Febrile-range hyperthermia worsens and hypothermia mitigates lung injury, and temperature dependence of lung injury is blunted by inhibitors of p38 mitogen-activated protein kinase (MAPK). Of the two predominant p38 isoforms, p38α is proinflammatory and p38β is cytoprotective. Here, we analyzed the temperature dependence of p38 MAPK activation, substrate interaction, and tertiary structure. Incubating HeLa cells at 39.5 °C stimulated modest p38 activation, but did not alter tumor necrosis factor-α (TNFα)-induced p38 activation. In in vitro kinase assays containing activated p38α and MAPK-activated kinase-2 (MK2), MK2 phosphorylation was 14.5-fold greater at 39.5 °C than at 33 °C. By comparison, we observed only 3.1- and 1.9-fold differences for activating transcription factor-2 (ATF2) and signal transducer and activator of transcription-1α (STAT1α) and a 7.7-fold difference for p38β phosphorylation of MK2. The temperature dependence of p38α substrate binding affinity, as measured by surface plasmon resonance, paralleled substrate phosphorylation. Hydrogen–deuterium exchange MS (HDX-MS) of p38α performed at 33, 37, and 39.5 °C indicated temperature-dependent conformational changes in an α helix near the common docking and glutamate: aspartate substrate-binding domains at the known binding site for MK2. In contrast, HDX-MS analysis of p38β did not detect significant temperature-dependent conformational changes in this region. We observed no conformational changes in the catalytic domain of either isoform and no corresponding temperature dependence in the C-terminal p38α-interacting region of MK2. Because MK2 participates in the pathogenesis of lung injury, the observed changes in the structure and function of proinflammatory p38α may contribute to the temperature dependence of acute lung injury.

Humans usually maintain body temperature within a narrow range, but regulated deviations occur as a result of normal circadian rhythm and in response to exogenous or endogenous pyrogens. Unregulated temperature deviations occur when normal thermoregulation fails or thermoregulatory effector mechanisms are overwhelmed by intentional or accidental environmental exposures. The biological effects of hyperthermia have been harnessed to improve neurologic outcome after cardiac arrest (1, 2) and more recently to reduce acute lung injury (3, 4). Hyperthermia has proven to be effective as adjuvant treatment against some forms of cancer (5), but it may worsen organ injury, including acute lung injury (6, 7). Changes in temperature within this clinically relevant hypo-to-hyperthermia range exert critical biological effects that impact cell survival, endothelial barrier function, coagulation, leukocyte trafficking, and inflammation (8–15). Within the lungs, temperature elevations accelerate pathogen clearance but also increase permeability pulmonary edema and neutrophil-mediated lung inflammation and injury (7). Within tumors, temperature elevations enhance recruitment of lymphocytes with anti-tumor activity (14). Despite the significant biological impact of hypo- and hyperthermia, gene expression analyses have shown that temperature shifts between 30 and 41 °C modify ≤4% of expressed genes (16–20) and ≤1% of miRNAs (21), suggesting that temperature shifts in this range affect only a narrow subset of signaling events.

Substantial preclinical data have implicated p38 mitogen-activated protein kinase (MAPK)2 in the pathogenesis of acute respiratory distress syndrome/acute lung injury (10, 11, 22–34) and other inflammatory diseases (35–39). The p38 family comprises four serine/threonine protein kinases of which p38α is

---

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; ATF2, activating transcription factor-2; CD, common docking; ED, glutamate:aspartate; DSF, differential scanning fluorimetry; ERK, extracellular signal-regulated kinase; FRH, febrile-range hyperthermia; HDX-MS, hydrogen–deuterium exchange mass spectrometry; HSP27, heat shock protein 27; IVK, in vitro kinase; MK2, MAPK-activated protein kinase; Rmax, response units at equilibrium; SPR, surface plasmon resonance; STAT, signaling transducer and activator of transcription; TNF, tumor necrosis factor; TRPV4, transient receptor potential cation channel subfamily V member 4; HMVEC, human microvascular lung endothelial cell; PDB, Protein Data Bank; ANOVA, analysis of variance; MANOVA, multivariate ANOVA.
the proinflammatory isoform (40, 41) and p38β and p38γ are cytoprotective (42, 43). Among the many biological processes regulated by p38α, endothelial and epithelial barrier function (10), leukocyte trafficking (11), and cytokine expression (35) are central to pathogenesis of acute respiratory distress syndrome/acute lung injury. We have previously identified the p38 signaling pathway as a potentially temperature-sensitive pathway that impacts heat shock protein expression and secretion (44, 45), endothelial barrier function, and neutrophil migration (10, 11). We showed that SB203580, a catalytic inhibitor that only inhibits p38α and p38β among the p38 isoforms, reduces the effect of febrile-range hyperthermia (FRH) on TNFα-induced endothelial leak, neutrophil transendothelial migration, and LPS-induced lung injury (10, 11). Other laboratories have shown that hypothermia reduces TNFα-induced activation of p38- and heat shock protein (HSP27)-dependent endothelial permeability, possibly through activation of dual-specificity protein phosphatase 1 (DUSP-1) expression (13).

The p38 MAPKs are classically activated through a canonical three-kinase module that dually phosphorylates p38 on the threonine and tyrosine in the TXY motif common to all MAPKs, but under certain conditions, p38 activation can occur through autophosphorylation of the threonine in the TXY motif (46, 47). Once activated, p38 translocates to both the nucleus and the cytoskeleton and can phosphorylate a broad range of substrates that have both pro- and anti-inflammatory actions (48). Substrate and function specificity of p38 kinase activity is achieved through selective binding of substrates to specific motifs on p38, including a substrate-docking groove located between the C- and N-terminal lobes and opposite the catalytic site (49). The nature of substrate-specific interactions with the p38 substrate-docking domains likely determines the phosphorylation efficiency for each substrate and the downstream biological effects.

The purpose of this study was to determine how shifts in temperature within a clinically relevant range modify p38 signaling. We show that changing the reaction temperature between 33 and 39.5 °C causes a conformational change within the substrate-binding groove of p38α but not p38β with an associated substrate-specific change in p38α substrate binding and kinase function. Binding affinity and phosphorylation rate of p38α for MK2 relative to ATF2 and STAT1α increased at 39.5 °C and decreased at 33 °C.

**Results**

**Temperature dependence of p38 activation**

We previously showed that exposure to FRH (~39.5 °C) causes a modest increase in levels of activated p38 MAPK in whole-lung tissue homogenates in mice *in vivo* (11) and human microvascular lung endothelial cells (HMVECs) *in vitro* (10, 11). In this study, we found that incubating HeLa cells at 39.5 °C stimulated an increase in phosphorylated p38 that peaked ~2-fold above baseline after 30–60 min and returned to baseline by 2–4 h (Fig. 1A and Fig. S4). THP1 and BEAS2B cell lines and primary cultured HMVECs exhibited a similar transient activation of p38 in response to hyperthermia (Fig. 1C and Fig. S4). Unlike p38, there was no detectable ERK activation in the hyperthermic HeLa cells (Fig. 1A and Fig. S4). In contrast with hyperthermia alone, stimulating HeLa cells with 10 ng/ml TNFα activated a rapid, transient ~25-fold increase in phosphorylated p38 that was similar at 33, 37, and 39.5 °C (Fig. 1D and Fig. S4).

