to isotonic medium before LPS stimulation. Cellular protein was harvested and subjected to Western blot analysis for the dually phosphorylated active forms of p38 and extracellular signal-related kinase (ERK) 1/2. TNF production was determined by an L929 bioassay, and stress fiber polymerization was evaluated by confocal microscopy. Preconditioning of macrophages with NaCl or mannitol resulted in dose-dependent reduction in ERK 1/2 phosphorylation with no effect on p38 phosphorylation. Urea preconditioning had no effect on either mitogen-activated protein kinase. A dose-dependent attenuation of TNF production was seen with NaCl and mannitol preconditioning (p < 0.05), but not with urea. NaCl and mannitol preconditioning resulted in failure of LPS-induced stress fiber polymerization, whereas urea did not. Extracellular hypertonic conditions (i.e., NaCl and mannitol) have an immunomodulatory effect on macrophages, demonstrated through failure of optimal stress fiber polymerization, ERK 1/2 activity, and TNF production. Intracellular hypertonic conditions (i.e., urea) had no significant effect. Hypertonic saline or mannitol resuscitation, therefore, may help protect against multiple-organ dysfunction syndrome as a result of this reduced proinflammatory responsiveness. The Journal of Immunology, 2002, 168: 1389–1396.

Fluid resuscitation after trauma is essential for the restoration of circulating plasma volume. However, the characteristics of the optimal fluid have been debated over the years. Initially, debate centered over the use of crystalloid vs colloid (1). Recently, debate has focused on the possible benefits of crystalloid in the form of hypertonic saline. Hypertonic saline, in addition to reducing the amount of fluid used for resuscitation, has been shown to have other benefits. This includes a demonstrated reduction in third space sequestration (2). This reduction in extravascular volume should have potential benefits in patients with severe head and pulmonary injury (3). Investigators have also shown additional physiological benefits, such as improved cardiac contractility and improved microvascular perfusion (4). In addition to these physiological benefits, hypertonic resuscitation has been demonstrated to result in a significantly improved survival over isotonic resuscitation in experimental models of hemorrhage and is associated with improved pulmonary and hepatic function (5). Clinically, a survival advantage has been demonstrated in certain subgroups of trauma patients resuscitated with hypertonic saline (6).

However, the mechanisms underlying these findings have only been partially elucidated and are thought to function in part through a modulation of the innate immune response (7). Despite its inhibitory role on ultimate neutrophil functioning after trauma and hemorrhage, hypertonic saline is known to cause activation of several inflammatory mediated cell signaling mechanisms (8). Rizoli et al. (9) demonstrated a marked reduction in neutrophil sequestration and lung damage after hypertonic resuscitation using a model of posthemorrhage-induced acute lung injury (ALI) and adult respiratory distress syndrome (ARDS). This reduction in neutrophil function was associated with an improvement in overall mortality and pulmonary injury. In contrast, Junger et al. (7) showed enhanced in vitro T cell proliferation after hypertonic treatment on PBMCs obtained from trauma patients. These apparent paradoxical effects demonstrate the variability of potential therapeutic interventions on the various components of the inflammatory response.

Despite the broad range of investigations, the effect of hypertonic resuscitation on the macrophage remains unknown. Activation of the proinflammatory phenotype in tissue-fixed macrophages after trauma and hemorrhage plays a critical role in the orchestrating of the host inflammatory response and subsequent tissue injury (10). Thus, macrophages are thought to contribute to the development of ALI, ARDS, and multiple-organ dysfunction syndrome (MODS) (11). Hyperosmotic stimuli of mononuclear cells are known to activate the mitogen-activated protein kinase (MAPK) signaling cascade, including p38 and extracellular signal-related kinase (ERK) 1/2 (12). Similar MAPK activation occurs after inflammatory stimuli such as endotoxin. These signaling kinases have been demonstrated to be essential for optimal inflammatory gene responses and production of effector proteins, such as TNF-α, and are thought to contribute to the development of ARDS (13, 14). However, the effect of hypertonic preconditioning on...
HYPERTONIC PRECONDITIONING INHIBITS MACROPHAGES

Macrophage inflammatory gene up-regulation and subsequent reaction to an inflammatory stimulus is unknown.

