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Temperature can prime human peripheral blood neutrophils in a p38MAPK α -dependent manner

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
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Summary

Introduction:

Previous *ex vivo* experiments by others suggest that elevated body temperature can prime the respiratory burst of human neutrophils. The mechanism of the priming phenomenon induced by temperature has not been addressed so far. Furthermore, the priming temperature range was not defined.

Materials and Methods:

In the present study we explored, under *in vitro* conditions, the influence of febrile-range temperatures on reactive oxygen species (ROS) generation by human peripheral blood neutrophils. ROS production was measured using whole-blood luminol-dependent chemiluminescence. Two elements of signal transduction pathways, calcium and p38 mitogen-activated protein kinase α (p38MAPK α), frequently underlying neutrophil priming were also examined. Calcium levels in the cytosol of resting and fMLP-stimulated isolated neutrophils were measured with the Fura-2AM spectrofluorimetric method. The activity of p38MAPK α was assessed indirectly with a specific inhibitor of the kinase, SB 203580.

Results:

The study revealed a priming effect at 38°C toward human peripheral blood neutrophil ROS production. Any concomitant effect on calcium response was not observed. Instead, experiments with SB 203580, a specific inhibitor of p38MAPK α , pointed to an increased activity of the kinase as a molecular background of temperature-induced priming. However, the priming effect of temperature was confined to 38°C, while higher temperatures proved to exert no effect (39 and 40°C) or even inhibited ROS generation by neutrophils (43°C).

Conclusions:

Our study suggests a heterogeneous influence of temperature on human neutrophil functioning, including the priming of the cells by a low-febrile-range temperature. It also suggests a p38MAPK α -dependent molecular background of the priming phenomenon.

Key words:

chemiluminescence • reactive oxygen species • SB 203580 • intracellular calcium

Abbreviations:

[Ca²⁺]_{cyt} – cytosolic calcium ion concentration, **CL** – whole-blood luminol-dependent chemiluminescence, **DPH** – 1, 6-diphenyl-1, 3, 5-hexatriene, **fMLP** – N-formyl-methionyl-leucyl-phenylalanine, **Fura-2AM** – acetomethoxyl ester of Fura-2, **MS** – mixture solution for CL, **NADPH oxidase** – reduced nicotinamidadeninucleotide oxidase, **PBS** – phosphate-buffered saline, **PMA** – phorbol myristate acetate, **p38MAPK** – p38 mitogen-activated protein kinase, **R_i** – ratio of fluorescence intensities of calcium-bound Fura-2AM to calcium-free Fura-2AM, **ROS** – reactive oxygen species.

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INTRODUCTION

Priming of neutrophils consists of enhancing their response to various stimulators, including chemoattractants and opsonized bacteria. Priming is of great importance in respect to neutrophil function regulation. First of all it is required for effective neutrophil stimulation. On the other hand, most of the known priming factors exert their action locally and thus limit neutrophil function to the place of infection, preventing their unnecessary activation. This is important because even localized neutrophil response can cause damage to surrounding tissues. Some tissue damage is an inevitable cost of the host defense offered by neutrophils, but it can exceed defense benefits in the case of excessive or inadequate neutrophil stimulation. Tissue injury results mainly from toxicity of neutrophil-derived reactive oxygen species (ROS), with minor participation of proteases liberated from the cells during degranulation³⁷. Neutrophils are implicated in the pathogenesis of many human diseases, including adult respiratory distress syndrome²⁹, cigarette smoking-induced pulmonary emphysema¹⁴, ischemia/reperfusion injury⁸, and rheumatoid arthritis⁹. Malicious consequences of neutrophil activity are also observed in the case of sepsis and other systemic inflammatory responses. Thus knowledge about the priming phenomenon seems clinically valuable and it may have an impact on therapeutic strategies.

The molecular basis responsible for the priming phenomenon is related to the preactivation of some extracellular signal transduction pathways⁶. Among these are the most common targets for priming agents of human neutrophils, namely the pathways containing some mitogen-activated protein kinases (i.e. p38MAPK α and ERK1/ERK2), sphingosine kinase, and calcium ions^{17, 24}. p38MAPK α is a member of the stress-activated protein kinases subfamily of MAPKs, p38MAPKs. Two of the four isoforms of p38MAPKs identified so far, p38 α and p38 δ , occur in human neutrophils^{15, 26}. p38MAPK α signals from chemoattractant receptors to the cell interior and is suggested to participate in the priming effect of lipopolysaccharide (LPS) and tumor necrosis factor (TNF)- α towards human neutrophils^{12, 26, 31, 36, 40}. Interestingly also some physicochemical factors such as osmotic⁴ or heat shock³⁵ can also prime ROS and leukotrien production, respectively, via p38MAPK.