**p38α activation at febrile temperature does not occur via autophosphorylation**

To determine the potential contribution of autophosphorylation to the p38 activation observed in hyperthermia-stimulated cells, we first determined whether p38 catalytic activity was required for p38 activation in HeLa cells incubated at 39.5 °C by pretreating with the p38 catalytic inhibitor, SB203580 (Fig. 1E and Fig. S4). Pretreating HeLa cells with 10 μM SB203580 caused only a slight decrease in p38 activation after 30-min exposure to 39.5 °C that was similar in magnitude to its effect on basal and TNFα-stimulated p38 activation. To further assess the potential for p38 to activate via autophosphorylation, we measured phosphorylation of recombinant p38α and p38β incubated in cell-free *in vitro* kinase reactions at various temperatures between 33 and 41 °C in the absence of other kinases (Fig. 1F and Fig. S4). Neither p38α nor p38β exhibited temperature-dependent phosphorylation during a 30-min incubation at temperatures between 33 and 41 °C.

**Temperature dependence of p38:substrate interactions**

We next analyzed the temperature dependence of p38α and p38β kinase activity for specific substrates in *in vitro* kinase assays performed at 33, 37, or 39.5 °C (Fig. 2 A–C and Fig. S5). The initial phosphorylation rate of MK2 by p38α, measured over the first 10 min of the reaction, was 14.5-fold greater at 39.5 °C than at 33 °C compared with a 3.1-fold temperature-dependent difference for ATF2 and 1.9-fold difference for STAT1α. For p38β, the phosphorylation rate of MK2, ATF2, and STAT1α was 7.7–2.4-, and 2.1-fold greater at 39.5 °C than at 33 °C. For p38α, the phosphorylation rate of MK2 relative to ATF2 was 4.7-fold greater at 39.5 °C than at 33 °C. For p38β, the ratio of MK2/ATF2 phosphorylation rate was 3.2 greater at 39.5 °C than at 33 °C.

We used SPR to quantify substrate binding affinity for immobilized, unphosphorylated recombinant p38α and p38β at 33, 37, and 39.5 °C (Fig. 3 A–D) and Fig. S1 (A and B). Based on the dissociation constant ($K_d$) values derived from the SPRs, binding affinity was approximately 3 orders of magnitude higher for ATF2 than MK2 for both p38α and p38β, but the temperature dependence of p38 binding was very different for the two substrates. The binding affinity of p38α to MK2 was 2.7-fold greater at 39.5 °C than at 33 °C, whereas its binding affinity for ATF2 was 33% lower at 39.5 °C versus 33 °C. For p38β, binding affinity to MK2 was 2-fold greater at 39.5 °C than at 33 °C, whereas its binding affinity for ATF2 was similar at all three temperatures. The temperature-dependent changes in p38 binding to and phosphorylation of MK2 occurred well below the melting temperatures for p38α and p38β, which were 47 and 48.3 °C, respectively, compared with 53.1 °C for ERK2 (Fig. 3F).
Analysis of temperature-dependent p38 conformational changes using HDX-MS

To understand how temperature-dependent conformational changes in p38 might explain the substrate-specific effect of temperature on kinase function, we performed HDX-MS for unphosphorylated p38 and p38 at 33, 37, and 39.5 °C. The analysis yielded 149 peptides with 97.8% sequence coverage for p38α and 85 peptides with 96.7% sequence coverage for p38β (Fig. S2). The HDX-MS analysis of p38α structure at 33 °C was in overall agreement with previous studies (50, 51), recapitulating the exchange-protected core (αE and αF for the C-terminal lobe, β3-αC-β4 for the N-terminal lobe) and the high deute-
rium uptake of loops and peripheral regions (glycine-rich loop or the MAPK insert) (Fig. S2). Moreover, the qualitative agreement extends to catalytically important motifs, such as the activation lip, which appears largely labile and readily exchangeable; the DFG motif; and the substrate P+1 binding pocket within the active site. HDX-MS analysis at 33 °C demonstrated regions displaying a bimodal isotopic envelope typical of EX1 kinetics that is indicative of large, slow, and cooperative conformational changes. Bimodal isotopic envelopes such as those that are clearly seen in Fig. 4A for p38α at 39.5 °C arise when a contiguous stretch of residues unfold in a concerted manner and remain unfolded long enough for all of the exposed amide hydrogens to exchange nearly completely before refolding (52, 53). This feature, which was not reported in a previous HDX-MS analysis of p38α performed at 10° (50, 51), became more pronounced as the temperature increased from 33 to 39.5 °C. Four peptide regions in p38α exhibited temperature-dependent EX1-type kinetics, residues 130–145, 207–215, 281–288, and 300–306. The most intense and fastest EX1-type kinetics were located around residues 130–145 and involved ~7 amide hydrogens encompassing the C-terminal end of αE. Fig. 4A shows the stacked spectra with the bimodal isotopic envelopes for peptide 130–145 (αE) of p38α (left) and p38β (right). For p38α, the exchange-prone species was not detected until 2 h of deuterium incubation at 33 °C, when it accounted for ~33% of the overall isotopic envelope. At 37 and 39.5 °C, the exchange-prone envelope was initially detected by 10 min, and by 2 h it had reached 64 and 83% of the total isotopic envelope, respectively. For p38β, the EX1 kinetics were much slower and less temperature-sensitive; the exchange-prone envelope was significantly populated only after a 2-h incubation at 39.5 °C and not at all at lower temperatures. The EX1 kinetics of αE are summarized in Fig. 4B, which shows decay of the exchange-protected envelope as a function of deuterium incubation time for both p38α and p38β at each of the three temperatures. For p38α, the half-life of exchange on αE was 85 and 60 min at 37 and 39.5 °C, respectively, and >2 h at 33 °C. For p38β, the half-life was >2 h at all three temperatures. Mapping peptide 130–145 (in red) to the known structure of p38α in Fig. 4C shows it to span αE within the substrate-binding groove between the CD and ED motifs. The C terminus of MK2 (yellow) is shown binding to the same region of the substrate-binding groove (PDB entry 2OKR) (54).

The conformational changes observed in peptide 130–145 of p38α were accompanied by somewhat slower but similarly temperature-dependent EX1 kinetics of conformational unfolding in adjacent α-helical segments spanning residues 207–215 (αF), 281–288 (αH), and 300–306 (αI) of p38α, each involving ~6–8 amide hydrogens (Fig. S3). The half-life for deuteration at 39.5 °C was ~60 min for residues 207–215, 281–
Temperature-dependent p38α MAPK structure and function

A. 

\[
\begin{array}{|c|c|c|}
\hline
K_D (\mu M) & R_{max} (RU) & \chi^2 (RU^2) \\
\hline
1.27 & 10.62 & 0.0334 \\
\hline
\end{array}
\]

\(p38\alpha: 33^\circ C\)

B. 

\[
\begin{array}{|c|c|c|}
\hline
K_D (\mu M) & R_{max} (RU) & \chi^2 (RU^2) \\
\hline
1.73 & 20.5 & 0.101 \\
\hline
\end{array}
\]

\(p38\beta: 33^\circ C\)

C. 

\[
\begin{array}{|c|c|c|}
\hline
K_D (\mu M) & R_{max} (RU) & \chi^2 (RU^2) \\
\hline
0.644 & 15.01 & 0.124 \\
\hline
\end{array}
\]

\(p38\alpha: 37^\circ C\)

D. 

\[
\begin{array}{|c|c|c|}
\hline
K_D (\mu M) & R_{max} (RU) & \chi^2 (RU^2) \\
\hline
0.474 & 12.06 & 0.181 \\
\hline
\end{array}
\]

\(p38\alpha: 39.5^\circ C\)

E. 

\[
\begin{array}{|c|c|c|}
\hline
\text{Substrate} & 33^\circ C & 37^\circ C & 39.5^\circ C \\
\hline
\text{p38\alpha} & 1.27 & 0.644 & 0.474 \\
\text{ATF2} & 0.0018 & 0.0025 & 0.0027 \\
\text{p38\beta} & 1.73 & 0.992 & 0.874 \\
\text{ATF2} & 0.0031 & 0.0023 & 0.0024 \\
\hline
\end{array}
\]

F. 

