Maximal Human Neutrophil Priming for Superoxide Production and Elastase Release Requires p38 Mitogen-Activated Protein Kinase Activation

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Hypothesis: Neutrophil priming has been implicated in the development of multiple organ failure, although the precise intracellular mechanisms that regulate neutrophil priming remain unclear. Our previous work characterized platelet-activating factor (PAF) priming of human neutrophils for concordant superoxide anion (O2−) generation and elastase degranulation. The p38 mitogen-activated protein kinase (MAPK) is activated by PAF stimulation. We hypothesized that PAF-induced human neutrophil priming for O2− and elastase release is mediated via the p38 MAPK pathway.

Design: Isolated neutrophils from 6 human donors were preincubated with the specific p38 MAPK inhibitor SB 203580 (1 µmol/L) or buffer (control) for 30 minutes. Cells were then primed with PAF (200 nmol/L), followed by receptor-dependent (N-formyl-methionyl-leucyl-phenylalanine, 1 µmol/L) or receptor-independent phorbol myristate acetate (PMA, 100 ng/mL) activation.

Setting: Urban trauma research laboratory.

Patients: Healthy volunteer donors of neutrophils.

Main Outcome Measures: Maximal rate of O2− generation was measured by superoxide dismutase-inhibitable reduction of cytochrome c and elastase release by the cleavage of N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide.

Results: SB 203580 significantly attenuated the generation of O2− and release of elastase from neutrophils activated with N-formyl-methionyl-leucyl-phenylalanine but not with PMA. Independent of the activator receptor status, SB 203580 almost completely blocked the exaggerated neutrophil cytotoxic response due to PAF priming.

Conclusions: The p38 MAPK pathway is required for maximal PAF-induced neutrophil priming for O2− production and elastase degranulation. Therefore, the MAPK signaling cascade may offer a potential therapeutic strategy to preempt global neutrophil hyperactivity rather than attempt to nullify the end products independently.

The neutrophil is a pivotal cellular mediator in the pathogenesis of the adult respiratory distress syndrome (ARDS) and postsurgery multiple organ failure (MOF). In the 2-event inflammatory model of organ injury,1,2 the first event is priming of the immune response. We focused on the neutrophil as a cellular surrogate of the systemic hyperinflammatory immune response. Priming of circulating neutrophils causes nonselective adherence to endothelium. The second event provokes activation of these adherent neutrophils, leading to the indiscriminate release of cytotoxic reactive oxygen metabolites and proteases into the neutrophil-endothelial cell microenvironment, which results in endothelial damage, capillary leak, and ultimately, end-stage organ dysfunction.3 In animal models, this destructive neutrophil priming via platelet-activating factor (PAF) appears to be a central process for gut ischemia and reperfusion-induced lung injury.4 Further clinical data have implicated PAF and similar lipid mediators in early postsurgery neutrophil priming associated with the development of ARDS and MOF.5,7

In the laboratory, we have previously characterized human neutrophil priming via PAF as a concordant event resulting in the up-regulation of adhesion molecules on the cell surface, and the simultaneous generation of superoxide anion (O2−) and release of elastase after activation.8 This concordant functional response suggests a possible common priming signal transduction pathway, but intracellular signaling pathways involved in neutrophil priming have only begun to be elucidated. Intracellular PAF signal transduction appears to be mediated via tyrosine phosphorylation and
MATERIALS AND METHODS

MATERIALS

1-α-phosphatidylcholine, β-acyl-g-O-alkyl (PAF), N-formylmethionyl-leucyl-phenylalanine (fMLP), cytochrome c, superoxide dismutase (SOD), N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (AAPV-pNA), N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPV-CK), dextran, and Triton X-100 were purchased from Sigma-Aldrich Corp (St Louis, Mo). Ficoll-Hypaque was obtained from Pharmacia Biotech (Uppsala, Sweden). A kinetic microplate reader (THERMOMax) and software (Softmax) were purchased from Molecular Devices (Menlo Park, Calif). SB 203580 was supplied by John C. Lee, PhD, at SmithKline Beecham Pharmaceuticals (King of Prussia, Pa). SB 203580 was dissolved in dimethyl sulfoxide and diluted in normal saline containing 0.25% human serum albumin (Abbott Laboratories, North Chicago, Ill).

SAMPLE COLLECTION

After providing informed consent via a protocol approved by the University of Colorado Health Sciences Center Combined Affiliated Institutional Review Board, Denver, 6 healthy human volunteers donated venous blood through a sterile 21-gauge butterfly needle into a heparinized syringe (10 U of heparin per milliliter of blood).