Increase in neutrophil cytosolic calcium level is an early step involved in signal transduction from chemoattractant receptors, separate from the p38MAPK α pathway^{1, 25}. Enhanced resting calcium levels as well as enhanced calcium response to stimulation also seem to be responsible for the action of an

array of priming agents, e.g. LPS, interleukin (IL)-8, and platelet activating factor^{17, 38, 39}.

Ex vivo studies on human neutrophils suggest that elevated body temperature can prime their production of reactive oxygen species, i.e. prime their respiratory burst^{16, 23}. However, the priming temperature range is unknown. Similarly, the mechanism of temperature-induced respiratory burst priming waits for elucidation. In the present study to define the priming ability of clinically relevant temperatures toward human neutrophils, the effect of febrile temperatures on the respiratory burst of human peripheral blood neutrophils was examined. The effect at 43°C, representing heat-shock temperatures, was also addressed. Additionally, p38 mitogen-activated protein kinase α (p38MAPK α) and calcium transients in neutrophils were examined as potentially involved in the temperature-dependent priming of the cells.

MATERIALS AND METHODS

Reagents

Acetomethoxyl ester of Fura-2 (Fura-2AM), ethylene glycol-bis-(2-aminoethylether)-tetraacetic acid, and Triton X-100, were all purchased from Serva (Heidelberg, Germany). Luminol and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were from Sigma (St. Louis, USA). SB 203580 was obtained from Tocris Cookson Ltd. (Bristol, UK). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes Inc. (Eugene, USA).

Subjects

Sixty-four healthy volunteers, 39 females and 25 males aged 19–35 years (22.6 ± 3.9), were donors of peripheral venous blood. The blood was taken from the antecubital vein using the Vacuette EDTA K3 blood withdrawal system. Blood counts were performed with a Micros OT 45 hematological analyzer (ABX Diagnostics, France). Donors answered a questionnaire on cigarette smoking, infections during the 3 months preceding the examination, and medications in use. Smokers, reconvalescents from infectious disease, and subjects on any medication were excluded from the study. Additionally, the non-inflammatory status of donors at the time of examination was ascertained by total white blood cell count and microscopically assessed differential count.

ROS production measurement

ROS production by neutrophils was measured using the whole-blood luminol-dependent chemilumines-

cence (CL) method. Whole-blood chemiluminescence is considered to have the same validity as chemiluminescence of isolated neutrophils (ICL) in detecting neutrophils in a primed state. However, whole-blood chemiluminescence is superior to ICL in terms of higher reproducibility of results and lower susceptibility to usual laboratory sample handling^{10, 18}.

Whole-blood chemiluminescence was measured according to Kukovetz et al.²¹ in a BIO-ORBIT 1251 luminometer with automated dispenser (Turkù, Finland) controlled by PC-based Multiuse software. Briefly, chemiluminescence of 3 µl whole-blood suspended in a 947 µl mixture solution (MS) of Ringer solution without Ca²⁺, 1.4 mM luminol solution, 5% (w/v) glucose solution, and distilled water was measured. At 60 sec of CL measurement, neutrophils were stimulated with automatically dispensed 50 µl of 2×10^{-4} M fMLP (final concentration 10^{-5} M) or 50 µl of 740 ng/ml phorbol myristate acetate (PMA; final concentration 37 ng/ml). The total measurement time was 10 min.