\[
\begin{array}{|c|c|}
\hline
\text{Melting temperature (°C)} & 55 \\
\hline
\text{p38\alpha} & * \\
\text{p38\beta} & * \\
\text{ERK2} & \\
\hline
\end{array}
\]
summarizes percent deuteration after 2 h for all three regions. For regions 207–215 and 281–288 in p38 (39.5 °C). In agreement with our past report (49) and those that typically occur in response to serious infections, we focused on p38 signaling because it is known to participate in the regulation of endothelial barrier function (23, 26, 55), inflammation, and neutrophil recruitment (11, 30, 56) and because previous studies suggested that p38 signaling is suppressed by hypothermia (10, 11). We studied temperatures representative of currently used therapeutic hypothermia protocols (33 °C) and those that typically occur in response to serious infections (39.5 °C). In agreement with our past report (11), we found that exposing cell cultures to 39.5 °C stimulated a modest and transient p38 activation in multiple cell types. Based on a previous report that a p38α mutant with increased activation loop flexibility underwent spontaneous autophosphorylation in Escherichia coli grown at 32 °C but not at 21 °C (57), we analyzed whether WT p38α might exhibit similar conformational changes that allow autophosphorylation at temperatures >37 °C. Whereas the p38 catalytic inhibitor, SB203580, can inhibit other kinases besides p38 (58), its inability to block FRH-stimulated p38 phosphorylation when present at a concentration above the IC_{50} for both p38α and p38β suggests that p38 catalytic activity is not required for its phosphorylation in these conditions. Furthermore, neither WT p38α nor p38β exhibited autophosphorylation in in vitro kinase reactions at temperatures up to 41 °C even with p38 protein present at 5.3 μM and the ATP concentration much greater than the reported K_m for ATP (59). Finally, HDX-MS analysis failed to show temperature dependence of the p38α activation loop. Canonical p38 activation following stimulation of HeLa cells with TNFα was not significantly affected by shifting temperature between 33 and 39.5 °C.

Having shown that clinically relevant shifts in temperature had only modest effects on p38 activation, we analyzed whether clinically relevant hypo- or hyperthermia might alter p38 kinase function. We focused this analysis on p38α and p38β, the two most widely expressed isoforms, because SB203580, a p38 catalytic inhibitor with activity limited to the α and β isoforms of p38, blocks hyperthermia effects on endothelial permeability and lung injury (10, 11). To avoid ambiguity caused by overlap and redundancies among multiple p38 isoforms and other MAPK signaling pathways (60), we analyzed the effect of temperature shifts on structure and function of purified recombinant proteins in cell-free reactions. We focused on two proinflammatory p38 substrates, MK2 and ATF2, which differ in cell location, function, and their molecular interaction with p38α. MK2 is a cytoplasmic kinase that modifies posttranscriptional regulation of proinflammatory cytokine expression and regulates endothelial permeability and neutrophil extravasation by phosphorylating HSP27 and modifying the actin cytoskeleton (61). ATF2 is a transcription factor that activates expression of multiple genes that regulate inflammation, cell cycle progression, and cell death (62). The substrate-docking domains of p38α confer substrate selectivity by increasing the phosphorylation efficiency of bound substrates (49). Structural analysis of p38α:MK2 complexes has shown that the C terminus of MK2 binds to the substrate-docking groove stretching between the ED and CD domains of the p38α (54). The structure of the p38α:ATF2 complex has not yet been solved, but mutational analysis suggests that ATF2 binds to the DEF pocket of p38α, which is distinct from the MK2-binding site (57).

In cell-free IVK reactions with recombinant dually phosphorylated p38α, the maximal rate of MK2 phosphorylation was 14.5-fold greater at 39.5 °C than at 33 °C, which was 4.7-fold greater than the temperature dependence of ATF2 phosphorylation. In these reactions, the ATP concentration was below the ~25 μM K_m for ATP (59), and the initial reaction rates were measured during the first 10 min of the reaction. Even if substrate limitation caused slowing of substrate phosphorylation prior to the 10-min sample collection, the more rapid reactions would be most impacted, thereby underestimating the effect of temperature on MK2 phosphorylation by p38α. For p38β, MK2 phosphorylation was 7.7-fold greater at 39.5 °C than at 33 °C, which was 3.2-fold greater than the difference for ATF2 phosphorylation. The narrow physiologically and clinically relevant temperature range studied is below the denaturation temperature for p38 as detected by the differential scanning fluorimetry (DSF) assay and below the optimal temperature for p38α and p38β catalytic activity for all three substrates tested (63). The similar modest increase in phosphorylation rate for STAT1α

Figure 3. Temperature dependence of p38 substrate binding is isoform- and substrate-dependent. Unphosphorylated p38α (A and C) or p38β (B and D) were covalently bound to the surface of a CM5 chip, and 0–3 μM MK2 (A and B) or 0–1 μM ATF2 (C and D) were injected at 33, 37, and 39.5 °C. The surface was washed with buffer at the same temperature, and the dissociation of analyte–ligand complexes was followed over time. Because MK:p38 association and dissociation occurred rapidly, K_D for p38/MK2 binding was determined using a steady-state affinity model (A and B). The y-axis shows response units at equilibrium (R_{eq}). The estimated K_{D,} maximal response (R_{max}), and the x^2 value for curve fitting are shown for each condition. E, calculated K_{D,} values for p38:MK2 and p38:ATF2 binding from SPR analysis at 33, 37, and 39.5 °C. F, melting temperature for p38α, p38β, and ERK2 by DSF; bar graph of 24 measurements each; mean ± S.D.; *, p < 0.0001 versus ERK2; †, p = 0.03 versus p38β.
Temperature-dependent p38α MAPK structure and function

and ATF2 observed over the 6.5 °C experimental temperature range is consistent with previously described temperature dependence of enzyme activity and with the classical and equilibrium models (63) and the macroscopic rate theory (64) of temperature dependence. However, these models of temperature-dependent enzyme catalytic activity do not explain the disproportionately large temperature dependence for MK2 phosphorylation. Therefore, we reasoned that the excess effect of hyperthermia on MK2 phosphorylation might be due to enhanced MK2 binding to its substrate-specific docking domain on p38α (54).

We used SPR to directly measure the temperature dependence of p38 substrate-binding affinity. Based on prior studies demonstrating that unphosphorylated and dually phosphorylated p38α have a similar binding affinity for MK2 (65), we immobilized unphosphorylated recombinant p38α or p38β to CM5 chips within the SPR flow cell and adjusted the temperature of the circulating buffer containing full-length MK2 or ATF2 to 33, 37, or 39.5 °C. Affinity of p38α:MK2 binding increased, whereas p38α:ATF2 binding affinity decreased as the reaction temperature was increased from 33 to 39.5 °C. The ratio of p38α:MK2 to p38α:ATF2 binding affinity was 4-fold greater at 39.5 °C than at 33 °C compared with a 2-fold difference for binding of the two substrates to p38β. The temperature dependence of selective p38:substrate binding is consistent with the results of the in vitro kinase reactions and with the known contribution of the p38 substrate-docking domains to substrate phosphorylation efficiency and specificity (49). We note that a prior study using SPR, stopped-flow fluorescence, and isothermal calorimetry found K_D values for p38:MK2 binding to range from 1 to 106 nM, depending on the method used and buffer salt concentration, compared with our observed K_D values of 1.27 and 0.474 μM at 33 and 39.5 °C, respectively (65). The NaCl concentration used in our SPR assay, 150 mM, was intermediate between the concentrations used in the prior study, and we used the same full-length MK2 and p38α variants. Our recombinant p38α and MK2 proteins could be activated in vitro and phosphorylated MK2 and HSP27, respectively (data not shown), suggesting that they were properly folded. The major methodologic difference between the prior and present studies was the use of a subphysiologic (25 °C) assay temperature in the prior study. To the best of our knowledge, our study is the first to measure p38:substrate binding affinity at physiologic temperatures.