In addition to MAPK activation, hypertonic saline is known to cause significant morphological changes to cells. These changes result in striking cytoskeletal rearrangements, which have been implicated as important for spatial positioning and optimal interactions of inflammatory mediated cellular signaling components (15). What is unknown is the duration of these rearrangements after exposure to hypertonic environments and whether they play a role in subsequent inflammatory stimuli-mediated signaling.

Tolerance, a term used to describe reduced cellular responsiveness to a stimulus, can be induced in mononuclear cells by pretreating cells with endotoxin (16). Pretreatment results in inhibition of MAPK and TNF-α production to subsequent endotoxin exposure (17). This led us to question whether hypertonic preconditioning, which results in similar MAPK activity to that after initial endotoxin exposure, would result in a similar reduction in inflammatory induced cellular signaling, as is demonstrated with endotoxin tolerance. In addition, we questioned whether this effect is specific to hypertonic saline or if other hypertonic conditions such as those created by urea and mannitol could result in similar changes. Finally, we hoped to resolve whether any distinct morphological change within the cytoskeleton could be attributed to hypertonic preconditioning. We hypothesized that tolerance induction may potentially explain, in part, the improved outcome noted after hypertonic resuscitation for shock through the reduction in the subsequent host immunoinflammatory response.

Materials and Methods

Reagents

Escherichia coli 0111:B4 LPS was obtained from Sigma-Aldrich (St. Louis, MO). Crystalline NaCl (J. T. Baker, Phillipsburg, NJ) was dissolved in sterile pyrogen-free water at a concentration of 2 M. Endotoxin contamination was tested by the Limulus Amebocyte Lysate assay (E-TOXATE kit, Sigma-Aldrich) and was found to be <0.05 ng/ml.

Cell isolation and treatment

Alveolar macrophages were obtained from male 1.5- to 2.0-kg New Zealand White rabbits by a bronchoalveolar lavage technique. Animals were housed in standard care facilities at the vivarium of Harborview Medical Center Research and Training Building (Seattle, WA), fed rabbit chow and water as needed, and used within 48 h of delivery. Animal use protocols were accepted by the Animal Care Committee of the University of Washington and met the standards of National Institutes of Health for animal care and use. Animals were euthanized with an overdose of pentobarbital and met the standards of National Institutes of Health for animal care and use. Animals were accepted by the Animal Care Committee of the University of Washington and met the standards of National Institutes of Health for animal care and use. Animals were euthanized with an overdose of pentobarbital for bronchoalveolar lavage. The lungs were gently lavaged six times with 40-ml aliquots of normal saline. The combined lavage fluid was centrifuged and resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) with 100 μg/ml gentamicin. Macrophages were then plated immediately in tissue culture-treated plates at 5 × 10^6 cells/ml. The macrophages were allowed to adhere for 60 min before the initiation of experimental protocols.

Selected cells were then treated with varying concentrations of NaCl, mannitol, or urea from 40 to 100 mM for 4 h (medium osmolarity of 355–525 mOsm). Treated cells were then washed and returned to fresh isotonic medium. Control and pretreated cells were then stimulated with 100 ng/ml LPS for a period of time between 0 and 60 min. Total cellular protein was electrophoresed in 10% SDS-PAGE gel and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was blocked for 1 h at room temperature with 5% BSA or 5% milk and then incubated with a HRP-conjugated anti-dual phosphorylated p38 (Cell Signaling, Beverly, MA) or anti-dual phosphorylated ERK 1/2 (Cell Signaling) for 1 h at 4°C, respectively. The blot was developed using the SuperSignal chemiluminescent substrate (Pierce) and exposed on film (KAR-5; Kodak, Rochester, NY). Densitometry was performed by the NIH:image program to quantitate optical density. All gels were reblotted for total ERK1, ERK2, and p38 to confirm equal loading.