NEUTROPHIL ISOLATION

Neutrophils were isolated from heparinized blood samples as described.8 Briefly, following dextran sedimentation of erythrocytes, the upper layer was removed and centrifuged at 200g for 10 minutes. The pellet was resuspended in phosphate-buffered saline and overlaid on 5 mL of Ficoll-Hypaque. This gradient was centrifuged at 400g for 30 minutes and the supernatant was discarded, leaving red blood cells and neutrophils in the bottom layer. Hypotonic lysis was performed twice at 4°C to remove contaminating red blood cells, and the neutrophils were resuspended in Krebs-Ringer phosphate with dextrose at pH 7.35 to a final concentration of 2.5 × 10⁷ cells/mL. The final cell population was more than 98% neutrophils by differential staining, and more than 99% viable by trypan blue exclusion.

O₂⁻ ASSAY

Generation of O₂⁻ by neutrophils was measured by SOD-inhibitable cytochrome c reduction in 96-well microplates. Isolated neutrophils were preincubated with the specific p38 MAPK inhibitor SB 203580 (1 µmol/L) or Krebs-Ringer phosphate with dextrose buffer (control) for 30 minutes in a shaking water bath at 37°C. SB 203580 (100 µmol/L in vitro) is a pyridinyl imidazole that binds to and blocks the activity of p38 MAPK in a highly selective manner.19,20 The concentration of SB 203580 chosen is based on previous work demonstrating that this dose effectively blocks p38 MAPK activity in neutrophils and inhibits neutrophil reactive oxygen metabolite production and adhesion.21,22 Neutrophil viability was again verified by trypan blue exclusion after preincubation with SB 203580, and was found to be unchanged at 99%. Of the pretreated neutrophils, 3.75 × 10⁵ were then added to individual wells of a microtiter plate. The PAF was added to the experimental wells to make a final concentration of 200 nmol/L, and incubation at 37°C was continued for 20 minutes (Figure 2). The fMLP activation alone stimulated neutrophil O₂⁻ generation (0.9 ± 0.3 nmol of O₂⁻/3.75 × 10⁶ cells per minute) above basal levels (0.03 ± 0.02 nmol of O₂⁻/3.75 × 10⁵ cells per minute). In addition, as demonstrated in previous work by neutrophil priming and activation correlated with the 2-event model in vivo. In vitro, our investigations into the neutrophil priming and activation sequence used PAF as a primer and fMLP as an activating stimulus. Human fMLP receptor is an example of a G protein–linked chemotactic receptor documented to activate the p38 MAPK signaling pathway.13,14,17 This well-characterized stimulant (a component of bacterial cell walls) was therefore used to investigate neutrophil receptor-dependent activation of O₂⁻ generation (Figure 1). The fMLP activation alone stimulated neutrophil O₂⁻ generation (0.9 ± 0.3 nmol of O₂⁻/3.75 × 10⁶ cells per minute) above basal levels (0.03 ± 0.02 nmol of O₂⁻/3.75 × 10⁵ cells per minute). This fMLP-stimulated O₂⁻ response was significantly inhibited by the specific p38 MAPK kinase inhibitor SB 203580 (0.4 ± 0.1 nmol of O₂⁻/3.75 × 10⁵ cells per minute). In addition, as demonstrated in previous work from our laboratory, PAF priming resulted in significantly increased production of O₂⁻ (7.9 ± 0.6 nmol of O₂⁻/3.75 × 10⁵ cells per minute) compared with fMLP stimulation alone. This PAF O₂⁻ priming response was similarly inhibited by SB 203580 (4.5 ± 0.5 nmol of O₂⁻/3.75 × 10⁵ cells per minute) but not completely blocked to basal levels.

Similar data were obtained with measured neutrophil elastase release using the receptor-dependent activator fMLP (Figure 2). The fMLP activation alone stimulated elastase release (17.9% ± 1.2% total neutrophil elastase) above basal levels (9.4% ± 0.9%). This fMLP-stimulated elastase response was significantly inhibited...
by SB 203580 (12.3% ± 1.2% total neutrophil elastase). When the cells were initially primed with PAF, the resultant elastase release was significantly increased (42.5% ± 3.9% total neutrophil elastase). This PAF elastase priming response was also inhibited by SB 203580 (23.9% ± 3.1%) but, again, not completely blocked to basal levels. These data demonstrate that p38 MAPK partially mediates PAF priming and fMLP activation of neutrophils for both O₂⁻ production and elastase release. However, since fMLP activates p38 MAPK, one cannot discriminate the effect of inhibiting p38 MAPK on PAF priming from its effect on fMLP activation.

