Fever-Like Temperatures Affect Neutrophil NF-κB Signaling, Apoptosis, and ANCA-Antigen Expression

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The neutrophil is pivotal to ANCA vasculitis pathogenesis. Fever frequently complicates ANCA diseases. This study investigated the effects of short-term heat exposure on apoptosis in neutrophils that were treated with LPS, GM-CSF, IL-8, and dexamethasone. All compounds delayed apoptosis. Heat abrogated the apoptosis-delaying effect of LPS without affecting constitutive apoptosis or delayed apoptosis by GM-CSF, IL-8, or dexamethasone. The heat effect was dose dependent over the 39 to 42°C range. NF-κB but not extracellular signal–regulated kinase, p38 mitogen-activated protein kinase (MAPK), or phosphatidylinositol 3-kinase/Akt controlled LPS-delayed apoptosis. Furthermore, LPS-induced IκBα degradation, DNA binding, and NF-κB–dependent gene transcription activation were abrogated by short-term heat. When core temperatures were raised to 40.5°C for 30 min in mice, LPS-induced neutrophil NF-κB activation also was prevented. Short-term heat removed heat-shock protein 90 from the IκB kinase complex, resulting in failure of LPS-induced IκB kinase activation. Despite delayed apoptosis, ANCA antigen expression was increased in LPS-treated neutrophils. ANCA antigen increase was prevented by p38 MAPK inhibition and by heat exposure. Heat exposure did not inhibit LPS-induced p38 MAPK phosphorylation. Instead, apoptosis-mediated p38 MAPK degradation was accelerated, thereby decreasing the p38 MAPK that was available for LPS-mediated ANCA antigen upregulation. These data suggest that fever-like temperatures modulate neutrophil behavior in this disease.

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Neutrophil apoptosis is an important mechanism for inflammation resolution (1). ANCA vasculitis is the archetype example of a neutrophil-mediated renal disease (2) in which neutrophil apoptosis has pathogenic implications (3). Apoptosis renders neutrophils less sensitive to ANCA (4) and results in "eat-me" signals to eliminate the cells to resolve inflammation (5). However, neutrophil apoptosis also may have deleterious effects. Apoptotic neutrophils increase their proteinase 3 (PR3) and myeloperoxidase (MPO) expression (4,6–8). In fact, injection of either apoptotic human or rat neutrophils in mice and rats induces ANCA generation (9,10). These ANCA induced respiratory bursts in human neutrophils (9). Moreover, ANCA can opsonize apoptotic neutrophils. Consequently, ANCA-loaded apoptotic cell phagocytosis is accelerated and phagocytes are activated (8,11). Conceivably, increased ANCA antigen presentation on apoptotic neutrophils and dysregulated clearance are important tolerance-breaking factors for self-antigens. These mechanisms are well established in lupus erythematosus (12). Active autoimmune diseases are accompanied by fever, particularly with accompanying infections that may occur during immunosuppressive treatment (13–16). Numerous mediators, including cytokines and glucocorticoids, modulate neutrophil apoptosis (17–20). The vast majority of laboratory investigations are conducted at 37°C. However, during systemic and local inflammation neutrophils become exposed to increased temperatures. Therefore, the effect of heat exposure should be considered in studies on neutrophil responses. We recently showed that short-term heat exposure affects intracellular signal transduction in neutrophils that are exposed to TNF-α (21). In this study, we tested the hypothesis that short-term heat exposure promotes apoptotic cell death in cytokine- and corticosteroid-challenged neutrophils.

Materials and Methods

GM-CSF and IL-8 were obtained from R&D Systems (Wiesbaden-Nordenstedt, Germany). LPS, propidium iodide, dexamethasone, and Ficoll-Hypaque were from Sigma (Deisenhofen, Germany). Dextran was from Amersham Pharmacia (Amsterdam, Netherlands), the α-actin antibody (C-2) and the polyclonal rabbit antibodies against IκBα (C-21) and IκB kinase γ (IKKγ; FL419) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb to IKKα was from Becton Dickinson (Heidelberg, Germany), the polyclonal antibody to IKKβ was from Cell Signaling (Frankfurt am Main, Germany), the PR3 mAb 12.8 was from CLB (Amsterdam, Netherlands), 4A5 was from Wieslab (Lund, Sweden), and the mAb to MPO was from Dako (Hamburg, Germany). Horseradish peroxidase–labeled donkey anti-rabbit IgG was from Amersham (Braunschweig, Germany), HBSS, PBS, RPMI 1640, and trypan blue were from Biochrom (Berlin, Germany), and the ApoAlert Annexin V Apoptosis Kit was from Clontech (Palo Alto, CA). FCS was purchased from Life Technologies BRL (Karlsruhe, Germany). Phospho-specific antibodies to p38 mitogen-activated protein kinase

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(MAPK), extracellular signal–regulated kinase (ERK), and Akt were from Cell Signaling. Endotoxin-free reagents and plastic disposables were used in all experiments.

Preparation and Culture of Human Neutrophils

Neutrophils from healthy human donors were isolated from heparinized whole blood as described previously (22). Samples were incubated at 37°C in 5% CO_2 in polypropylene tubes without agitating (Becton Dickinson). The final cell concentration was 5×10^6 cells/ml. Stimulation was done with 100 ng/ml LPS, 20 ng/ml GM-CSF, 100 nM IL-8, and 10^{-7} M dexamethasone, respectively. The cell viability was detected in every cell preparation by trypan blue exclusion and found to be >99%. The percentage of neutrophils after isolation was >95% by Wright-Giemsa staining and by light microscopy.

In Vitro *Heat Exposure*

Fresh neutrophils at 5×10^6 cells/ml HBSS were incubated for 60 min at 37°C or were exposed to 38, 39, 40, 41, or 42°C. We used Thermo blocks from Eppendorf (Hamburg, Germany). A digital thermometer checked the temperature (Testo 900). After samples were cooled to 37°C, a >95% cell viability was determined by trypan blue exclusion.

Whole-Body Hyperthermia in Mice and Isolation of Bone Marrow Neutrophils

Female 8- to 10-wk-old C57Bl/6 mice were anesthetized with an isoflurane gas (Zeijtun, Malta) and placed on a heating pad (Effenberger, Pfaffing, Germany) until their rectal temperatures reached 40.5°C. Core body temperature was monitored using a probe and was maintained at 40.5 ± 0.5°C for 30 min. The control group was at 36.5 ± 0.5°C. To prevent dehydration, the mice received 1 ml of sterile 0.9% saline subcutaneously between the scapulae after anesthesia. The mice recovered for 1 h, ate and drank *ad libitum*, and behaved normally after the manipulation. The mice then were killed, femurs and tibias were dissected, and the bone marrow was flushed with ice-cold sterile PBS without calcium and magnesium. Neutrophils were isolated further by Ficoll-Hypaque density gradient centrifugation and red blood cell lysis. The cell viability was >99% according to trypan blue exclusion. The neutrophil isolation was >95% by Wright-Giemsa staining. The Berlin Animal Review Board (reg. 0261/02) approved all protocols.