TNF production

Alveolar macrophages treated with 100 ng/ml of LPS were incubated overnight, with and without hypertonic preconditioning (as described above). Supernatants were then harvested for TNF analysis. Macrophage TNF production was measured using the biologic cytotoxicity assay with transformed mouse fibroblasts (National Cancer Tissue Culture clone L929; American Type Culture Collection, Manassas, VA). In brief, L929 cells were pretreated with actinomycin D (Sigma-Aldrich) 1 μg/ml for 15 min in RPMI 1640 supplemented with 5% horse serum (HyClone Laboratories, Logan, UT) and added at 5 × 10^4 cells/well to serial dilutions of the supernatants collected from the stimulated macrophages. The plates were then incubated in 5% CO2 at 37°C. After 24 h, the cells were washed with PBS and then fixed and stained in 0.1% crystal violet in 20% methanol.

Dried monolayers were solubilized in 0.1 M of sodium citrate in 50% methanol, and absorbance was read at 550 nm with a kinetic microplate reader (Molecular Devices, Menlo Park, CA). One unit of TNF activity was defined as that which produced 50% cytosis of the L929 monolayer. A linear regression was performed to determine the point between serial dilutions where the 50% cytosis endpoint occurred. The E. coli 0111:B4 LPS showed no direct toxicity against the L929 cell line.

Stress fiber polymerization

Cells treated with 100 ng/ml LPS for 15 min, with and without hypertonic preconditioning with 100 mM of NaCl, 100 mM of mannitol, or 100 mM of urea, were subjected to stress fiber analysis via confocal microscopy. After their stimulation, cells were warmed in PBS and fixed with 3.7% formaldehyde solution. Cells were then permeabilized with 0.1% Triton X-100. Cells were then blocked with 1% BSA and stained with FITC-conjugated phalloidin (Molecular Probes, Eugene, OR) for 30 min. Cells were then washed and mounted. Cells were then examined using confocal microscopy (Bio-Rad, Hercules, CA).

Cell viability

Representative cell populations from each condition were subjected to trypan blue exclusion, confirming >95% viability in all conditions.

Statistical analysis

Values are expressed as means ± SEM. Group means are compared by ANOVA with post hoc testing by Fisher’s least significant difference. Paired or unpaired comparisons between various experimental groups and corresponding LPS-treated group were analyzed by paired and unpaired t tests, respectively. A p value of 0.05 or less was considered significant.

Results

LPS stimulation activates ERK 1/2 and p38

The activation of ERK 1/2 and p38 was investigated after 100 ng/ml of LPS stimulation of rabbit alveolar macrophages. Activation was determined by using specific Abs to the dually phosphorylated active form of both ERK 1/2 and p38. Western blot analysis of time course studies demonstrated maximal phosphorylation of ERK 1/2 and p38 at 30 min (data not shown).

NaCl preconditioning inhibited LPS-induced ERK 1/2 activity

Rabbit alveolar macrophages were preconditioned with NaCl for 4 h at various concentrations between 40 and 100 mM. After their preconditioning, cells were returned to isotonic medium and stimulated with 100 ng/ml of LPS. ERK 1/2 activity was then determined using a specific Ab, which recognized the dually phosphorylated active form. Western blot analysis demonstrated a similar maximal phosphorylation of ERK 1/2 at 30 min (data not shown). However, when compared with control, a dose-sensitive reduction
in maximal phosphorylation was noted with increasing concentration of NaCl used for preconditioning (Fig. 1A). Despite this reduction in activation, total levels of either ERK1 or ERK2 protein were unchanged from control cells (data not shown).

Mannitol preconditioning inhibited LPS-induced ERK 1/2 activity
Similar to cells preconditioned with NaCl, alveolar macrophages were preconditioned with various concentrations of mannitol between 40 and 100 mM. An inhibition in ERK 1/2 activation was noted with increasing doses of mannitol (Fig. 1B). ERK 1 and ERK 2 levels were unchanged from controls (data not shown).

NaCl preconditioning had no effect on LPS-induced p38 activity
In a similar fashion to ERK 1/2 activation, p38 activation was investigated after NaCl preconditioning. Activity of p38 was determined using a specific Ab to the dually phosphorylated active form and was analyzed by Western blot. Similar to ERK 1/2, maximal p38 activity occurred at 30 min after LPS under all preconditioning concentrations. However, unlike ERK 1/2, no significant effect on p38 activation could be demonstrated with any concentration of NaCl used in preconditioning (Fig. 2A).