Because the significant temperature-dependent changes in p38α:MK2 interaction occurred at temperatures ~8 °C below the melting temperature for p38α, we searched for localized, temperature-dependent conformational changes in p38α consistent with its functional changes using HDX-MS. We performed HDX-MS reactions at physiologic pH and over the physiologic temperature range of interest, 33–39.5 °C. We observed regions demonstrating EX1 kinetics, which we identified by the presence of a typical signature bimodal isotopic envelope with the progressive appearance of a distinct exchange-prone species concomitant with the progressive decay of the exchange-protected species. The slow kinetics and the cooperative nature (i.e. spanning multiple amide hydrogens) of the conformational interconversion has generally led to the phenomenon being ascribed to large changes in secondary (local unfolding), tertiary (local and global unfolding), or quaternary structure (subunit dissociation, protein/protein interfaces) (52, 53).

The prediction that the chemical HDX rate is temperature-dependent (66) has been experimentally confirmed and shown to modify the EX2 exchange, in which the chemical HDX rate is limiting. Analytical methods for HDX-MS have been developed to distinguish between temperature-dependent conformational information and direct effects of temperature on the chemical HDX rate over large temperature ranges (67, 68). In contrast with EX2 kinetics, for EX1 kinetics, the rate of conformational changes/fluuctuations is rate-limiting rather than the temperature dependence of the chemical exchange rate, thereby preserving the characteristic behavior of EX1 kinetics if the rate of the underlying cooperative unfolding/conformational change is not heavily temperature-dependent.

The results of the HDX-MS analysis of p38 are consistent with a localized temperature-dependent cooperative unfolding of four helices in p38α that form a tertiary structure element located within a region of known direct contact between p38α and MK2 (54). Importantly, this analysis did not show conformational changes in any other p38α domain, including the DEF pocket, which is required for ATF2 phosphorylation (57), or the p38α catalytic site. In contrast with the temperature-dependent changes in localized unfolding of p38α, there was no significant temperature-dependent difference in percentage of deuterium uptake in p38β.

The results of the SPR, HDX-MS, and IVK assays are consistent with a temperature-dependent conformational change in p38α localized to one of its substrate-docking domains that confers efficiency and specificity to substrate phosphorylation (49) and with the previously demonstrated structure of the p38α:MK2 complex (54). The absence of corresponding temperature dependence within the C-terminal p38α-interacting

Figure 4. HDX-MS analysis of temperature-dependent conformational changes in p38 MAPK. A, signature EX1 bimodal spectra of peptide segment 130–145 of the p38α p38β (right). The bimodal isotopic envelope (dark blue) was deconvoluted into an exchange-protected envelope (green) or exchange-prone envelope (light blue). B, for segment 130–145, the relative decay of the percentage exchange-protected envelope is plotted for p38α (solid lines) and p38β (dashed line) at 33 °C (turquoise), 37 °C (purple), or 39.5 °C (red) as a function of deuterium incubation time. C, space-filling model of p38α showing spatial relationship of segment 130–145 (α2, red) to the CD (yellow) and ED (brown) motifs and the MK2 C-terminal regulatory domain (orange) lying within the substrate-docking groove of p38α. D, the relative proportion of the percentage exchange-protected envelope after 2 h of deuterium incubation is plotted as a function of temperature for p38α (solid) and p38β (dashed) for segment 207–215 (green), 281–288 (yellow), and 300–306 (blue). E, difference plots are plotted for p38α (top) and p38β (bottom). The differences in percentage deuteration between 39.5 and 33 °C after 10 s (orange), 10 min (blue), and 2 h (purple) deuterium incubation were plotted as a function of the peptide segments from the N to C terminus based on the first residue of the segment. Vertical color bars, peptide segments that displayed EX1 bimodal behavior and are color-coded according to D. Segments with significant differences for which do not display EX1 bimodal behavior are denoted by vertical back arrows. F, structural representation of temperature-dependent peptide segments of p38α (top; PDB entry 5UOJ) and corresponding segments from p38β (bottom; PDB entry 3GC8) using the same color coding as in D and E.
Temperature-dependent p38α MAPK structure and function

Figure 5. Temperature-dependent deuteration kinetics for the C-terminal p38α-interacting region of MK2. HDX-MS analysis of MK2 reveals no temperature dependence in the C-terminal p38α-interacting region. A and B, stack spectra of peptidic segment 371–382. The undeuterated control is shown in A. The kinetics of HDX are shown in B at the indicated reaction temperature and deuterium incubation times. The isotopic distribution is shown in red, and the centroid is indicated by the vertical green line. C, relative kinetic deuterium uptake at 33, 37, and 39.5 °C for the peptidic segments 369–382, 371–382, 371–387, and 383–399, covering the entire p38α-interacting region of MK2.

region of MK2 indicates that the temperature dependence of p38α:MK2 binding and MK2 phosphorylation is attributable to temperature-dependent changes in the conformation of p38α itself.

To our knowledge, the only other signaling pathway that has been shown to contribute to lung injury and exhibit temperature dependence in the clinically relevant range is transient receptor potential cation channel subfamily V member 4.
Temperature-dependent p38α MAPK structure and function

(TRPV4), which is expressed on pulmonary endothelium and is responsive to both mechanical stretch and clinically relevant shifts in temperature (69).

In summary, we showed that increases in temperature in a range between clinically relevant hypothermia and febrile-range hyperthermia cause localized conformational changes within the p38α substrate-binding groove known to interact with the proinflammatory substrate MK2 and are associated with increased binding and phosphorylation of MK2. Considering the known contribution of the p38-MK2-HSP27 pathway to the pathogenesis of acute lung injury (23), the observed temperature responsiveness of p38α might explain the previously described temperature dependence of pulmonary endothelial barrier dysfunction, cytokine expression, and acute lung injury (7, 11, 70). These results also demonstrate the importance of analyzing signaling processes at temperatures at which they occur in vivo and demonstrate that localized conformational changes in certain kinases may modify the pattern of substrate phosphorylation without substantially altering global catalytic function.

Experimental procedures

Chemicals, recombinant proteins, and antibodies

Rabbit anti-phospho-MK2 (Thr-334; catalog no. 3041), rabbit anti-phospho-STAT1 (Ser-727; catalog no. 8826), and rabbit anti-dually phosphorylated (Thr-180/Tyr-182; catalog no. 9215) p38α/β were obtained from Cell Signaling Technologies (Danvers, MA). Mouse anti-human p38α (catalog no. 7972) and rabbit anti-monophosphorylated (Thr-180; catalog no. 101758) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Goat anti-rabbit Ig-IRDye 800 conjugate (catalog no. 926-32211) and goat anti-mouse Ig-IRDye 700 conjugate (catalog no. 926-88070) were obtained from LI-COR. All plasmids containing N-terminal His-tagged coding sequences were transformed in E. coli BL21, grown in lysogeny broth medium, induced with 1 mM isopropyl 1-thio-D-galactopyranoside (Thermo Fisher Scientific) for 4 h, and proteins were purified using cobalt columns (TALON™, Clontech) according to the manufacturer’s protocol, and purified proteins were confirmed by SDS-PAGE and immunoblotting as described previously (71). N-terminal His/HA-dually tagged p38α and p38β were expressed from pRSetA plasmid (Thermo Fisher Scientific) as described previously (71). Human dually phosphorylated N-terminal His-tagged p38α was expressed from pETDuet plasmid (EMD-Millipore) containing the untagged sequence expressing the constitutively active human MAPK kinase-6 (MKK6) mutant (S207G/T211G) in the second multicloning site as described (pETDuet-p38α) (71). We created a similar expression plasmid for dually phosphorylated p38β by exchanging the p38β-coding sequence for the p38α-coding sequence in pETDuet-p38α to create pETDuet-p38β. The coding sequences for ATF2 and MK2 were optimized for bacterial expression, synthesized (GenScript, Piscataway, NJ), and ligated between the BamHI and HindIII sites in pRSetA. The p38α and p38β protein expressed from the pETDuet plasmid was confirmed to be >80% dually phosphorylated by MALDI-TOF in the University of Maryland School of Pharmacy Proteomics Center.