Apoptosis

Flow cytometry after either annexin V staining or ethanol permeabilization and propidium iodide staining was used to assess apoptosis (4). Cells were analyzed using a FACScan (Becton Dickinson), and 10,000 events per sample were collected in listmode using CellQuest Pro software.

Flow Cytometry, Western Blotting, and Immunoprecipitation

PR3 and MPO immunostaining and flow cytometry were performed as described previously (4). For Western blotting, samples were incubated for 5 min at 95°C in loading buffer (250 mM Tris-HCL [pH 6.8] with 4% SDS, 20% glycerol, 0.01% bromphenol blue, and 10% β-mercaptoethanol). Five to 20 μg of protein was electrophoresed on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with TBS-T/5% skim milk for 1 h and incubated overnight with the indicated primary antibodies, followed by a horseradish peroxidase–labeled secondary Ab. The blot was developed in a chemiluminescence substrate (ECL; Amersham) and exposed to an x-ray film. For immunoprecipitation, lysates from 2.5×10^7 neutrophils were prepared (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, and 10% glycerol, supplemented with 2 mM diisopropylfluorophosphate, 10 mM dithiothreitol, 8 mM β-glycerophosphate, and a protease inhibitor cocktail; Roche, Penzberg, Germany). Before 1.25 μg of anti-IKKα antibody was added, extracts were precleared with Protein A–Sepharose (Amersham) for 30 min at 4°C. Antibody binding was carried out overnight at 4°C, before Protein A–Sepharose was added for 2 h. Twenty microliters of 2× loading buffer were added to the A-Sepharose immunocomplex followed by boiling for 5 min at 95°C. Samples were subjected to 8% SDS-PAGE, and Western Blot analysis was performed.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Extracts were prepared and electrophoretic mobility shift assay was performed as described previously (22). Nuclear extracts were incubated with 20,000 cpm of a 22-bp oligonucleotide that contained the NF-κB consensus sequence that had been labeled with [α-32]ATP T4 polynucleotide kinase. Probes were subjected to electrophoresis on native 5% polyacrylamide gels and autoradiographed. The oligonucleotides for H_2K were as follows: Forward primer 5′-GATCCAGGGCTGGGGATTCCCCATCTCCACAGG-3′, reverse primer 5′-GATCCCTGTGGAGATGGGGAATCCCCAGCCCTG-3′.

IκB Kinase Activity Assay

Whole-cell lysates were prepared as described previously (22). Cell equivalents were used for immunoprecipitation in lysis buffer. Before 1 μg of monoclonal IKKα antibody was added, extracts were precleared with Protein A–Sepharose. The next day, 25 μl of Protein A–Sepharose was added for an additional 2 h. Fifteen microliters of kinase buffer including purified recombinant IκBα and 3 μCi [γ-32]ATP was added to the A-Sepharose immunocomplex, and the kinase reaction was incubated for 20 min at 37°C. Samples were boiled and subjected to 12% SDS-PAGE and analyzed by autoradiography. Equal loading was confirmed by IKKα Western blot.

Quantitative Reverse Transcription–PCR

Total RNA were isolated according to a Qiagen protocol including DNase treatment. Quantitative reverse transcription–PCR (RT-PCR) was performed using Taqman technology (Applied Biosystems, Weiterstadt, Germany) as described previously (22). The following oligonucleotides were used for IκBα: Forward primer 5′-CCCTGTAATGGCCGGACTG-3′, reverse primer 5′-AGGAGTGACACCAGGTCAGGA-3′, and the probe Fam 5′-CCTTCACCTCGCAGTGGACCTGC-3′ Tamra. For TNF-α, we used human TNF-α (hTNF-α) 86F 5′-GGTGCTTGTTCCTCAGCCTC-3′ and hTNF-α 150R 5′-CAGGCAGAAGAGCGTGGTG-3′. RT-PCR and quantification were performed using the Taqman 5700 (Applied Biosystems) (22). For RNA quantification, the fluorescence signal was measured at each PCR cycle, and the increase in the fluorescence normalized reporter signal (RN) was documented in an amplification plot. Using nontemplate controls, the threshold was set in the log phase to subtract unspecific fluorescence signals. The sample cycle threshold (Ct) values were determined. Ct-value difference (ΔCt) was used to calculate the factor of differential expression ($2^{\Delta Ct}$). Results were analyzed according to the standard curve method.

Statistical Analyses

Results are given as mean ± SEM. Comparisons between multiple groups were done using Kruskal Wallis tests. Specific differences between multiple groups then were determined by use of a Bonferroni *post hoc* test on the ranked values.

Results

Short-Term Heat Exposure and Neutrophil Apoptosis

We exposed neutrophils to 42°C for 60 min or kept cells of the same preparation at 37°C. All cells were adjusted for 30 min at 37°C, thereafter followed by incubation with LPS, GM-CSF, IL-8, or dexamethasone. After 20 h, apoptosis was assessed. Figure 1 shows that under 37°C, LPS, GM-CSF, IL-8, and dexamethasone delayed constitutive apoptosis. Fever-like temperature spikes inhibited LPS-delayed apoptosis at 20 h. In contrast, heat exposure did not affect constitutive apoptosis and also had no effect on delayed apoptosis by GM-CSF, IL-8, and dexamethasone. We next titrated a dose-response curve to assess temperature effects on LPS-delayed apoptosis. Figure 1C demonstrates that exposure to increased temperatures between 37 and 42°C progressively abrogated LPS-delayed apoptosis. Inhibition became significant already at 40°C.