Blood was added to the MS immediately after withdrawal. Before measurement the samples were preincubated for 15 min in a water-bath held at the examination temperature (43°C or one of febrile temperatures, i.e. 38, 39, 40°C) and at 37°C (control samples) and then all samples were preincubated for the next 10 min at 37°C in the thermostatically controlled measuring chamber of the luminometer. Duration of exposure to the examination temperature (15 min) was based on literature and was a compromise between CL sensitivity limitations and sufficient exposure to stress^{10, 31}. The temperature of the water-bath was monitored with a mercury thermometer. The temperature of the samples was continuously monitored during the preincubation period with a Multimeter BM 511X thermocouple (Brymen Technology Corp., Taiwan). Sample temperatures were steady within $\pm 0.2^\circ\text{C}$ and were equal to that of the water-bath/luminometer chamber during the first minute of the incubation time regardless of the examined temperature. Measurements at every temperature were done in duplicate. Resting CL (mean CL over the first 60 s of measurement), peak CL after stimulation, and integral CL (area under the CL-time curve for the total 10 min of measurement) were assessed. Results are mean values from duplicate measurements calculated per 1000 neutrophils and presented in CL arbitrary units (AU) for resting and peak CL, and as $\text{AU} \times \text{sec}$ for integral CL. Because monocytes and eosinophils (the cells, besides neutrophils, responsible for whole-blood chemiluminescence) together constituted on average $2.9\% \pm 1.5\%$ of the total white cell count (mean $2.0\% \pm 1.1\%$ and

$0.8\% \pm 0.8\%$ for monocytes and eosinophils, respectively), while the mean neutrophil count was $62.0\% \pm 8.6\%$ of the whole, chemiluminescence of whole-blood in our experiments derived mainly from the neutrophil activity¹⁹.

Cytosolic Ca²⁺ measurement

Ca²⁺ cytosol level was measured in neutrophils isolated according to the Boyum method³ from blood of the same tube as for fMLP-stimulated CL measurements and in parallel to them. The purity of the isolated neutrophils was at least 95% and their viability exceeded 98% each time, as assessed by the trypan blue exclusion test. The isolated neutrophils were loaded with Fura-2AM. Afterwards, the neutrophils were exposed to temperature, suspended in Ca²⁺-low (100 nM) or Ca²⁺-rich (1 mM) medium, and then the cytosolic Ca²⁺ concentration was measured spectrofluorometrically as previously described²⁷ in a Perkin-Elmer Luminescence Spectrometer LS-50B (Norwalk, CT, USA) operating at a 510-nm emission wavelength and at 340-nm and 380-nm excitation wavelengths. The spectrometer was controlled by a PC equipped with the FL Data Manager (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England). Measurements were performed at 37°C for 7 min each. At 60 s of the measurement, 1 µl of 6×10^{-5} M fMLP (final concentration 10^{-7} M) was manually added to the sample. The measurements were carried out once for each temperature. Some experiments in Ca²⁺-rich medium were extended on cytosolic Ca²⁺ level measurement in the presence of 1 mM Ni²⁺ outside the cells. Ni²⁺ was manually added to the sample as 120 mM NiCl₂ (5 µl) just before measurements.

The duration of exposure to temperature, sample volumes during exposure, and the methods and parameters of water-bath and sample temperature monitoring were the same as those described above for CL.

All calcium results are reported as the ratio of fluorescence intensities for calcium-bound to calcium-free Fura-2AM (I_{+Ca}/I_{-Ca}), which changes directly with absolute Ca²⁺ cytosol concentration. In contrast to absolute Ca²⁺ cytosol concentration calculations, I_{+Ca}/I_{-Ca} (R_i) is almost independent of physicochemical factors²⁸. Thus, using R_i seems advantageous under the present experimental conditions, where changes in sample temperature could influence their physicochemical properties. As in the CL assessment, 3 parameters of cytosolic Ca²⁺ level (R_i) in neutrophils suspended in both Ca²⁺-rich and Ca²⁺-low medium were taken into account, i. e. resting level

(resting R_i), peak value (peak R_i), and the area under the R_i -time curve for the total measurement time (area R_i).

Influence of p38MAPK α -specific inhibition on primed neutrophil ROS production

SB 203580, a specific inhibitor of p38MAPK α in human neutrophils^{7, 13}, was used to determine the role of the kinase in the priming of ROS production observed for the preincubation temperature of 38°C. In this respect, the inhibitory concentration of SB 203580 decreasing chemiluminescence by 50% (IC_{50}) of samples preincubated at 38°C (priming temperature) and 37°C (reference temperature) was established.

Samples containing whole blood and MS were preincubated at 38°C and at the reference temperature without SB 203580 (control samples) or with the inhibitor present in 1 of 3 concentrations (1, 10, or 100 μ M). Timing and conditions of temperature exposure as well as its monitoring were the same as described earlier. Details of sample composition are presented in the Table 1.