Cell culture

HMVECs were purchased from Promocell (Heidelberg, Germany), maintained in endothelial cell growth medium MV2, used at passages 3–10, and studied at postconfluence according to the supplier’s protocol as we have described previously (10, 11). The THP1 human monocyte cell line (ATCC catalog no. TIB202) was maintained in RPMI 1640 medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, pH 7.3, penicillin, streptomycin, 0.05 mM β-mercapto-ethanol, and 10% defined fetal bovine serum (FBS) (Gibco). HeLa cells (ATCC catalog no. CCL-2) were cultured in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, 1 mM sodium pyruvate, 2 mM l-glutamine, penicillin, streptomycin, and 10% FBS. BEAS2B cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium with high glucose (Gibco Thermo Fisher) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, pH 7.3, and 5% heat-inactivated FBS. Prior to experimental exposures, THP1 cells were differentiated by treating with 5 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 24 h, washing with PBS, and culturing at 37 °C in phorbol 12-myristate 13-acetate–free medium for an additional 24 h.

Immunoblotting

Cells were lysed in radioimmunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and protease and phosphatase inhibitors (Roche Applied Science), and lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membrane, blocked with 5% nonfat dry milk, and probed with primary Abs against an antibody that recognizes monophosphorylated and dually phosphorylated p38α/β and total p38α/β. Bands were detected using secondary Abs conjugated to IR fluorophores (LI-COR) and IR fluorescence imaging (Odyssey; LI-COR), and the data were quantified using the LI-COR image analysis software.

In vitro kinase assay

The temperature dependence of p38 substrate phosphorylation was analyzed by in vitro kinase assay in 20 μl of reaction volume containing 75 ng of active dually phosphorylated p38α or p38β (~100 nM), 85–170 ng of substrate proteins (~100 nm), and 5 μCi of [γ-32P]ATP with final ATP concentration 83 nM in kinase buffer (50 mM Tris-HCl, 10 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35, pH 7.5). Reactions were performed at 33, 37, or 39.5 °C for 10, 30, 60, or 120 min and then terminated by adding an equal volume of 2× SDS-PAGE sample buffer. The reaction mixtures were separated by SDS-PAGE. The gels were dried, and the radioactive phosphate incorporation into substrates was measured by phosborimaging on a Typhoon FLA 7000 (GE Healthcare Life Sciences). The data were quantified using ImageQuant TL (GE Healthcare Life Sciences) and expressed as relative values. Autophosphorylation of p38α and p38β was measured in the same in vitro kinase reactions as described for substrate phosphorylation, except each reaction contained 2 μg of unphosphorylated p38 protein (2.6 μM) and nonradioactive ATP at a final concentration of 5 mM and then...
were immunoblotted using an antibody that detects both monophosphorylated and dually phosphorylated p38α/β.

**Surface plasmon resonance analysis of protein–protein interactions**

Protein–protein interactions of p38 with substrates were investigated by SPR analysis utilizing a Biacore T200 instrument (GE Healthcare). Unphosphorylated p38α or p38β was covalently bound to the surface of flow cell 2 and flow cell 4 of a CM5 chip to a final level of 1200 and 1400 RU, using the NHS-EDC kit from GE Healthcare. Flow cells 1 and 3 were treated as blanks. MK2 (0–3 μM in 120 μl of HBS-EP buffer consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) Surfactant P20) and ATF2 (0–1 μM in 120 μl of HBS-EP buffer) were injected into flow cells 1–4 at 33, 37, and 40 °C. The surface was then washed with buffer at the same temperature for 3 min, and the dissociation of analyte–ligand complexes was followed over time. The surfaces of the flow cells were regenerated by injecting 15-μl aliquots of 15 mM glycine, pH 1.75, and the process was repeated. Curve fitting and calculation of \(K_d\) was performed using Biacore BLAeval version 2.0 software as we have described previously (72). Values from the reference flow cell were subtracted to obtain the values for specific binding. For ATF2, the \(K_d\) was calculated using the Langmuir 1:1 binding model using Equation 1,

\[
K_0 = \frac{k_d}{k_a} \quad (\text{Eq. 1})
\]

where \(k_a\) and \(k_d\) are the dissociation and association rate constants, respectively (Fig. 3 C and D) and Fig. S1). Because p38:MK2 association and dissociation were rapid, we determined \(K_d\) values for MK2 binding using a steady-state affinity model and Equation 2,

\[
R_{eq} = \frac{K_d C_{\text{max}}}{K_a C + 1} \quad (\text{Eq. 2})
\]

where the value of \(K_a\) is the inverse of \(K_d\), \(R_{eq}\) is the equilibrium concentration at the analyte concentration (C), and \(R_{\text{max}}\) is the maximal binding response. The value of \(K_a\) was calculated by fitting a plot of the equilibrium response (\(R_{eq}\)) against the analyte concentration (C) to this equation, and \(K_d\) was calculated as the inverse of \(K_a\). The experimental data were fitted using numerical integration methods with an iterative approximation algorithm to find the best solution to the equation. The closeness of the fitted and the experimental data were evaluated using a \(\chi^2\) defined in Equation 3,

\[
\chi^2 = \frac{\sum (r_i - \bar{r}_i)^2}{n - p} \quad (\text{Eq. 3})
\]

where \(r_i\) is the fitted value, \(\bar{r}_i\) is the corresponding experimental value, \(n\) is the number of data points, and \(p\) is the number of fitted parameters. Both 1:1 binding curves (Fig. 3 C and D) and Fig. S1 (A and B)) and steady-state binding curves (Fig. 3, A and B) are shown.

**DSF**

The melting temperatures of recombinant p38α, p38β, and ERK2 were determined using DSF (73). SYPRO orange (Invitrogen; diluted 1:1000 in 10 mM HEPES, 150 mM NaCl, pH 7.5) and 1 mM unphosphorylated recombinant human p38α, p38β, or ERK2 were added to 96-well PCR plates. The plates were mixed, sealed, and centrifuged at 1000 rpm for 1 min, and a melting curve was performed using an Applied Biosystems real-time PCR instrument. The melting point was determined from the first derivative curve as described (73).

**Hydrogen:deuterium exchange mass spectrometry**

The coverage maps for p38α, p38β, and MK2 were obtained from undeuterated 20 μM recombinant proteins in 10 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT diluted 1:10 with ice-cold quench buffer (100 mM glycine, 15 mM tris(2-carboxyethyl)phosphine, pH 2.5). The quenched controls were injected into a Waters HDX nanoAcquity UPLC (Waters, Milford, MA) with in-line pepsin digestion (Waters Enzyme BEH pepsin column). Peptidic fragments were trapped on an Acquity UPLC BEH C18 peptide trap and separated on an Acquity UPLC BEH C18 column. A 7-min, 5–35% acetonitrile (0.1% formic acid) gradient was used to elute peptides directly into a Waters Synapt G2 mass spectrometer. MSE data were acquired with a 20–30-V ramp trap CE for high-energy acquisition of product ions with continuous lock mass (Leu-Enk) for mass accuracy correction. Peptides were identified using the ProteinLynx Global Server 2.5.1 with additional filtering of 0.3 fragments/residue using DynamX 3.0 (Waters).