**RECEPTOR-INDEPENDENT NEUTROPHIL ACTIVATION**

Recognizing that fMLP activates p38 MAPK, independent of PAF, the receptor-independent activator PMA was used to determine whether p38 MAPK mediates PAF priming of the neutrophil functional response (Figure 3). The PMA activation alone stimulated neutrophil O₂⁻ generation (2.8 ± 0.7 nmol of O₂⁻/3.75 × 10⁵ cells per minute) above basal levels (0.03 ± 0.02 nmol of O₂⁻/3.75 × 10⁵ cells per minute). However, in contrast to fMLP receptor-dependent activation, the O₂⁻ response stimulated by PMA receptor-independent activation was not significantly inhibited by the specific p38 MAPK inhibitor SB 203580 (2.3 ± 0.5 nmol of O₂⁻/3.75 × 10⁵ cells per minute). The PAF priming followed by PMA activation resulted in significantly increased production of O₂⁻ (5.4 ± 0.9 nmol of O₂⁻/3.75 × 10⁵ cells per minute) compared with PMA stimulation alone. This PAF priming of the respiratory burst was inhibited by SB 203580 (2.4 ± 0.4 nmol of O₂⁻/3.75 × 10⁵ cells per minute), decreasing it to the same level produced with PMA stimulation alone. Therefore, PMA receptor-independent activation discriminates the effect of p38 MAPK inhibition on PAF priming from activation.

Similar data were obtained with measured neutrophil elastase release using PMA activation (Figure 4). The PMA activation alone stimulated elastase release (29.4% ± 2.4% total neutrophil elastase) above basal levels (9.4% ± 0.9%). This PMA-stimulated elastase response was not significantly inhibited by SB 203580 (25.9% ± 4.6% total neutrophil elastase). When the cells were initially primed with PAF, the resultant elastase release in response to PMA was significantly increased (43.6% ± 3.9% total neutrophil elastase). This PAF elastase priming response was also nearly completely inhibited by SB 203580 (25.9% ± 4.6% total neutrophil elastase) down to the same level released with PMA stimulation alone. These data, using receptor-independent activation, demonstrate that p38 MAPK is required for maximal PAF priming of neutrophils for both O₂⁻ production and elastase release.
Investigation into the pathogenesis of MOF has implicated the neutrophil as a key cellular mediator. Our laboratory and clinical work focused on the mechanisms responsible for early postinjury neutrophil hyperactivity. These studies and others have further elucidated the neutrophil priming-activation sequence that forms the framework for the 2-event model of postinjury MOF. Animal studies have invoked PAF-induced neutrophil priming as a pivotal event for gut ischemia and reperfusion-induced lung injury. Our clinical studies also suggest that PAF and similar inflammatory lipid mediators contribute to postinjury neutrophil priming and the development of MOF. Further in vitro studies on neutrophil functional response demonstrate that PAF primes these cells concordantly for superoxide production and elastase release. Enhanced protease degranulation may be even more important than reactive oxygen metabolites for neutrophil-mediated tissue injury. Although traditional therapy has concentrated on nullifying cellular end products (antioxidants or antiproteases), these therapies have largely been unsuccessful. We therefore investigated more proximal aspects in this cascade of events. Specifically, our efforts focused on signal transduction pathways involved in the concordant neutrophil priming response to determine whether a common intracellular signaling pathway existed. Elucidating the cellular signaling that determines the neutrophil primed state and its functional response may allow the development of strategies targeted to modify the systemic inflammatory response. Intervention at the level of signal transduction may prove to be more effective clinically than manipulation of neutrophil priming end products.

Figure 1. Superoxide anion (O$_2^-$) production from isolated neutrophils in response to platelet-activating factor (PAF)-induced priming and receptor-dependent activation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Data are expressed as mean ± SEM from 6 donors. Superoxide Vmax equals nanomoles of O$_2^-$/3.75 $\times$ 10$^5$ cells per minute. Asterisk indicates P = .002 compared with control value. Dagger indicates P = .001 compared with fMLP control value.