Short-Term Heat Exposure and Antiapoptotic Signaling

Figure 2 shows that LPS activated ERK, p38 MAPK, and phosphatidylinositol 3-kinase (PI3-K)/Akt, respectively. Dexamethasone did not activate any pathway and therefore is not shown. When we exposed cells to increasing temperatures from 37 to 42°C for 60 min before LPS stimulation, we found slightly decreased LPS-induced activation of ERK and PI3-K/Akt with increasing temperature, whereas p38 MAPK phosphorylation was not affected. We then performed a time course of ERK, p38 MAPK, and PI3-K/Akt activation after 60 min of exposure to either 37 or 42°C. These experiments showed some reduction of ERK and PI3-K/Akt activation at the peak of 15 min after LPS. GM-CSF– and IL-8–induced activation of the three pathways was included for comparison. The data demonstrate no heat effect, indicating that signaling in response to both cytokines was still functioning, excluding toxic cell damage by heat. Both of these cytokines showed more rapid activation kinetics. The respective peak activation is given. Also, no toxic cellular damage was observed when viability was tested after heat exposure by trypan blue exclusion (data not shown).

We next studied inhibitors of ERK, p38 MAPK, and PI3-K/Akt on apoptosis. Preincubation with these inhibitors did not affect constitutive or LPS-delayed apoptosis (data not shown). However, in two parallel experiments, preincubation with the inhibitors blocked LPS-induced ERK, p38 MAPK, and Akt activation, indicating that signaling pathways other that ERK, p38 MAPK, and PI3-K/Akt control delayed apoptosis by LPS. We then studied the effect of short-term heat exposure on NF-κB activation in LPS-treated neutrophils. We showed previously that inhibition of NF-κB by a two-component small peptide that consists of a protein transduction domain and NEMO binding domain abrogated LPS-delayed apoptosis (22). Figure 3A indicates that LPS resulted in IκBα degradation under 37°C control conditions and that previous heat exposure decreased the LPS-induced IκBα degradation dose dependently. Inhibition was clearly apparent at 40°C. Using 42°C, we performed a time-course study that indicated that inhibition occurred at all time points between 2 and 30 min (Figure 3B). In parallel experiments, GM-CSF and IL-8 failed to activate NF-κB under both 37°C control conditions and after short-term heat. We confirmed the inhibiting effect of heat exposure on LPS-induced NF-κB activation by electromobility shift assay (Figure 3C) and by quantitative RT-PCR experiments that assessed IκBα mRNA expression, because IκBα is transcribed in an NF-κB–depen-

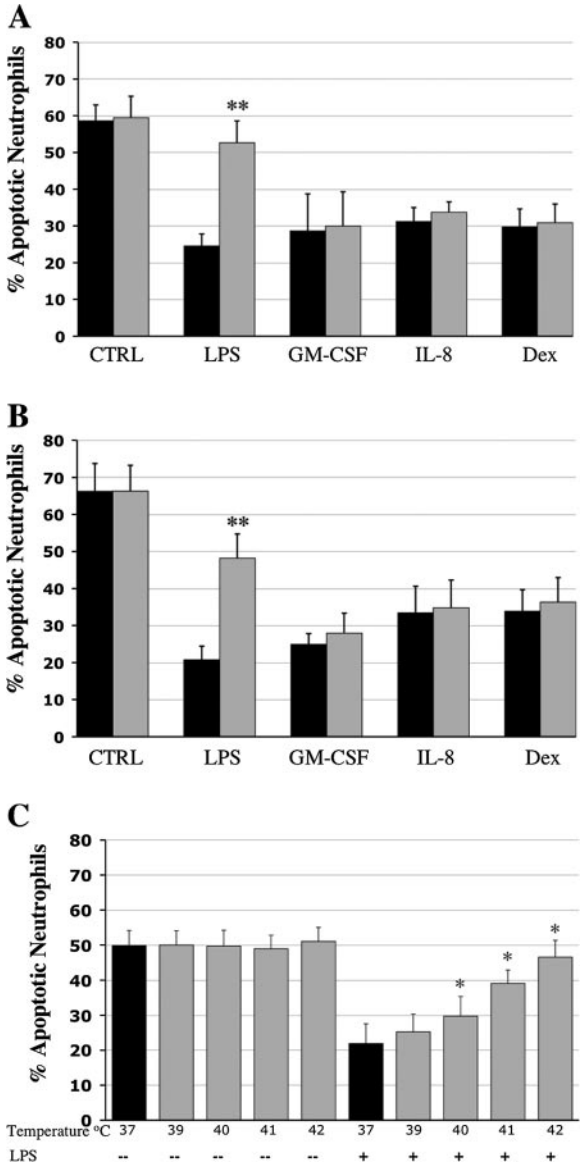

Figure 1. Short-term heat exposure affects neutrophil apoptosis. After exposure to 42 (▨) or 37°C (■) for 60 min, samples were incubated with buffer control (CTRL), 100 ng/ml LPS, 20 ng/ml GM-CSF, 100 nM IL-8, and 10^{-7} M dexamethasone (Dex). After 20 h, cells were either stained with Annexin V (A) or permeabilized in ethanol and stained with propidium iodide (B). Short-term exposure to 42°C abrogated delayed apoptosis by LPS but not delayed apoptosis by all other stimuli ($n = 5$; $^{**}P < 0.01$). (C) Increasing temperature effects on LPS-delayed neutrophil apoptosis by flow cytometry. After exposure of cells to 37, 39, 40, 41, and 42°C for 60 min, samples were incubated with buffer control or 100 ng/ml LPS. Apoptosis was measured after 20 h using propidium iodide and flow cytometry ($n = 6$). Short-term heat dose-dependently abrogated LPS-delayed apoptosis ($^{*}P < 0.05$).

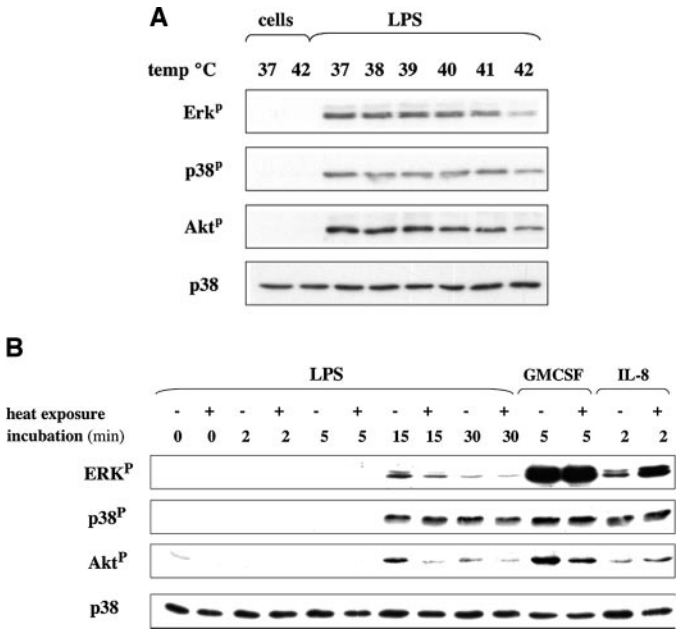

Figure 2. Short-term heat exposure effects on extracellular signal–regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3-K)/Akt activation. (A) After either 37°C or increasing temperatures up to 42°C for 60 min, neutrophils were stimulated with 100 ng/ml LPS. (B) In addition, a time-course study for LPS-induced activation after either 37 or 42°C was performed. Stimulation with 20 ng/ml GM-CSF or 100 nM IL-8 was included for comparison. After indicated time points, cells were harvested for Western blotting with phospho-specific antibodies to ERK, p38 MAPK, and PI3-K/Akt ($n = 3$). Short-term heat exposure inhibited LPS-induced activation of ERK and PI3-K/Akt, without affecting p38 MAPK.