Duplicate hydrogen:deuterium exchange reactions were performed for each condition by diluting 20 μM p38α or p38β in 10 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT diluted 1:10 deuterium buffer (10 mM HEPES, 99.99% D₂O, 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT); incubating in a sand block set to 33, 37, or 39.5 °C; and monitored with a digital temperature probe. All reaction components were equilibrated for 5 min at the respective temperatures prior to initiation of the reactions. Reactions were quenched at various times (10 s, 1 min, 10 min, 1 h, and 2 h) with an equal volume of ice-cold 100 mM glycine buffer, 10 mM tris(2-carboxyethyl)phosphine, pH 2.5. Back-exchange correction was performed against fully deuterated controls acquired by incubating 3 μl of p38 protein in 27 μl of 10 mM HEPES, 99.99% D₂O, 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT containing 4 M deuterated guanidine DCl for 2 h prior to quenching. Fully deuterated control proteins did not exhibit any temperature-dependent deviations when incubated at 33, 37, and 39.5 °C. For 38β, the deuterium uptake reactions and fully deuterated controls were performed as follows. 5 μl of protein was diluted with 45 μl of D₂O buffer and quenched with 50 μl of quench buffer. All other experimental acquisition parameters were similar to those of p38α.

The deuterium uptake by the identified peptides over time and for the fully deuterated controls was analyzed using Water’s DynamX 3.0 software. The normalized percentage of deuterium uptake (%D) at an incubation time \(t\) for a given peptide is given by Equation 4,
where \( m_t \) is the centroid mass at incubation time \( t \), \( m_o \) is the centroid mass of the fully deuterated control, and \( m_d \) is the centroid mass of the fully deuterated control. Percentage deuteration difference plots (\( \Delta D \)) were generated using the percentage deuteration calculated. Confidence intervals for the \( \Delta D \) plots were determined using the method outlined by Houde et al. (74) using the duplicate runs at 10 s, 10 min, and 2 h, adjusted to percentage deuteration using the fully deuterated controls. Confidence intervals (98%) were plotted on the \( \Delta D \) plots as horizontal dashed lines. EX1-type cooperative unfolding was analyzed using HX-Express2 (52). MK2 controls were performed as described for p38 with the following differences. Coverage maps were obtained from 5 \( \mu \)l of 2.5 mg/ml MK2 diluted 1:20 in ice-cold quench buffer with 1.5 M guanidine HCl. Data acquisition was performed on a Synapt G2Si equipped with HDX technology and temperature-controlled sample chambers. Peptides were identified using ProteinLynx Global Server 3.0.3. Deuterium incubation reactions were performed for 10 s, 10 min, and 2 h at 33, 37, and 39.5 °C, the same as for p38 with the following exceptions. Sample pre-equilibration and hydrogen exchange reactions were performed in the temperature-controlled sample chambers. 5 \( \mu \)l of MK2 was diluted with 45 \( \mu \)l of 1× PBS (99.99% D\(_2\)O), pH 7.4, and quenched with 70 \( \mu \)l of ice-cold quench (100 mM glycine buffer, 2.5 M guanidine HCl) prior to manual injections.

**Statistical methods**

Data are presented as mean ± S.E. Differences among more than two groups were analyzed by applying a Tukey honestly significant difference test to a one-way analysis of variance (ANOVA). Differences in the phosphorylation rates between substrates and between p38 isoforms were analyzed by multi-ANOVA (MANOVA). Differences with \( p < 0.05 \) were considered significant.

**Author contributions**—D. D., P. L. W., and J. D. H. data curation; D. D., P. L. W., P. S., and J. D. H. formal analysis; D. D., P. L. W., M. E. T., A. N., Y. Z., P. S., and J. D. H. investigation; D. D., P. L. W., M. E. T., and J. D. H. methodology; D. D., D. J. W., P. S., and J. D. H. writing-review and editing; P. S. and J. D. H. conceptualization; P. S. and J. D. H. funding acquisition; P. S. and J. D. H. project administration; J. D. H. supervision; J. D. H. writing-original draft.

**Acknowledgments**—Amy Defnet provided technical assistance. Additional support was provided by the University of Maryland Baltimore, School of Pharmacy Mass Spectrometry Center (SOP1841-IQB2014).

**References**

1. Hypothermia after Cardiac Arrest Study Group (2002) Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. *N. Engl. J. Med.* 346, 549–556 CrossRef Medline

2. Bernard, S. A., Gray, T. W., Buist, M. D., Jones, B. M., Silvester, W., Gutteridge, G., and Smith, K. (2002) Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N. Engl. J. Med.* 346, 557–563 CrossRef Medline

3. Villar, J., and Slutsky, A. S. (1993) Effects of induced hypothermia in patients with septic adult respiratory distress syndrome. *Resuscitation* 26, 183–192 CrossRef Medline

4. Slack, D. F., Corwin, D. S., Shah, N. G., Shanholz, C. B., Verceles, A. C., Netzer, G., Jones, K. M., Brown, C. H., Terrin, M. L., and Hasday, J. D. (2017) Pilot feasibility study of therapeutic hypothermia for moderate to severe acute respiratory distress syndrome. *Crit. Care Med.* 45, 1152–1159 CrossRef Medline

5. Mallory, M., Gogineni, I., Jones, G. C., Greer, L., and Simone, C. B., 2nd (2016) Therapeutic hyperthermia: the old, the new, and the upcoming. *Crit. Rev. Oncol. Hematol.* 97, 56–64 CrossRef Medline

6. Hasday, J. D., Garrison, A., Singh, I. S., Standiford, T., Ellis, G. S., Rao, S., He, J.-R., Rice, P., Frank, M., Goldblum, S. E., and Viscardi, R. M. (2003) Febrile-range hyperthermia augments pulmonary neutrophil recruitment and amplifies pulmonary oxygen toxicity. *Ann. J. Pathol.* 162, 2005–2017 CrossRef Medline

7. Rice, P., Martin, E., He, J.-R., Frank, M., DeTolla, L., Hester, L., O’Neill, T., Manika, C., Benjamin, I., Nagarsekar, A., Singh, I., and Hasday, J. D. (2005) Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental Gram-negative bacterial pneumonia. *J. Immunol.* 174, 3676–3685 CrossRef Medline

8. Nagarsekar, A., Greenberg, R. S., Shah, N. G., Singh, I. S., and Hasday, J. D. (2008) Febrile-range hyperthermia accelerates caspase-dependent apoptosis in human neutrophils. *J. Immunol.* 181, 2636–2643 CrossRef Medline

9. Nagarsekar, A., Tulapurkar, M. E., Singh, I. S., Atamas, S. P., Shah, N. G., and Hasday, J. D. (2012) Hyperthermia promotes and prevents respiratory epithelial apoptosis through distinct mechanisms. *Am. J. Respir. Cell Mol. Biol.* 47, 824–833 CrossRef Medline

10. Shah, N. G., Tulapurkar, M. E., Damarla, M., Singh, I. S., Goldblum, S. E., Shapiro, P., and Hasday, J. D. (2012) Febrile-range hyperthermia augments reversible TNF-α-induced hyperpermeability in human microvascular lung endothelial cells. *Int. J. Hyperthermia* 28, 627–635 CrossRef Medline

11. Tulapurkar, M. E., Almutairi, E. A., Shah, N. G., He, J. R., Puche, A. C., Shapiro, P., Singh, I. S., and Hasday, J. D. (2012) Febrile-range hyperthermia modifies endothelial and neutrophilic functions to promote extravasation. *Am. J. Respir. Cell Mol. Biol.* 46, 807–814 CrossRef Medline

12. Sutcliffe, I. T., Smith, H. A., Stanimirovic, D., and Hutchison, J. S. (2001) Effects of moderate hypothermia on IL-1β-induced leukocyte rolling and adhesion in pial microcirculation of mice and on proinflammatory gene expression in human cerebral endothelial cells. *J. Cereb. Blood Flow Metab.* 21, 1310–1319 CrossRef Medline