CL of all samples preincubated at the same temperature, i.e. 38 or 37°C, were measured simultaneously in a Wallac 1420 Multilabel Counter multi-channel luminometer (Perkin-Elmer Life Sciences, USA), so that 4 channels were used each time. The luminometer was controlled by a PC equipped with original Wallac 1420 Manager, version 2 software. Each sample had a volume of 284 μ l during measurement and contained 3 μ l of whole blood (Table 1). The measurements were performed at 37°C and CL was measured every 12 s for 1 s. The total measurement time approximated 12 min. At 60 s of measurement, 10^{-5} M FMLP or 37 ng/ml PMA was added.

Finally, the concentration of SB 203580 decreasing CL by 50% (IC_{50}) was determined at both 38 and 37°C on the basis of the SB 203580 inhibitory index calculated from peak CL using the formula:

$$X_{inh} = 100\% (CL_{max+inh} / CL_{max})$$

where $CL_{max+inh}$ is the peak CL of samples preincubated with inhibitor and CL_{max} the peak CL of control samples (without inhibitor).

Influence of temperature on neutrophil membrane fluidity

Exposure of neutrophils to elevated temperature may change their membrane fluidity, and this may persist during some period after exposure. Therefore, the membrane fluidity of the neutrophils exposed to the highest tested temperature, 43°C, was examined when 10 min of exposure at 43°C had elapsed. Thus the timing of the membrane fluidity measurements was the same as that of the other measurements, i.e. ROS production and cytosolic Ca^{2+} level measurements.

Freshly isolated cells were suspended in PBS without calcium (2×10^6 cells per 1 ml). Membrane fluidity was measured with the DPH fluorescence anisotropy method³³. Briefly, the neutrophils were labeled with 2 μ M DPH for 30 min at 37°C in a water-bath. Then, DPH-labeled cells were exposed to 43°C for 15 min and for next 10 min to 37°C in, respectively, the water-bath and the thermally equilibrated holder of the LS-50 spectrofluorometer equipped with polarizers (Perkin-Elmer, Norwalk, CT, USA) where DPH fluorescence measurements were next performed. Excitation and emission wavelengths were 365 and 430 nm, respectively. Control samples were subjected after DPH loading to 37°C for a total preincubation period of 25 min.

Statistical analysis

Both the effects of temperature on calcium cytosol levels as well as on CL were assessed statistically using the Wilcoxon test. In any case of statistically significant difference compared with control temperature, the Spearman rank test was performed to evaluate the correlation between changes in CL (experiments with FMLP as stimulator) and that in Ca^{2+} cytosol levels. In Spearman analysis, changes in CL and Ca^{2+} levels were presented as the quotients of

Table 1. Composition of samples in experiments with SB 203580, a specific inhibitor of p38MAPK α in neutrophils

Final concentration of SB 203580 (μ M)	MS (μ l)	Whole-blood (μ l)	Solution of SB 203580 in DMSO (μ l)	DMSO (μ l)	Volume taken for measurement (μ l)
0	1124	12	0	5.0	284
1	1124	12	0.5 ^a	4.5	284
10	1124	12	5.0 ^a	0	284
100	1011	120	4.5 ^b	0	285 ^c

^a 2.5 μ M SB 203580, ^b 25 μ M SB 203580, ^c diluted with MS to 284 μ l before measurement.

the value for the examined temperature and the value for the reference (control) at 37°C. The Wilcoxon test was also used for statistical analysis of membrane fluidity measurement results. The significance threshold for all statistics was set at $p < 0.05$.

RESULTS

ROS production measurements

Neutrophils preincubated at any temperature responded to both fMLP and PMA with an increase in CL (Fig. 1). Resting CL data were pooled from experiments with fMLP and PMA and analyzed together. Resting CL for the preincubation temperature of 38°C was higher by $27.17\% \pm 12.79\%$ as compared with the reference temperature of 37°C ($p = 0.046$, mean $\pm$ SE, $n = 12$). No difference in resting CL was observed at 39 and 40°C where the parameter was $109.67\% \pm 13.48\%$ and $83.33\% \pm 11.01\%$, respec-