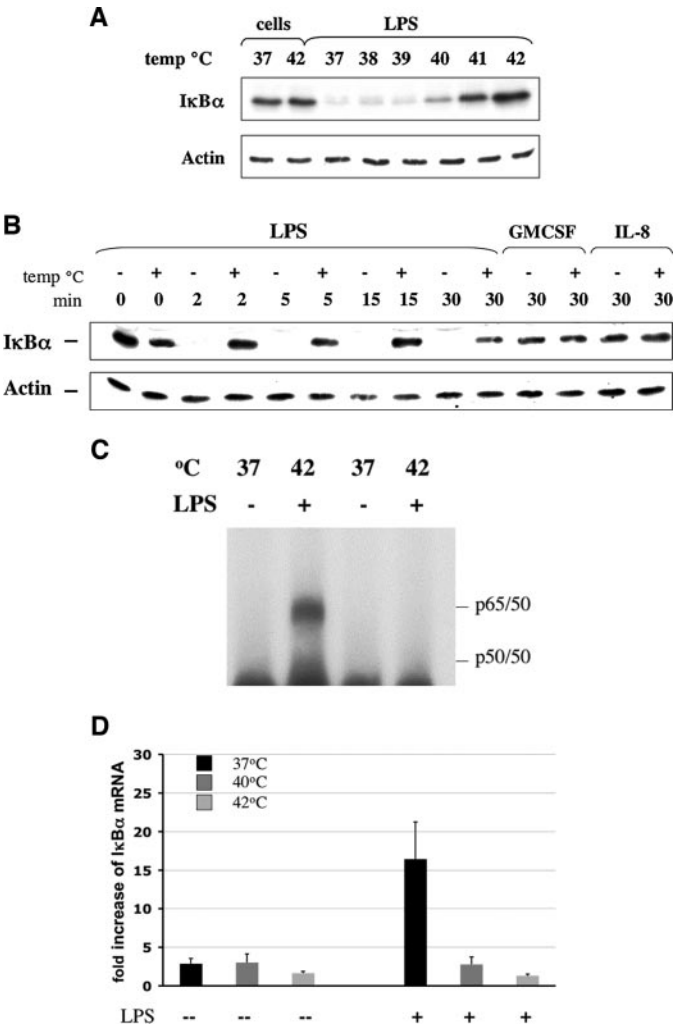

Figure 3. Short-term heat affects NF-κB activation. (A) After either 37°C or increasing temperatures up to 42°C for 60 min, neutrophils were incubated with buffer or 100 ng/ml LPS for 30 min. Samples were assayed for IκBα degradation ($n = 3$). (B) A time-course study compared the effect of 37 and 42°C on IκBα degradation after 2, 5, 15, and 30 min of LPS stimulation ($n = 5$). (C) Effect of 37 *versus* 42°C on NF-κB activation by electrophoretic mobility shift assay ($n = 3$). (D) IκBα mRNA expression was assessed by Taqman reverse transcription–PCR (RT-PCR) after neutrophil exposure to 37, 40, and 42°C ($n = 2$). Collectively, these experiments indicate that heat exposure abrogates LPS-induced NF-κB activation.

dent manner (Figure 3D). Collectively, these data indicate that short-term heat exposure inhibits NF-κB activation, and two independent assays suggest that this effect occurs already at 40°C.

Effect of Short-Term Temperature Increases in Mice

We investigated whether increasing whole-body temperature of mice to 40.5°C for 30 min inhibits NF-κB activation in neutrophils that are treated with LPS *ex vivo*. Mice were anesthetized and body temperature was either raised to 40.5°C for 30 min or kept at 36.5°C. The mice were allowed to recover for 60 min at room temperature. Mice were killed, and neutrophils were harvested from the bone marrow. Western blotting demonstrated that LPS-induced IκBα degradation was prevented in neutrophils from mice that were exposed to 40.5°C. Quantification of six independent experiments was performed by optical densitometry (Figure 4).

Short-Term Heat Exposure Effects on IKK Complex

Heat-shock proteins (HSP) can bind directly to IKK subunits and affect IKK activity. We studied HSP90 and HSP70 expression as well as composition and activity of the IKK complex in neutrophils after exposure to either 37 or 42°C for 60 min. We found no change in HSP90 and HSP70 protein expression up to 4 h after heat exposure (data not shown). In addition, the inhibitory effect of heat exposure on NF-κB activation occurred when cycloheximide was used to block protein *de novo* synthesis. In parallel experiments, 2.5 μg/ml cycloheximide prevented recurrence of IκBα after degradation, indicating that the concentration used in this study was sufficient to block protein *de novo* synthesis (data not shown). Together, these data suggest that the effect of heat exposure on NF-κB activation was independent of HSP.

Binding of HSP90 to IKKα and IKKβ is essential for IKK

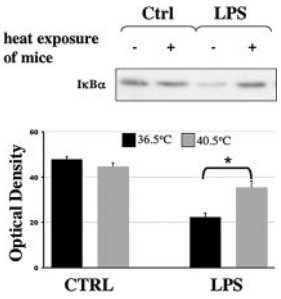

Figure 4. Short-term fever-like temperatures in mice affected *ex vivo* NF-κB activation after LPS. After either raising core body temperature of mice to 40.5°C for 30 min or keeping temperature at 36.5°C, bone marrow neutrophils were isolated and incubated with buffer or 100 ng/ml LPS, respectively. After 30 min, samples were assayed for IκBα degradation ($n = 6$). A typical Western blot experiment from six is depicted, and quantification by optical density measurements of the scanned IκBα bands is provided ($^*P < 0.05$).

activation. We therefore investigated the effect of heat exposure on composition of the IKK complex. Precipitation of IKKα revealed that all three IKK were present in the complex after 37°C as well as after exposure to 42°C for 60 min (Figure 5A). In contrast, HSP90 was detected in the IKKα precipitates at 37°C but was almost undetectable after cells were exposed to 42°C for 60 min. In each of these four experiments, the Western blots were retested with an IKKα antibody, demonstrating that similar amounts of IKKα were precipitated. Densitometric analysis of HSP90 protein is provided in Figure 5B. We next assessed the effect of short-term heat exposure on the function of the IKK complex. By kinase activity assays, we demonstrate that previous heat exposure of neutrophils to either 40 or 42°C abrogated IKK activation in response to LPS (Figure 5C).