Mannitol preconditioning had no effect on LPS-induced p38 activity
Similar to the effect of NaCl, no significant effect on the activity of p38 was found by Western blot analysis after mannitol preconditioning (Fig. 2B).

Urea preconditioning had no effect on either p38 or ERK 1/2 activity induced by LPS
Unlike cells preconditioned with either mannitol or NaCl, no effect was noted on ERK 1/2 activity induced by LPS using Western blot analysis when compared with control (Fig. 1C). In addition, p38 activity determined by Western blot analysis showed no change from control responses after 30 min of LPS stimulation (Fig. 2C).

LPS stimulates TNF-α production
LPS stimulation of alveolar macrophage overnight resulted in significant TNF production as determined by L929 bioassay activity.

Either NaCl or mannitol preconditioning led to a reduction in TNF-α production
Noting the effect of NaCl and mannitol on signal transduction through ERK 1/2, we evaluated the effect of this preconditioning on TNF production. Cells preconditioned with as little as 40 mM NaCl resulted in a 60% reduction in TNF production as analyzed by L929 bioassay on overnight supernatants. This response was also noted to be dose sensitive, with increasing doses resulting in further reduction in TNF production (Fig. 3A). Similarly, mannitol preconditioning resulted in reduction in TNF production. However, this effect was not as strong as NaCl, with only a 40% reduction noted with 40 mM mannitol preconditioning. In addition, this effect was also noted to be dose responsive (Fig. 3B).

Urea preconditioning had no effect on LPS-induced TNF-α production
Urea, unlike NaCl or mannitol but similar to the effect on ERK 1/2, had no effect on TNF production at any concentration from 40 to 100 mM when compared with cells stimulated with only LPS (Fig. 3C).

LPS stimulation leads to stress fiber polymerization
The potential contributions of stress fibers and cell shape were analyzed by FITC-conjugated phalloidin staining of actin stress fibers. After 15 min of 100 ng/ml of LPS stimulation, significant stress fiber polymerization was noted when compared with control cells (Fig. 4, A and B).
Either NaCl or mannitol led to inhibition of LPS-induced stress fiber polymerization. However, cells preconditioned with 100 mM NaCl or mannitol did not demonstrate any significant polymerization after 15 min of LPS stimulation and appeared similar to unstimulated control cells (Fig. 4, C and D).

Urea preconditioning had no effect on LPS-induced stress fiber polymerization. Unlike NaCl or mannitol, no significant effect was noted as a result of urea preconditioning on LPS-induced stress fiber formation. Endotoxin stimulation of urea-preconditioned cells demonstrated...
similar morphological changes to those seen with endotoxin stimulation alone (Fig. 4E).

**NaCl and mannitol preconditioning demonstrated no effect on LPS-induced ERK 1/2 activity 20 h after initial hypertonic preconditioning**

NaCl or mannitol preconditioning demonstrated a dose-dependent inhibition of LPS-induced ERK 1/2 activity if stimulated after initial hypertonic preconditioning. Therefore, rabbit alveolar macrophages were incubated in isotonic medium after initial hypertonic preconditioning for 20 h before LPS stimulation. Cells were then stimulated with 100 ng/ml LPS overnight. Supernatants were harvested and analyzed for TNF-α by an L929 bioassay. No significant difference in LPS-induced TNF-α production could be demonstrated between hypertonic preconditioned cells and non-preconditioned cells (Fig. 5).

**Discussion**

Hypertonic resuscitation has been shown to be effective in restoring circulating plasma volume after a hypovolemic challenge, with an improvement in overall survival (2, 18). The factors underlying this improvement are poorly characterized. Hypertonic saline, in addition to several beneficial physiological effects, provides a significant immunomodulating role (5, 7). This effect has been extensively studied in neutrophils (19, 20) and lymphocytes (21),

![Figure 4](image-url)

**Figure 4.** Confocal microscopy for FITC-conjugated phalloidin rabbit alveolar macrophages. **A**, Tissue culture-fixed alveolar macrophage with minimal stress fiber polymerization. **B**, Rabbit alveolar macrophage stimulated with *E. coli* 0111:B4 LPS for 15 min before formaldehyde fixing and staining. Cell shown representative of cells treated with LPS, demonstrating significant stress fiber polymerization with pseudopodia formation. **C**, Alveolar macrophage preconditioned with 100 mM of NaCl before *E. coli* 0111:B4 LPS treatment in isotonic medium. Cell demonstrates lack of appropriate stress fiber polymerization after LPS stimulation. **D**, Similarly, mannitol-preconditioned cell demonstrating lack of stress fiber polymerization after LPS. **E**, Urea-preconditioned cell demonstrating no significant morphological change when compared with control cells stimulated with LPS.