tively, as compared to that for reference samples ($p > 0.05$, mean $\pm$ SE, $n = 12$). Preincubation at 43°C resulted in resting CL decrease to $67.05\% \pm 9.06\%$ of resting CL for 37°C ($p = 0.046$, mean $\pm$ SE, $n = 12$). Peak and integrate CL at 38°C after fMLP stimulation was as high as $150.00\% \pm 24.28\%$ and $149.33\% \pm 22.28\%$, of these values at 37°C, respectively ($p = 0.027$ for both parameters, mean $\pm$ SE, $n = 6$). There was no difference in fMLP-stimulated CL at 39°C compared with the corresponding control samples ($104.00\% \pm 14.39\%$ and $102.50\% \pm 12.67\%$ of control samples CL for peak and integrate CL, respectively; $p > 0.05$, mean $\pm$ SE, $n = 6$). Although not statistically significant, peak and integral CL parameters both tended to be lower for samples preincubated at 40°C than those preincubated at the control temperature ($94.00\% \pm 9.53\%$ and $94.00\% \pm 9.58\%$ for peak and integral CL, respectively; $p > 0.05$, mean $\pm$ SE, $n = 6$). Peak CL and integral CL at 43°C were significantly decreased and they were $82.05\% \pm 14.09\%$ and

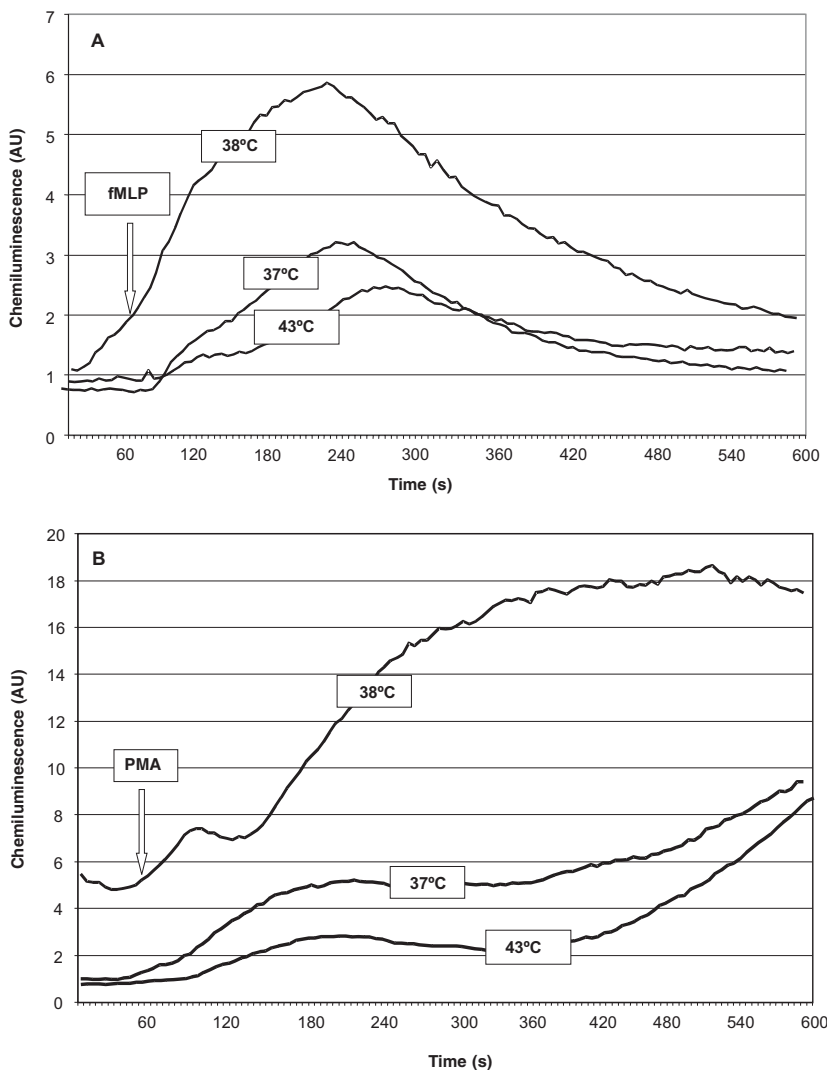


Figure 1. Representative tracings of CL after: **A** – stimulation with fMLP (10^{-5} M) obtained for priming (38°C), inhibiting (43°C), and reference (37°C) preincubation temperatures. The arrow indicates the time of adding fMLP; **B** – stimulation with PMA (37 ng/ml) obtained for priming (38°C), inhibiting (43°C), and reference (37°C) preincubation temperatures. The arrow indicates time of adding PMA.

76.00%±11.49%, respectively, of corresponding values at 37°C ($p=0.046$ for both, mean ±SE, $n=6$).