13. Yang, D., Xie, P., Guo, S., and Li, H. (2010) Induction of MAPK phosphatase-1 by hypothermia inhibits TNF-α-induced endothelial barrier dysfunction and apoptosis. *Cardiovasc. Res.* 85, 520–529 CrossRef Medline

14. Fisher, D. T., Chen, Q., Skitzki, J. J., Muhitch, J. B., Zhou, L., Appenheimer, M. M., Vardam, T. D., Wei, E. L., Passanese, J., Wang, W. C., Gollnick, S. O., Dewhirst, M. W., Rose-John, S., Repasky, E. A., Baumann, H., and Evans, S. S. (2011) IL-6 trans-signaling licenses mouse and human tumor plasminogen activator production culture. *Cardiovasc. Res.* 91, 262–267 CrossRef Medline

15. Singh, I. S., and Hasday, J. D. (2012) Hyperthermia promotes and prevents respiratory epithelial apoptosis in human cerebral endothelial cells. *J. Immunol.* 188, 2636–2643 CrossRef Medline

16. Bedoya-López, A., Estrada, K., Sanchez-Flores, A., Ramirez, O. T., Altimarano, C., Segovia, L., Miranda-Rios, J., Trujillo-Roldán, M. A., and Valdez-Cruz, N. A. (2016) Effect of temperature downshift on the transcriptional responses of Chinese hamster ovary cells using recombinant human tissue plasminogen activator production culture. *PLoS One* 11, e0151529 CrossRef Medline

17. Kariya, A., Tabuchi, Y., Yonoki, T., and Kondo, T. (2013) Identification of common gene networks responsive to mild hyperthermia in human cancer cells. *Int. J. Mol. Med.* 32, 195–202 CrossRef Medline

18. Sonna, L. A., Kuhlmeier, M. M., Carter, H. C., Hasday, J. D., Lilly, C. M., and Fairchild, K. D. (2006) Effect of moderate hypothermia on gene expression by THP-1 cells: a DNA microarray study. *Physiol. Genomics* 26, 91–98 CrossRef Medline

19. Sonna, L. A., Wenger, C. B., Flinn, S., Sheldon, H. K., Sawka, M. N., and Lilly, C. M. (2004) Exterional heat injury and gene expression changes: a...
Temperature-dependent p38α MAPK structure and function

DNA microarray analysis study, J. Appl. Physiol. 96, 1943–1953 CrossRef Medline

Tabuchi, Y., Takasaki, I., Wada, S., Zhao, Q. L., Horii, T., Nomura, T., Ohtsuka, K., and Kondo, T. (2008) Genes and genetic networks responsive to mild hyperthermia in human lymphoma U937 cells. Int. J. Hyperthermia 24, 613–622 CrossRef Medline

20. Potla, R., Singh, I. S., Atamas, S. P., and Hasday, J. D. (2015) Shifts in temperature within the physiologic range modify strand-specific expression of select human microRNAs. RNA 21, 1261–1273 CrossRef Medline

21. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270, 7420–7426 CrossRef Medline

22. Damarla, M., Hasan, E., Boueiz, A., Le, A., Pae, H. H., Montouchet, C., Kolb, T., Simms, T., Myers, A., Kayali, U. S., Gaestel, M., Peng, X., Reddy, S. P., Damiano, R., and Hassoun, P. M. (2009) Mitogen activated protein kinase activated protein kinase 2 regulates actin polymerization and vascular leak in ventilator associated lung injury. PLoS ONE 4, e6600 CrossRef Medline

23. Birukov, K. G., Jacobson, J. R., Flores, A. A., Ye, S. Q., Birukova, A. A., Verin, A. D., and Garcia, J. G. (2003) Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch. Am. J. Physiol. Lung Cell Mol. Physiol. 285, L785–L797 CrossRef Medline

24. Wang, Y. X., Xu, X. Y., Su, W. L., Wang, Q., Zhu, W. X., Chen, F., Jin, G., Liu, Y. J., Li, Y. D., Sun, Y. P., Gao, W. C., and Ruan, C. P. (2010) Activation and clinical significance of p38 MAPK signaling pathway in patients with severe trauma. J. Surg. Res. 161, 119–125 CrossRef Medline

25. Wang, Q., and Doerschuk, C. M. (2001) The p38 mitogen-activated protein kinase mediates cytoskeletal remodeling in pulmonary microvascular endothelial cells upon intracellular adhesion molecule-1 ligation. J. Immunol. 166, 6877–6884 CrossRef Medline

26. Garcia, J. G., Wang, P., Schaphorst, K. L., Becker, P. M., Borbiev, T., Liu, F., Birukova, A., Jacobs, K., Bogatcheva, N., and Verin, A. D. (2002) Critical involvement of p38 MAPK kinase in pertussis toxin-induced cytoskeletal reorganization and lung permeability. FASEB J. 16, 1064–1076 CrossRef Medline

27. Petrache, I., Birukova, A., Ramirez, S. I., Garcia, J. G., and Verin, A. D. (2003) The role of the microtubules in tumor necrosis factor-α-induced endothelial cell permeability. Am. J. Respir. Cell Mol. Biol. 28, 574–581 CrossRef Medline

28. O’Reilly, F. M., Casper, K. A., Otto, K. B., Sexton, A. A., and Swerlick, R. A. (2003) Regulation of tissue factor in microvascular dermal endothelial cells. J. Invest. Dermatol. 120, 489–494 CrossRef Medline

29. Wang, Q., Yerkumichov, M., Gaarde, W. A., Popoff, I. J., and Doerschuk, C. M. (2005) MKK3 and -6-dependent activation of p38 MAPK mediates cytoskeletal remodeling in pulmonary microvascular endothelial cells induced by ICAM-1 ligation. Am. J. Physiol. Lung Cell Mol. Physiol. 288, L359–L369 CrossRef Medline

30. Tremblay, P. L., Auger, F. A., and Huot, J. (2006) Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of MAPK pathways in neutrophils. J. Biol. Chem. 281, 42184–42199 CrossRef Medline

31. Thalhammer, T., McGrath, M. A., and Harnett, M. M. (2008) MAPKs and their relevance to arthritis and inflammation. Rheumatology (Oxf) 47, 409–414 CrossRef Medline

32. Chung, K. F. (2011) p38 mitogen-activated protein kinase pathways in asthma and COPD. Chest 139, 1470–1479 CrossRef Medline

33. Fisk, M., Gajendragadkar, P. R., Mäki-Petäjä, K. M., Wilkinson, I. B., and Cheriy, J. (2014) Therapeutic potential of p38 MAP kinase inhibition in the management of cardiovascular disease. Am. J. Cardiovasc. Drugs 14, 155–165 CrossRef Medline

34. Asaduzzaman, M., Wang, Y., and Thorlacius, H. (2008) Critical role of p38 mitogen-activated protein kinase signaling in septic lung injury. Mol. Cell. Biol. 33, 3728–3734 CrossRef Medline

35. Feng, Y. J., and Li, Y. Y. (2011) The role of p38 mitogen-activated protein kinase in the pathogenesis of inflammatory bowel disease. J. Dig. Dis. 12, 327–332 CrossRef Medline

36. Beardmore, V. A., Hinton, H. J., Eftychii, C., Apostolaki, M., Arnaka, M., Darragh, J., McRath, J., Carr, J. M., Armit, L. J., Clacher, C., Malone, L., Kollias, G., and Arthur, J. S. (2005) Generation and characterization of p38β (MAPK11) gene-targeted mice. Mol. Cell. Biol. 25, 10454–10464 CrossRef Medline

37. O’Keefe, S. J., Mudgett, J. S., Cupo, S., Parsons, J. N., Chartrain, N. A., Fitzgerald, C., Chen, S. L., Lowitz, K., Rasa, C., Visco, D., Lucell, S., Carballo-Jane, E., Owens, K., and Zaller, D. M. (2007) Chemical genetics define the roles of p38α and p38β in acute and chronic inflammation. J. Biol. Chem. 282, 34663–34671 CrossRef Medline