![Figure 5](image-url)

**Figure 5.** LPS-induced TNF-α production returns 20 h after initial hypertonic preconditioning with either NaCl or mannitol. Hypertonic preconditioning with either NaCl or mannitol was demonstrated to attenuate LPS-induced TNF-α production. However, maintaining cells in isotonic medium for 20 h after initial hypertonic preconditioning, as previously described, demonstrated no difference with either NaCl (**A**) or mannitol (**B**) in LPS-induced TNF-α production when compared with cells stimulated with LPS without preconditioning 20 h later. TNF production expressed in U/ml (1 U = 40 pg). *, Statistically significant difference from LPS-treated control cells; p < 0.05. Data representative of five experiments.
however the effect on the macrophage and its subsequent response to an inflammatory stimulus has been lacking.

The macrophage is a regulatory inflammatory cell causing the recruitment and activation of numerous proinflammatory phenotypes, such as the neutrophil, to areas of infection and tissue injury (22). This action is critical to the normal host immune response to inflammatory stimuli. It performs this action by producing a plethora of cytokines and chemokines, with TNF-α being a central mediator (23). In addition, macrophages produce a number of other factors, such as tissue factor and prostanoids, which result in microcirculatory thrombosis (24). The macrophage, along with the recruited activated neutrophil, release high levels of reactive oxygen intermediates and proteases, which contribute to endothelial injury and capillary leak (25). In addition, stress, such as hemorrhage, before an inflammatory stimulus, such as endotoxin, results in an exaggerated macrophage-induced inflammatory response (26). This response is associated with markedly elevated cytokine levels and inflammatory responsiveness (27). As a result of this aberrant response, significant tissue injury develops, as seen clinically in the development of ARDS and MODS (11).

Hypertonic saline, when used for resuscitation, results in a dramatic initial increase in serum osmolarity. Recent studies have demonstrated that after initial resuscitation with hypertonic saline, serum osmolarities can transiently reach levels of up to 500 mOsm and can remain elevated for 4 h with a serum osmolarity of 350 mOsm (9). Using these in vivo data, alveolar macrophages were subjected to hypertonic preconditioning for 4 h with 50–100 mOsm of NaCl, mannitol, or urea, resulting in medium osmolarities of 355–525 mOsm, respectively. Although the maximum dose is probably not clinically relevant, it allowed the study of the dose effect response and any respective toxicity of the various hypertonic pretreatments. Although hypertonic preconditioning affected macrophage function, this was not a result of global toxicity or cell death. As demonstrated by trypan blue exclusion, >95% cell viability was maintained under all hypertonic conditions. In addition, there was no effect on the ability of the cells to activate p38 MAPK. This in vitro model, therefore, allowed the study of hypertonic preconditioning with various hypertonic agents on endotoxin-induced inflammatory signaling in the central mediating inflammatory cell, the macrophage.

Important to both normal and excessive inflammatory signaling within the macrophage is the activation of the MAPK family (12). Optimal activation of both ERK 1/2 and p38 has been demonstrated to be essential for inflammatory mediated gene transcription and translation (28). Although previous work has demonstrated activation of both p38 and ERK 1/2 after initial exposure to hypertonic saline, our data demonstrate that, after hyperosmotic preconditioning, a significant modulation of endotoxin-induced macrophage MAPK signaling occurred (8, 29, 30). Hypertonic preconditioning with extracellular stress, either NaCl or mannitol, significantly reduced the activation of ERK 1/2 to subsequent endotoxin stimulation. However, no significant change in p38 activity was demonstrated. Although this MAPK inhibition is selective to ERK 1/2, it is consistent with in vitro models of endotoxin tolerance, demonstrating repetitive inflammatory stimuli causing inhibition of MAPK activity (17, 31). However, a major difference is the selective nature of MAPK inhibition by extracellular hypertonic stress compared with a nonselective global MAPK inhibition by endotoxin tolerance. This selective inhibition, unlike endotoxin tolerance, leads to an inhibition of macrophage function without complete abolishment of inflammatory mediated MAPK signaling.