Figure 2. Elastase release from isolated neutrophils in response to platelet-activating factor (PAF)-induced priming and receptor-dependent activation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Data are expressed as mean ± SEM from 6 donors. Elastase release equals percentage of total neutrophil elastase content (Triton X-100 cell lysis). Asterisk indicates P = .005 compared with control value. Dagger indicates P = .003 compared with fMLP control value.

Figure 3. Superoxide anion (O$_2^-$) production from isolated neutrophils in response to platelet-activating factor (PAF)-induced priming and receptor-independent activation with phorbol myristate acetate (PMA). Data are expressed as mean ± SEM from 6 donors. Superoxide Vmax equals nanomoles of O$_2^-$/3.75 $\times$ 10$^5$ cells per minute. Asterisk indicates P = .01 compared with control value. Dagger indicates P = .03 compared with PMA control value.

Figure 4. Elastase release from isolated neutrophils in response to platelet-activating factor (PAF)-induced priming and receptor-independent activation with phorbol myristate acetate (PMA). Data are expressed as mean ± SEM from 6 donors. Elastase release equals percentage of total neutrophil elastase content (Triton X-100 cell lysis). Asterisk indicates P = .004 compared with control value. Dagger indicates P = .01 compared with PMA control value.
parallel signal processors: extracellular signal-related kinase 1/2 (ERK 1/2 or p42/44 MAPK), c-Jun amino-terminal kinase and stress-activated protein kinase (JNK/ SAPK), and p38 MAPK. Cross-talk between these parallel pathways is crucial to the coordinated responses of cells, resulting in an integration of multiple effector mechanisms.

Recent work has demonstrated that at least 2 of these MAPK pathways are involved in neutrophil cytotoxicity. ERK 1/2 were the first cloned and well-characterized mammalian MAPKs, and are generally associated with mitogenesis and cellular differentiation. Previous work in our laboratory revealed that blockade of ERK 1/2 had no effect on O$_2^-$ production, and actually increased elastase degranulation from fMLP-activated neutrophils. The p38 MAPK is the second MAPK pathway delineated in neutrophils and is activated by many agonists, including tumor necrosis factor α, lipo-polysaccharide, and granulocyte-macrophage colony-stimulating factor. Returning to our neutrophil priming and activation paradigm, we and others previously demonstrated that neutrophil activation with fMLP results in p38 MAPK activation. Nick et al have documented the activation of p38 MAPK after exposure of neutrophils to PAF and its association with a number of functional neutrophil responses. However, the concordant neutrophil functional response associated with the neutrophil primed state, and its associated signaling cascade, has not been clarified.

We began our investigations into the neutrophil priming signaling cascade using the well-characterized, receptor-dependent chemoattractant fMLP as the activating stimulus. The O$_2^-$ and elastase functional responses to fMLP activation are decreased about 44% and 69%, respectively, by specific inhibition of p38 MAPK. This inhibition may indicate that, although fMLP primarily functions via the p38 MAPK pathway, other signaling events are also required, and further downstream kinases may be activated to bypass the selective blockade caused by SB 203580. These results agree with other investigators who have documented that fMLP-induced neutrophil O$_2^-$ production and migration, adherence, and interleukin 8 production can be at least partially inhibited by selective p38 MAPK inhibition (SB 203580 or SK&F 86002). The PAF priming followed by fMLP activation was also inhibited by SB 203580 but, again, other signaling cascades were able to partially circumvent the inhibition. Furthermore, we could not delineate the selective effects of p38 MAPK inhibition on PAF priming vs the effects on fMLP activation.

We therefore used the receptor-independent activator PMA to determine if p38 MAPK mediates PAF priming of the neutrophil functional response, independent of its effects on fMLP activation. As reported by others and documented in our experiments, PMA-induced O$_2^-$ production and elastase release were not inhibited by SB 203580. Therefore, PMA does not rely on p38 MAPK signal transduction for either the resulting neutrophil respiratory burst or the degranulation response. Indeed, PMA has been shown to activate the Raf kinases, MEK-1 and MEK-2, p42/44 ERK MAPKs, and p38 MAPK and, therefore, has many available down-stream kinases to bypass any selective blockade of p38 MAPK. The PAF priming followed by activation with PMA resulted in a significantly increased neutrophil functional response measured for both O$_2^-$ production and elastase release. This PAF priming was completely inhibited by SB 203580. Therefore, PAF appears to have a much more limited set of downstream kinases to activate compared with either fMLP or PMA. Figure 5 schematically represents these proposed intracellular neutrophil signal transduction cascades with multiple areas of cross-talk and intercommunication.