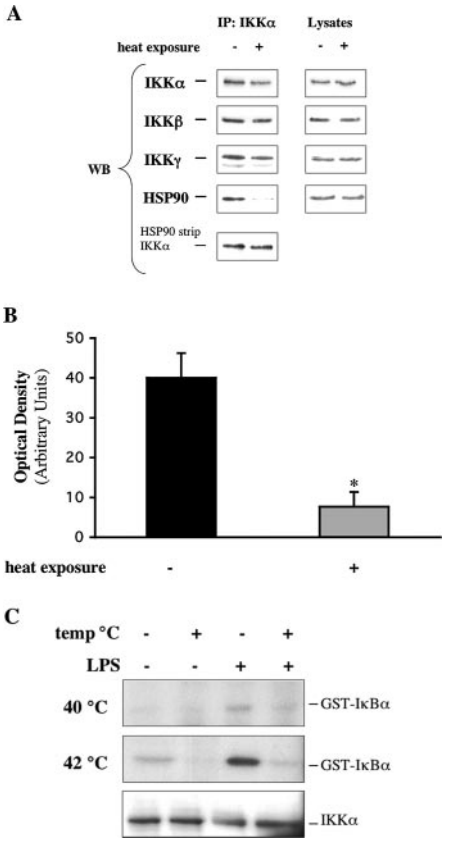

Figure 5. Heat exposure affects the composition and the activity of the IκB kinase (IKK) complex. (A) Neutrophils were exposed to either 37 or 42°C for 60 min. After 30 min at 37°C, IKKα was precipitated and electrophoresed on an 8% SDS gel, and Western blotting was performed with the indicated antibodies ($n = 4$). The data demonstrate that complex formation between IKKα, β, and γ was not altered by heat exposure. However, heat-shock protein 90 (HSP90) was displaced from the complex after exposure of neutrophils to 42°C. (B) Densitometry shows that HSP90 precipitated with IKKα was significantly reduced after heat exposure ($n = 4$; $^*P < 0.05$). (C) For assessing IKK activity, neutrophils were exposed to 37, 40, or 42°C for 60 min before stimulation with 1 μg/ml LPS (+) or buffer control (−). Whole-cell lysates were immunoprecipitated with a monoclonal IKKα antibody. Precipitates were incubated with 1 μg of GST-IκBα and 3 μCi of [γ-32]ATP. Samples were boiled and subjected to 12% SDS-PAGE and autoradiography. Equal loading was confirmed by IKKα Western blot.

Short-Term Heat Exposure Effects on TNF-α mRNA and ANCA Antigen Expression

To study the effect of short-term heat exposure on neutrophil functions that may have implications above and beyond the neutrophil, we first assayed TNF-α mRNA expression that itself is controlled by NF-κB. We observed that LPS increased TNF-α mRNA and that this effect was abrogated by previous heat exposure to either 40 or 42°C *in vitro* (Figure 6).

We then studied the effect of short-term heat exposure on ANCA antigen expression after overnight LPS. Heat exposure to 42°C inhibited LPS-induced upregulation of membrane PR3 (Figure 7A) and MPO (Figure 7B). LPS-induced upregulation of mPR3 occurred mostly in the nonapoptotic membrane proteinase 3 (mPR3)-positive neutrophil population (Figure 7C). Apoptosis was assessed in parallel experiments. Heat did not affect constitutive apoptosis (47 ± 4 *versus* 49 ± 4%), whereas delayed apoptosis by LPS was abrogated by previous exposure to 42°C (22 ± 9 *versus* 46 ± 3%). We also found that exposure to a lower temperature of 40°C inhibited upregulation of mPR3 expression in the presence of LPS (374 ± 82 MFI without heat exposure *versus* 245 ± 76 mean fluorescence intensity (MFI) after 40°C) without having an effect on mPR3 expression in unstimulated cells (203 ± 66 *versus* 207 ± 71 MFI; $n = 3$). Again, abrogation of the LPS-delaying effect on apoptosis was confirmed in parallel (data not shown). In two parallel experiments, we showed that the LPS-induced increase in PR3 expression at 37°C was decreased by 114 ± 16% with 10 μM of the p38 MAPK inhibitor SB202190 and confirmed that the LPS delay on apoptosis was not prevented by SB202190 (constitutive apoptosis was 49 ± 6% in the absence and 45 ± 5% in the presence of SB202190; these numbers were 21 ± 1 and 23 ± 3% for LPS-treated samples). It is interesting that unstimulated cells that were cultured for 20 h showed 214 ± 49 MFI for mPR3 staining when an isotype control antibody was added and

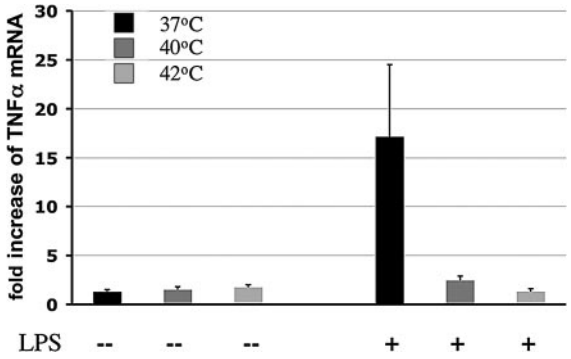

Figure 6. Short-term heat exposure affects TNF-α mRNA expression. After cell exposure to 37, 40, or 42°C for 60 min *in vitro*, neutrophils were incubated with buffer or 100 ng/ml LPS for 30 min. Samples were assayed for TNF-α mRNA expression by Taqman RT-PCR ($n = 2$). These experiments indicate that heat exposure abrogates LPS-induced TNF-α generation.