This led to further speculation on what factors specific to both NaCl and mannitol were responsible for this selective MAPK inhibition. Because both NaCl and mannitol do not freely cross the cell membrane, the question arises whether this is due directly to a cellular recognition of hypertonicity (i.e., specific receptors) created by these agents or to the cellular shrinkage induced by the increased extracellular tonicity (32). To answer this fundamental question, urea, which is a membrane-permeable agent and does not cause cellular shrinkage, was used as a hypertonic preconditioning agent (33, 34). Urea, when used for preconditioning, did not demonstrate any significant effect on endotoxin-induced MAPK activity. These data would support that hypertonic-induced cellular shrinkage appears to be the mechanism underlying this selective ERK 1/2 inhibition.

Consistent with the selective inhibition of ERK 1/2 by extracellular hypertonic preconditioning was the significant attenuation of endotoxin-induced TNF-α production caused by either NaCl or mannitol preconditioning. As expected, urea, which had no effect on MAPK signaling, had no significant effect on endotoxin-induced TNF-α production, although a nonsignificant trend was observed. Although previous work has demonstrated a similar finding, the mechanism causing this reduction has only now been partly elucidated (35, 36). Our data demonstrate a selective inhibition of ERK 1/2 by extracellular hypertonic preconditioning, which appears to partly explain the reduction in TNF-α. Previous work has shown that, for optimal TNF-α production, both p38 and ERK 1/2 activation are required, but selective inhibition of ERK 1/2 alone only partly accounts for the dramatic attenuation of TNF-α production demonstrated by extracellular hypertonic preconditioning (37). Previous explanations for attenuation of inflammatory mediator production after hypertonic preconditioning have centered on the production of counterregulatory factors leading to inhibition of TNF-α production. Factors that may play a role include IL-10, PG, and other currently unknown mediators (38, 39). Our laboratory, as well as others, has demonstrated that IL-10 production is increased after hypertonic preconditioning in mononuclear cells. Although thought to play a role in the inhibition of TNF-α, previous work by Oreopoulos et al. (36) demonstrated that blocking IL-10 membrane receptor binding did not significantly reverse attenuation of TNF-α production induced by hypertonic preconditioning. Therefore, at this time, the only consistent mechanism demonstrated to explain TNF-α-attenuated production is the selective inhibition of ERK 1/2 demonstrated in this study.

Because inhibition of macrophage inflammatory functioningcorrelated with cell shrinkage caused by extracellular hypertonic conditioning, the possible structural effects that could be attributed to hypertonic saline and mannitol were investigated. It is known that, after initial exposure of inflammatory cells to extracellular hypertonic conditions, cell shrinkage occurs as a result of the equilibration of osmolarity across the cell membrane through the loss of intracellular water (40, 41). This change in cell shape results in a significant change in the cytoskeletal framework (14). Previously, the cytoskeleton has been demonstrated to be important in endotoxin-induced cellular activation. Stress fiber polymerization after endotoxin stimulation is necessary for optimal cellular adhesion, migration, intracellular signaling, and gene transcription and translation (42). Recent work has demonstrated that inhibition of endotoxin-induced stress fiber polymerization by cytochalasin D and other inhibitors results in an inhibition of MAPK signaling and attenuation of TNF-α production in mononuclear cells (43–45). These effects are similar to those demonstrated by extracellular hypertonic conditioning. Given these findings, we studied the direct effect of hypertonic preconditioning on endotoxin-induced stress fiber polymerization by evaluating their formation under confocal microscopy. This study clearly demonstrates that preconditioned cells with either NaCl or mannitol had a marked reduction in endotoxin-induced actin polymerization. Although only a single
time point is demonstrated, maximal endotoxin-induced stress fiber polymerization occurred after 15 min of stimulation, a time point before maximal ERK 1/2 activity. This lack of appropriate stress fiber polymerization, therefore, could explain the inhibition of ERK 1/2 activation and TNF-α production after endotoxin stimulation, which is similar to that seen with depolymerization of actin stress fibers by cytochalasin D. Consistent with these findings is the lack of inhibitory effect by hypertonic preconditioning with urea on endotoxin-induced stress fiber polymerization. Taken together, these data indicate that the actin cytoskeleton may be the critical mediator affected by hypertonic preconditioning. This inhibition of appropriate stress fiber polymerization would result in a failure of optimal spatial positioning of kinases leading to inhibition of appropriate intracellular interactions, thus leading to down-regulation of proinflammatory gene signaling.