Collectively, these data demonstrate that maximal PAF-induced neutrophil priming for O$_2^-$ production and elastase degranulation requires p38 MAPK signal transduction. Therefore, p38 MAPK appears to serve a pro-inflammatory role in the neutrophil. Further work in pulmonary endothelial cells also demonstrated a pro-inflammatory role for p38 MAPK, since inhibition of this signaling pathway results in decreased adhesion molecule expression. Therefore, inhibition of p38 MAPK activity may offer a potential therapeutic target to reduce neutrophil-mediated hyperinflammation and, ultimately, postinjury MOF and ARDS by altering both the neutrophil and endothelial cell response during systemic inflammatory response syndrome. We recognize that this is a complex system, and that MAPK inhibition may affect other immune cells such as T-cells and monocytes. However, these effects may add to the anti-inflammatory effects demonstrated on the neutrophil. In addition, 4 distinct isoforms of p38 MAPK have been identified in mammalian cells (the original p38α, as well as p38β, p38γ, and p38δ). Only the p38α and p38δ MAPK isoforms have been detected in neutrophils. Further elaboration of these intricate and complicated signal transduction pathways involved in the potentially destructive neutrophil-endothelial cell interaction may generate more specific inhibitors and afford the possibility to selectively modulate the inflammatory response.

This study was supported in part by grants P50GM49222 and T32GM08315 and Clinical Associate Physician Award.

Figure 5. Mitogen-activated protein kinase (MAPK) pathways in neutrophils in response to platelet-activating factor (PAF), N-formyl-methionyl-leucyl-phenylalanine (fMLP), and phorbol myristate acetate (PMA) that have been associated with activation of cytotoxic mechanisms. Specific pathways within the shaded box remain to be fully elucidated, and there are multiple areas of cross-talk and intercommunication. O$_2^-$ indicates superoxide anion; PKC, protein kinase C; and ERK, extracellular signal-related kinase.
Investigation into the pathophysiology of MOF has implicated the neutrophil as a key cellular mediator. We previously characterized PAF priming of human neutrophils as a concordant event resulting in adhesive neutrophils with maximal cytotoxic potential. Whereas traditional therapy has concentrated on extracellular events such as nullifying cellular end products, intervention at the level of signal transduction may prove to be more effective clinically. The concordant neutrophil functional response suggests a possible common priming signal transduction pathway, but precise intracellular signaling pathways involved in neutrophil priming remain unclear. Our data demonstrate that maximal PAF-induced neutrophil priming for O$_2^-$ production and elastase degranulation requires p38 MAPK signal transduction. The p38 MAPK thus appears to serve a proinflammatory role in the neutrophil, and inhibition of p38 MAPK activity may offer a potential therapeutic strategy to reduce global neutrophil-mediated hyperinflammation and ultimately, postinjury ARDS and MOF. Further elucidation of these complicated signal transduction pathways involved in the potentially destructive neutrophil-endothelial cell interaction may generate more specific inhibitors and afford the possibility to selectively modulate the inflammatory response.

REFERENCES

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DISCUSSION

Ronald V. Maier, MD, Seattle, Wash: I congratulate the authors on a continuation of their studies, trying to define the mechanisms involved in the excessive inflammatory response by the neutrophil. As was pointed out, it is key to the paradigm of the 2-hit phenomenon that priming—or perhaps even a better phrase would be reprogramming—of the neutrophil to exhibit a hyperinflammatory phenotype with increased expression of cell surface adhesion molecules and increased release of toxic oxidants and proteases, which are thought to underlie MOF—is crucial. Priming of the PMNs occurs with multiple stimuli, as the authors point out. In addition to the PAF, which is studied in this series, GM-CSF, TNF, and others are known to prime the neutrophil similarly. In addition, it is established in the literature that all of these agents are known to increase p38 MAP kinase activity. It is not illogical, as the authors chose in their hypothesis, that the increased activity of the neutrophil and priming may well be due to p38 activity. Thus, they selected a specific inhibitor of p38 to investigate the role in the priming.

They confirmed inhibition for both fMLP and PMA stimulation of primed cells. However, since fMLP also stimulates p38 directly, they could not separate fMLP priming vs activation, and dependence on p38. Thus, their use of the nonreceptor stimulus, PMA, and the near-complete inhibition of priming in this setting nicely confirm the presumed role for p38. I have 2 areas of questions.