220 ± 59 MFI when a neutralizing antibody to hTNF-α was added. In the presence of LPS, the mPR3 increased to 374 ± 90 MFI with the isotype control antibody and only to 311 ± 78 MFI with the neutralizing antibody to hTNF-α ($n = 3$). These data suggest that TNF-α is released from neutrophils that are cultured in the presence of LPS and that this TNF-α is responsible, at least in part, for an increase in mPR3 expression. These data indicate that LPS-mediated ANCA antigen upregulation depends on p38 MAPK and suggest that TNF-α, which was shown to use a p38 MAPK to increase mPR3 expression, was involved. However, heat exposure did not inhibit LPS-induced p38 MAPK activation (Figure 3). Instead, as shown in Figure 8, overnight incubation resulted in degradation of p38 MAPK protein. LPS inhibited p38 MAPK degradation. However, previous exposure of neutrophils to either 40 or 42°C diminished the LPS rescue effect on p38 MAPK protein degradation. Our data suggest that by preventing the apoptosis-delaying effect of LPS, heat exposure decreases the amount of p38 MAPK that is available for LPS-mediated ANCA antigen upregulation.

Discussion

Our study suggests that fever-like temperatures serve to regulate neutrophil fate and behavior in a clinically relevant manner. Apoptosis resolves neutrophil inflammation. ANCA vasculitis is a prototype neutrophil-mediated renal disease in which apoptosis has regulatory importance (4,6–11). Neutrophils are short-lived inflammatory cells that become exposed to cytokines and high temperatures during systemic fever and at sites of local inflammation. LPS, GM-CSF, IL-8, and dexamethasone delay neutrophil apoptosis (22–24). However, very few studies have addressed the effect of fever-like temperatures on human neutrophil signaling and functions. Werz *et al.* (25) found that short-term exposure of neutrophils to 45°C activated 5-lipoxygenase, a key enzyme for leukotriene synthesis. NADPH oxidase activity was either inhibited or increased (26,27). These studies used mostly temperatures above 43°C, which were well above the more physiologic temperatures used in our experiments. When we exposed neutrophils to

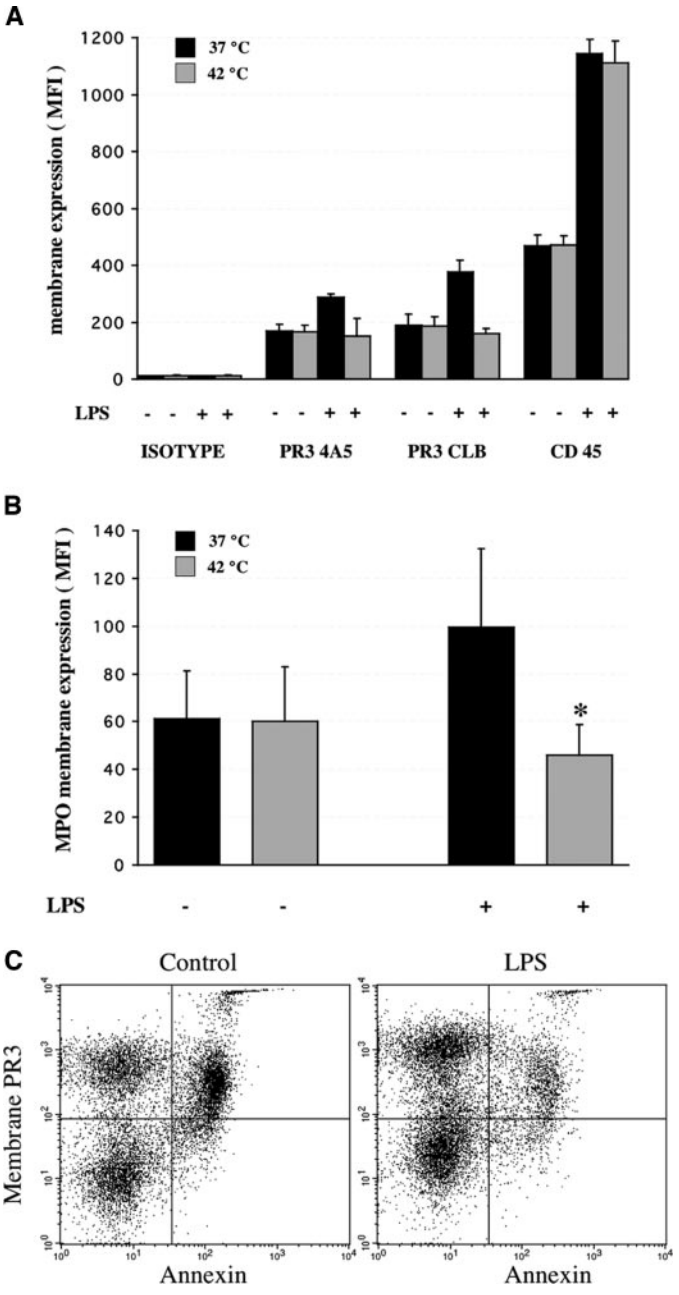

Figure 7. Short-term heat exposure affects ANCA antigen membrane expression. (A) After either 37 or 42°C for 60 min, neutrophils were incubated for 20 h with buffer or 100 ng/ml LPS. Samples were assayed for membrane proteinase 3 (mPR3) expression by flow cytometry using two different mAb to PR3, a mAb to CD45, and an isotype control antibody. (B) Evaluation of myeloperoxidase (MPO) membrane expression. (C) Double staining for membrane PR3 expression and apoptosis (Annexin) in control and LPS-treated neutrophils not exposed to heat. Heat exposure abrogated LPS-induced upregulation of PR3 and MPO expression ($n = 4$; $^*P < 0.05$). LPS-induced upregulation of mPR3 occurs mostly in the nonapoptotic mPR3-positive subpopulation.

increasing temperatures for only 60 min, we observed that the LPS-induced apoptosis delay was dose-dependently abrogated, already at 40°C. In contrast, constitutive apoptosis and delayed

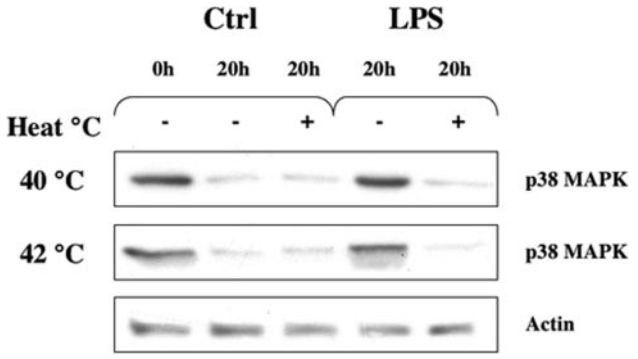

Figure 8. Short-term heat exposure affects p38 MAPK protein degradation. The amount of p38 MAPK protein was estimated by Western blotting. Neutrophil incubation (20 h) without and with previous short-term heat exposure decreased p38 MAPK protein. LPS prevented p38 MAPK degradation. This effect was abrogated by previous heat exposure to 40 and 42°C, respectively. An example from two independent experiments is shown.