Therefore, hypertonic resuscitation after hypovolemic stress may prevent inappropriate excessive inflammatory response to an inflammatory stimulus through inhibition of inflammatory mediated stress fiber polymerization, ERK 1/2 activity, and inflammatory mediator production. Inhibition of this response would result in reduced macrophage responsiveness, leading to a reduction in neutrophil recruitment and host injury as demonstrated by ALI, ARDS, and MODS.

However, the inhibitory effect of hypertonic preconditioning with either NaCl or mannitol on macrophage is only transient. In fact, return of macrophage function after hypertonic preconditioning occurred within 20 h of the initial hypertonic exposure. In vitro experiments not only demonstrated a return of LPS-induced intracellular signaling through ERK 1/2, but also LPS-induced TNF-α production. Although rabbit macrophages become less responsive with time ex vivo, this had no significant effect on ERK 1/2 activity and only diminished LPS-induced TNF-α production without hypertonic preconditioning by 25%. Despite this reduction, hypertonic preconditioning with either NaCl or mannitol followed by LPS 20 h later demonstrated a 2- to 10-fold increase in TNF-α production over cells stimulated with LPS shortly after hypertonic exposure. In addition, no significant difference could be demonstrated in LPS-induced TNF-α production between cells that underwent hypertonic preconditioning 20 h before and those that were never preconditioned. This clearly demonstrates a transient affect of hypertonic preconditioning on alveolar macrophages and thus would lead to speculation that the clinical use of hypertonic resuscitation would also be transient. In fact, previous work by Rizoli et al. (46) has demonstrated the loss of LPS-induced CD11b expression on neutrophils by hypertonic preconditioning returns if cells are maintained in an isotonic environment for 18 h before endotoxin stimulation. In addition, subsequent hypertonic preconditioning demonstrated a return of inhibition of LPS-induced CD11b expression within these cells. Taken together, these combined data demonstrate that the possible beneficial modulation of the innate immune response is transient for ~18–20 h and could return if cells are subjected to further preconditioning.

Therefore, this transient reduction in macrophage inflammatory functioning may partly explain the improvement in survival seen in in vivo models. This reduction, as previously mentioned, would inhibit the development of ARDS and MODS by inhibiting macrophage recruitment and activation of neutrophils and production of numerous proinflammatory mediators, such as TNF-α, which plays a critical role in this process (47). Thus, down-regulation of this inappropriate proinflammatory phenotype through the use of appropriate hypertonic resuscitation strategies may lead to reduced morbidity and mortality in the clinical setting.

The present study demonstrates that hypertonic preconditioning with either NaCl or mannitol clearly has an immunomodulatory effect on alveolar macrophages. This modulation results in inhibition of the proinflammatory phenotype, resulting in inhibition to endotoxin stimulation. Extracellular hypertonic preconditioning, such as that created with NaCl or mannitol, led to inhibition of endotoxin-induced stress fiber polymerization. As a result, intracellular spatial relations are affected, resulting in an inhibition of cellular signaling through ERK 1/2 and a significant reduction in inflammatory mediator production. This preconditioning leads to a transient down-regulation of the proinflammatory phenotype. Taken together, these findings further support a possible role for hypertonic solutions as a means to regulate an exaggerated inflammatory response, possibly leading to a reduction in the incidence of MODS and ARDS.

References

Downloaded from http://www.jimmunol.org/ by guest on April 12, 2017