The first is how convinced are the authors that this is a primary p38 activity? I know that is their whole premise, but I would ask for some further clarification.

Borsch Haubold in JBC last year showed that SB 203 blocks both Cox-1 and Cox-2 as well as it blocks p38. Our laboratory and others have shown that blocking cyclooxygenase downregulates arachidonic acid metabolites. And second, with that downregulation is a decrease in production and release of cytotoxic and other inflammatory mediators. While I don’t know if this mechanism is operative in neutrophils, I would presume that it is.

And, thus, do the authors have any data measuring the impact of their inhibitor on Cox activity or subsequent arachidonic acid metabolism and metabolite production? Could they reverse the inhibition by adding back an arachidonic acid metabolite such as PGE2, [prostaglandin E2], thus stimulating the pKA pathway and restoring activation by bypassing p38?

While this does not negate that there is a role for p38, it augments neutrophil priming up to 24 hours after injury and this, starting point in our investigations into this pathway.

The time course of priming, at least in the laboratory, looking at 1 population of cells: the priming response lasts about 30 minutes. Again, in the trauma patients, we have documented neutrophil priming up to 24 hours after injury and this, of course, involves more neutrophils entering the circulation. It is unclear right now at what time point we would have to block this type of signal transduction pathway to get a response. We are hoping to advance some of this knowledge using our animal model in gut ischemia reperfusion to try to elucidate those timing details.

I know it would not help to use Westerns to measure phosphorylation and thus the activation of p38 because the inhibitor blocks this activity, but do you have any functional data such as in-gel kinases showing that your inhibitor actually was reducing p38 activity in these cells?

And lastly, the authors propose that inhibition of signal pathway kinases, thus blocking the reprogramming and hyperactive response of the PMN, may be of clinical therapeutic importance and a better approach than attempting to block individual mediators such as oxidants and proteases; I concur with the authors. I think this is very appealing. But my question is, as the authors state, priming is known to occur within seconds of stimulation and very early in the disease process. Thus, prevention of priming is unlikely to be feasible in the clinical setting.

Why did the authors not perform a time course study? That is, why did they not use the inhibitor after priming but prior to stimulation, which would be much closer to the clinical setting? In addition, something that is very important before we go toward clinical trials is, what is the time course? How long does the priming last in these cells? Is it for 5 minutes? Do we have only a 5-minute window in which to interact? This has led, I think, to many problems in past clinical development of therapeutic interventions, and it is a very simple thing to do. What was your time window for intervention? How long does priming last?

Dr Patrnick: Thank you, Dr Maier, for the very insightful questions. I will try to address some of them, and I think further research that we are currently performing will provide more answers.

The first question was if we were convinced that this priming activity is truly from p38 activity. We did not look at Cox-1 or Cox-2 activity or evaluate that specific pathway in neutrophils. Furthermore, I am not sure if there have been studies evaluating the mechanisms of Cox-1 and Cox-2 in this cell line. There is confirmatory data concerning p38 itself. There are many isoforms, at least 4 of them have been identified, and these other signaling routes have the potential of being more amenable to selective inhibition. This also addresses 1 of your other questions, which is that, out of those 4 p38 isoforms identified, 2 have been identified in neutrophils. If more selective inhibitors can be developed for those isoforms, we might be able to more selectively inhibit the specific enzymes that are responsible for priming and, hopefully, avoid these more broader inhibitions that happen with the SB compound we use.

We do have previous gel data looking at phosphorylation of downstream products, and have shown that this inhibitor, at the concentration we use—1 micromolar—completely blocks the activation of downstream products, and the dose we used is based on previous dose-response experiments in our laboratory.

The clinical applicability of this data is a very important question, and the fact, as Dr Maier pointed out, that neutrophil priming in the trauma patients we studied occurs very early after injury—at least 3 hours is as early as we have been able to document it, and it could certainly be even earlier than that—and how that prevention will be feasible is something that we add later. And, again, this was a pretreatment model and the starting point in our investigations into this pathway.

The time course of priming, at least in the laboratory, looking at 1 population of cells: the priming response lasts about 30 minutes. Again, in the trauma patients, we have documented neutrophil priming up to 24 hours after injury and this, of course, involves more neutrophils entering the circulation. It is unclear right now at what time point we would have to block this type of signal transduction pathway to get a response. We are hoping to advance some of this knowledge using our animal model in gut ischemia reperfusion to try to elucidate those timing details.