apoptosis by GM-CSF, IL-8, and dexamethasone were not affected. That the latter compounds still delayed apoptosis indicates that the heat effect did not result in thermal injury but instead depended on the mediator challenging the neutrophil. Fever is common in autoimmune disease, with or without concomitant infection. The two largest randomized ANCA vasculitis treatment trials documented a high infection rate in patients who received immunosuppression (15,16). Moreover, in ANCA vasculitis, pyogenic, LPS-producing Gram-negative *Escherichia coli* and *Klebsiella* species were among the most common isolated pathogens during the first year of treatment (28).

We studied four pathways that are relevant to LPS-delayed neutrophil apoptosis, namely p38-MAPK, ERK, PI3-K/Akt, and NF-κB (22,24,29). Our results show that short-term heat exposure decreased LPS-induced ERK and PI3-K/Akt activation with only marginal effects on p38 MAPK. However, pharmacologic inhibitors of p38 MAPK, ERK, and PI3-K/Akt demonstrated that these pathways did not control LPS-delayed apoptosis. NF-κB provides another important LPS-induced neutrophil antiapoptotic pathway (30). We showed earlier that NF-κB is pivotal for LPS-delayed apoptosis using a small peptide that specifically inhibits the IKK complex (22). We found that NF-κB was strongly activated only by LPS but not by GM-CSF, IL-8, or dexamethasone. Short-term heat exposure completely prevented LPS-induced IκBα degradation, NF-κB DNA binding, and initiation of a NF-κB–dependent gene transcription. Importantly, we show that *in vivo* administration of a short fever spike in mice reproduced the effect of *in vitro* heat exposure. These data underscore the idea that increased body temperatures have a clinically important effect on neutrophil responses.

Increased HSP70 protein expression, either by heat or by overexpression, inhibits the NF-κB pathway (31,32). We found that neither HSP70 nor HSP90 protein was induced in a time frame in which heat-mediated NF-κB inhibition occurred. Moreover, we found that NF-κB inhibition did not require any *de novo* protein synthesis. Studies using the antitumor agent geldanamycin showed that the association of HSP90 with the IKK complex is essential for the activation of NF-κB (33,34). Our results indicate that short-term heat exposure resulted in dissociation of HSP90 from the IKK complex. In accordance with our data, Pittet *et al.* (35) also recently observed HSP90 dissociation from IKKα after heat. However, in contrast to our study, these investigators exposed cells to 43°C and used nonhuman epithelial cells, rather than neutrophils.

Our data have implications beyond neutrophil apoptosis. We observed that LPS-induced NF-κB–dependent gene transcription of TNF-α was blocked after short-term heat exposure. This finding suggests that fever-like temperatures may abrogate delivery of NF-κB–dependent proinflammatory molecules by neutrophils (36). Fewer proinflammatory molecules than TNF-α probably also would reduce the inflammatory response of cells other than neutrophils. In addition, we observed that membrane expression of ANCA antigens was increased in neutrophils that were incubated overnight with LPS. This observation was unexpected because apoptosis was shown to increase ANCA antigen expression (4,6–8), and LPS resulted in delayed apoptosis. It is interesting that increased membrane PR3 expression in LPS-treated neutrophils was dominantly observed in the nonapoptotic population. p38 MAPK upregulated LPS-induced ANCA antigen expression but did not mediate LPS-delayed apoptosis. The increase in ANCA antigen was mediated, at least in part, by TNF-α that was released from LPS-treated neutrophils. These data are in agreement with a recent study by Witko-Sarsat's group demonstrating that the mechanisms that lead to PR3 expression during stimulated degranulation and during apoptosis are different (37). Heat exposure, similar to p38 MAPK inhibition, prevented the increase in ANCA antigen expression in LPS-treated neutrophils. Our data suggest that the heat effect is mediated *via* p38 MAPK, although p38 MAPK activation was not inhibited by heat exposure. Instead, heat resulted in accelerated p38 MAPK protein degradation in LPS-treated neutrophils. Suzuki *et al.* (38) showed that apoptosis promotes caspase-mediated p38 MAPK cleavage and that this process was diminished when apoptosis was delayed. Therefore, our data are consistent with the idea that heat exposure, by preventing the apoptosis-delaying effect of LPS, decreases the amount of p38 MAPK that is available to mediate LPS-induced ANCA antigen upregulation.

Conclusion

Conceivably, decreased ANCA antigen expression after heat exposure and, therefore, less opsonization by ANCA, could promote noninflammatory disposal of apoptotic neutrophils in an LPS-rich inflammatory milieu. Heat-mediated effects on neutrophil ANCA activation warrant careful future study. We suggest that fever-like temperatures may have important roles in modulating inflammatory diseases. Combating fever may be counterproductive, compared with addressing disease-related mechanisms directly.