38. Ferrari, G., Terushkin, V., Wolff, M. J., Zhang, X., Valacca, P., Poggi, P., Pintucci, G., and Mignatti, P. (2012) TGF-β1 induces endothelial cell apoptosis by shifting VEGF activation of p38 (MAPK) from the prosurvival p38β to proapoptotic p38α. Mol. Cancer Res. 10, 605–614 CrossRef Medline

39. Tulapurkar, M. E., Ramarathnam, A., Hasday, J. D., and Singh, I. S. (2015) Bacterial lipopolysaccharide augments febrile range hyperthermia-induced heat shock protein 70 expression and extracellular release. J. Biol. Chem. 288, 2756–2766 CrossRef Medline

40. Liu, H., Yanamandala, M., Lee, T. C., and Kim, J. K. (2014) Mitochondrial p38β and manganese superoxide dismutase interaction mediated by estrogen in cardiomyocytes. PLoS One 9, e85272 CrossRef Medline

41. Gupta, A., Cooper, Z. A., Tulapurtur, M. E., Potla, R., Maiy, T., Hasday, J. D., and Singh, I. S. (2013) Toll-like receptor agonists and febrile range hyperthermia synergize to induce heat shock protein 70 expression and extracellular release. J. Biol. Chem. 288, 15469–15474 CrossRef Medline

42. Trempolcke, N., Dave-Coll, N., and Nebreda, A. R. (2013) SnapShot: p38 MAPK substrates. Cell 152, 924–924.e1 CrossRef Medline

43. Tanoue, T., Maeda, R., Adachi, E., and Ashwell, J. D. (2009) T cell receptor-mediated activation of p38α by mono-phosphorylation of the activation loop results in altered substrate specificity. J. Biol. Chem. 284, 15469–15474 CrossRef Medline

44. Mittlestadt, P. R., Yamaguchi, H., Appella, E., and Ashwell, J. D. (2009) T cell receptor-mediated activation of p38α by mono-phosphorylation of the activation loop leads to distinct substrate specificity. J. Biol. Chem. 284, 15469–15474 CrossRef Medline
55. Birukova, A. A., Birukov, K. G., Gorshkov, B., Liu, F., Garcia, J. G., and Verin, A. D. (2005) MAP kinases in lung endothelial permeability induced by microtubule disassembly. *Am. J. Physiol. Lung Cell Mol. Physiol.* **289**, L75–L84 CrossRef Medline
56. Cara, D. C., Kaur, J., Forster, M., McCafferty, D. M., and Kubers, P. (2001) Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis *in vivo*. *J. Immunol.* **167**, 6552–6558 CrossRef Medline
57. Tzarum, N., Komornik, N., Ben Chetrit, D., Engelberg, D., and Livnah, O. (2013) DEF pocket in p38α facilitates substrate selectivity and mediates autophosphorylation. *J. Biol. Chem.* **288**, 19537–19547 CrossRef Medline
58. Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15434–15439 CrossRef Medline
59. Frantz, B., Klatt, T., Pang, M., Parsons, J., Rolando, A., Williams, H., Tocci, M. J., O’Keefe, S. J., and O’Neill, E. A. (1998) The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. *Biochemistry* **37**, 13846–13853 CrossRef Medline
60. Hommes, D. W., Peppelenbosch, M. P., and van Deventer, S. J. (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* **52**, 144–151 CrossRef Medline
61. Qian, F., Deng, J., Wang, G., Ye, R. D., and Christman, J. W. (2016) Pivotal role of mitogen-activated protein kinase-activated protein kinase 2 in inflammatory pulmonary diseases. *Curr. Protein Pept. Sci.* **17**, 332–342 CrossRef Medline
62. Yu, T., Li, Y. J., Bian, A. H., Zuo, H. B., Zhu, T. W., Ji, S. X., Kong, F., Yin, D. Q., Wang, C. B., Wang, Z. F., Wang, H. Q., Yang, Y., Yoo, B. C., and Cho, J. Y. (2014) The regulatory role of activating transcription factor 2 in inflammation. *Mediators Inflamm.* **2014**, 950472 CrossRef Medline
63. Daniel, R. M., and Danzon, M. J. (2013) Temperature and the catalytic activity of enzymes: a fresh understanding. *FEBS Lett.* **587**, 2738–2743 CrossRef Medline
64. Arcus, V. L., Prentice, E. J., Hobbs, J. K., Mul holland, A. J., Van der Kamp, M. W., Ludney, C. R., Parker, E. J., and Schipper, L. A. (2016) On the temperature dependence of enzyme-catalyzed rates. *Biochemistry* **55**, 1681–1688 CrossRef Medline
65. Lukas, S. M., Kroe, R. R., Wildeson, J., Peet, G. W., Frego, L., Davidson, W., Ingraham, R. H., Pargellis, C. A., Labadia, M. E., and Werneburg, B. G. (2004) Catalysis and function of the p38α-MK2a signaling complex. *Biochemistry* **43**, 9950–9960 CrossRef Medline
66. Ogunseyan, I., Lento, C., and Wilson, D. J. (2018) Contemporary hydrogen deuterium exchange mass spectrometry. *Methods* **144**, 27–42 CrossRef Medline
67. Ouyeyi, O. A., Sours, K. M., Lee, T., Resing, K. A., Ahn, N. G., and Klinman, J. P. (2010) Temperature dependence of protein motions in a thermophilic dihydrofolate reductase and its relationship to catalytic efficiency. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 10074–10079 CrossRef Medline
68. Ouyeyi, O. A., Sours, K. M., Lee, T., Kohen, A., Resing, K. A., Ahn, N. G., and Klinman, J. P. (2011) Comparative hydrogen-deuterium exchange for a mesophilic vs thermophilic dihydrofolate reductase at 25 degrees C: identification of a single active site region with enhanced flexibility in the mesophilic protein. *Biochemistry* **50**, 8251–8260 CrossRef Medline
69. Hamanaka, K., Jian, M. Y., Weber, D. S., Alvarez, D. F., Townsley, M. I., Al-Mehdi, A. B., King, J. A., Liedtke, W., and Parker, J. C. (2007) TRPV4 initiates the acute calcium-dependent permeability increase during ventilator-induced lung injury in isolated mouse lungs. *Am. J. Physiol. Lung Cell Mol. Physiol.* **293**, L923–L932 CrossRef Medline
70. Shah, N. G., Tulapurkar, M. E., Almutairy, E. A., and Hasday, I. D. (2009) Febrile-range hyperthermia augments TNF-α induced permeability in human microvascular endothelial cells in the lung (hMVEC-L). *Am. J. Respir. Crit. Care Med.* **179**, A4014 CrossRef
71. Shah, N. G., Tulapurkar, M. E., Ramarathnam, A., Brophy, A., Martinez, R., 3rd, Hom, K., Hodges, T., Samadani, R., Singh, I. S., MacKerell, A. D., Jr., Shapiro, P., and Hasday, J. D. (2017) Novel non-catalytic substrate-selective p38α-specific MAPK inhibitors with endothelial-stabilizing and anti-inflammatory activity. *J. Immunol.* **198**, 3296–3306 CrossRef Medline
72. Burkhard, K. A., Chen, F., and Shapiro, P. (2011) Quantitative analysis of ERK2 interactions with substrate proteins: roles for kinase docking domains and activity in determining binding affinity. *J. Biol. Chem.* **286**, 2477–2485 CrossRef Medline
73. Niesen, F. H., Berglund, H., and Vedadi, M. (2007) The use of differential gen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. *J. Pharm. Sci.* **96**, 2071–2086 CrossRef Medline