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References

1. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 83: 865–875, 1989
2. Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, Falk RJ, Jennette JC: The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol* 167: 39–45, 2005
3. Esnault VL: Apoptosis: The central actor in the three hits that trigger anti-neutrophil cytoplasmic antibody-related systemic vasculitis. *Nephrol Dial Transplant* 17: 1725–1728, 2002
4. Kettritz R, Scheumann J, Xu Y, Luft FC, Haller H: TNF-alpha–accelerated apoptosis abrogates ANCA-mediated neutrophil respiratory burst by a caspase-dependent mechanism. *Kidney Int* 61: 502–515, 2002
5. Whyte MK, Meagher LC, MacDermot J, Haslett C: Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol* 150: 5124–5134, 1993
6. Gilligan HM, Bredy B, Brady HR, Hebert MJ, Slayter HS, Xu Y, Rauch J, Shia MA, Koh JS, Levine JS: Antineutrophil cytoplasmic autoantibodies interact with primary granule constituents on the surface of apoptotic neutrophils in the absence of neutrophil priming. *J Exp Med* 184: 2231–2241, 1996
7. Yang JJ, Tuttle RH, Hogan SL, Taylor JG, Phillips BD, Falk RJ, Jennette JC: Target antigens for anti-neutrophil cytoplasmic autoantibodies (ANCA) are on the surface of primed and apoptotic but not unstimulated neutrophils. *Clin Exp Immunol* 121: 165–172, 2000
8. Harper L, Cockwell P, Adu D, Savage CO: Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. *Kidney Int* 59: 1729–1738, 2001
9. Rauova L, Gilburd B, Zurgil N, Blank M, Guegas LL, Brickman CM, Cebecauer L, Deutsch M, Wiik A, Shoenfeld Y: Induction of biologically active antineutrophil cytoplasmic antibodies by immunization with human apoptotic polymorphonuclear leukocytes. *Clin Immunol* 103: 69–78, 2002
10. Patry YC, Trewick DC, Gregoire M, Audrain MA, Moreau AM, Muller JY, Meflah K, Esnault VL: Rats injected with syngenic rat apoptotic neutrophils develop antineutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 12: 1764–1768, 2001
11. Moosig F, Csernok E, Kumanovics G, Gross WL: Opsonization of apoptotic neutrophils by anti-neutrophil cytoplasmic antibodies (ANCA) leads to enhanced uptake by macrophages and increased release of tumour necrosis factor-alpha (TNF-alpha). *Clin Exp Immunol* 122: 499–503, 2000
12. Walport MJ: Lupus, DNase and defective disposal of cellular debris. *Nat Genet* 25: 135–136, 2000
13. Bradley JD, Brandt KD, Katz BP: Infectious complications of cyclophosphamide treatment for vasculitis. *Arthritis Rheum* 32: 45–53, 1989
14. Hoffman GS, Kerr GS, Leavitt RY, Hallahan CW, Lebovics RS, Travis WD, Rottem M, Fauci AS: Wegener granulomatosis: An analysis of 158 patients. *Ann Intern Med* 116: 488–498, 1992
15. Jayne D, Rasmussen N, Andrassy K, Bacon P, Tervaert JW, Dadoniene J, Ekstrand A, Gaskin G, Gregorini G, de Groot K, Gross W, Hagen EC, Mirapeix E, Pettersson E, Siegert C, Sinico A, Tesar V, Westman K, Pusey C: A randomized trial of maintenance therapy for vasculitis associated with antineutrophil cytoplasmic autoantibodies. *N Engl J Med* 349: 36–44, 2003
16. Etanercept plus standard therapy for Wegener's granulomatosis. *N Engl J Med* 352: 351–361, 2005
17. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80: 2012–2020, 1992
18. Cox G, Gauldie J, Jordana M: Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol* 7: 507–513, 1992
19. Kettritz R, Gaido ML, Haller H, Luft FC, Jennette CJ, Falk RJ: Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney Int* 53: 84–91, 1998
20. Takeda Y, Watanabe H, Yonehara S, Yamashita T, Saito S, Sendo F: Rapid acceleration of neutrophil apoptosis by tumor necrosis factor-alpha. *Int Immunol* 5: 691–694, 1993
21. Salanova B, Choi M, Rolle S, Wellner M, Scheidereit C, Luft FC, Kettritz R: The effect of fever-like temperatures on neutrophil signaling. *FASEB J* 19: 816–818, 2005
22. Choi M, Rolle S, Wellner M, Cardoso MC, Scheidereit C, Luft FC, Kettritz R: Inhibition of NF-kappaB by a TAT-NEMO-binding domain peptide accelerates constitutive apoptosis and abrogates LPS-delayed neutrophil apoptosis. *Blood* 102: 2259–2267, 2003
23. Cox G: Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* 154: 4719–4725, 1995
24. Klein JB, Rane MJ, Scherzer JA, Coxon PY, Kettritz R, Mathiesen JM, Buridi A, McLeish KR: Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 164: 4286–4291, 2000
25. Werz O, Burkert E, Samuelsson B, Radmark O, Steinhilber D: Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* 99: 1044–1052, 2002
26. Maridonneau-Parini I, Malawista SE, Stubbe H, Russo-Marie F, Polla BS: Heat shock in human neutrophils: Superoxide generation is inhibited by a mechanism distinct from heat-denaturation of NADPH oxidase and is protected by heat shock proteins in thermotolerant cells. *J Cell Physiol* 156: 204–211, 1993
27. Rosenspire AJ, Kindzelskii AL, Petty HR: Cutting edge: Fever-associated temperatures enhance neutrophil responses to lipopolysaccharide: A potential mechanism involving cell metabolism. *J Immunol* 169: 5396–5400, 2002

28. Koselj-Kajtna M, Koselj M, Rott T, Kandus A, Bren A: Infectious complications of immunosuppressive treatment for anti-neutrophil cytoplasm antibody-related vasculitis. *Transplant Proc* 34: 3001–3002, 2002
29. Frasch SC, Nick JA, Fadok VA, Bratton DL, Worthen GS, Henson PM: p38 mitogen-activated protein kinase-dependent and -independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. *J Biol Chem* 273: 8389–8397, 1998
30. McDonald PP, Bald A, Cassatella MA: Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* 89: 3421–3433, 1997
31. Feinstein DL, Galea E, Aquino DA, Li GC, Xu H, Reis DJ: Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation. *J Biol Chem* 271: 17724–17732, 1996
32. Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H, Feng Y, Han C, Zhou G, Rigby AC, Sharp FR: Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and impairing NF-kappa B survival signaling. *Genes Dev* 18: 1466–1481, 2004
33. Chen G, Cao P, Goeddel DV: TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell* 9: 401–410, 2002
34. Broemer M, Krappmann D, Scheidereit C: Requirement of Hsp90 activity for IkappaB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-kappaB activation. *Oncogene* 23: 5378–5386, 2004
35. Pittet JF, Lee H, Pespeni M, O'Mahony A, Roux J, Welch WJ: Stress-induced inhibition of the NF-kappaB signaling pathway results from the insolubilization of the IkappaB kinase complex following its dissociation from heat shock protein 90. *J Immunol* 174: 384–394, 2005
36. Pahl HL: Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18: 6853–6866, 1999
37. Durant S, Pederzoli M, Lepelletier Y, Canteloup S, Nusbaum P, Lesavre P, Witko-Sarsat V: Apoptosis-induced proteinase 3 membrane expression is independent from degranulation. *J Leukoc Biol* 75: 87–98, 2004
38. Suzuki K, Hasegawa T, Sakamoto C, Zhou YM, Hato F, Hino M, Tatsumi N, Kitagawa S: Cleavage of mitogen-activated protein kinases in human neutrophils undergoing apoptosis: Role in decreased responsiveness to inflammatory cytokines. *J Immunol* 166: 1185–1192, 2001