Similarly to experiments with fMLP, only preincubation at 38°C enhanced response to PMA stimulation comparing to control, increasing peak and integrate CL by 13.60%±5.23% and 13.02%±5.81%, respectively ($p=0.046$, mean ±SE, $n=6$). Non significant decrease in both parameters as compared to reference temperature was observed after PMA stimulation for preincubation at 39°C (83.60%±11.93% and 79.70%±7.40% for peak and integrate CL, respectively; $p>0.05$, mean ±SE, $n=6$) and 40°C (90.52%±10.98% and 86.52%±11.73% for peak and integrate CL, respectively; $p>0.05$, mean ±SE, $n=6$). Likewise in experiments with fMLP as stimulator, 43°C inhibited CL in response to PMA comparing to that for 37°C. Namely, peak and integrate CL for 43°C were only 75.77%±6.78% and 69.70%±7.42% of peak and integrate CL for 37°C, respectively ($p=0.028$ for both peak and integrate CL, mean ±SE, $n=6$).

Samples subjected to 38°C revealed non-significant brisker response to stimulation both with fMLP and PMA (Fig. 1; detailed data not shown).

In summary, the only temperature that proved to exert a priming effect compared with 37°C was 38°C, while 43°C seemed to be inhibitory.

Ca²⁺ cytosol level

In all samples, normal response to fMLP stimulation was observed. There were no differences in calcium levels with respect to any of the evaluated parameters between the control temperature and values for 38 or 43°C (data not shown). No difference was observed in the peak and resting values for 39°C (data not shown), while there was a statistically significant, although small, increase in the R_i area in Ca²⁺-rich medium at this preincubation temperature (102.00%±0.81% as compared to reference temperature; $p=0.046$, mean ±SE, $n=6$). Resting and area under curve values were statistically higher in neutrophils preincubated at 40°C in Ca²⁺-rich medium (105.83%±1.35%, $p=0.043$ and 103.50%±1.54%, $p=0.046$ for resting and area R_i , respectively, comparing to corresponding values for 37°C; mean ±SE, $n=6$). All remaining parameters for 40°C did not differ statistically as compared with controls (data not shown). The blockade of plasma membrane calcium channels with Ni²⁺ attenuated the increase both in resting R_i and integral R_i values for the preincubation temperature of 40°C compared with the control temperature (1.43±0.10 vs. 1.42±0.10 and 710.06±49.03 vs. 700.45±42.51, respectively resting R_i and area R_i

for 40 and 37°C in 1 mM Ni²⁺-containing medium; mean ±SD, $n=6$).

Correlation between changes in Ca²⁺ cytosol level and changes in CL

The only correlation observed was a strong positive correlation between changes in resting R_i (Ca²⁺-rich medium) and non-significant changes in resting CL (resting CL data only from experiments with fMLP) for 40°C ($r=0.94$, $p=0.007$). Changes in both parameters were assessed using the quotients of values for 40 and 37°C (see statistical analysis part). Because the quotients were all greater than 1.00 for calcium levels (1.05±0.03, mean ±SD) and generally lower than 1.00 for CL (0.83±0.27, mean ±SD), the positive correlation between the changes means the higher increase in calcium compensates for the lower decrease in CL.

Influence of p38MAPK α -specific inhibition on primed neutrophil ROS production

Figure 2 shows the influence of different concentrations (1, 10 and 100 μ M) of SB 203580 on fMLP- and PMA-stimulated CL. The inhibitor decreased CL for both stimulators in dose-dependent manner. However, PMA-stimulated CL was much less sensitive to inhibition of p38MAPK α comparing to that induced by fMLP ($p=0.041$, Mann-Whitney test). The concentration of SB 203580 decreasing CL by 50% (IC₅₀) was less than 1 μ M for 38°C and approximately 16 μ M for 37°C after stimulation with fMLP. The corresponding IC₅₀ values were near 100 μ M and much above 100 μ M after stimulation with PMA.

Membrane fluidity of neutrophils exposed to 43°C

No difference in the membrane fluidity of cells exposed to 43°C (0.297±0.009; mean ±SD, $n=6$) with that at 37°C (0.298±0.011; mean ±SD, $n=6$) was detected at 10 min of temperature exposure.

DISCUSSION

The present study revealed a priming effect at a temperature of 38°C on ROS generation by human neutrophils. This is indicated by increased peak and integral CL in response to fMLP and PMA stimulation compared with the control temperature. Resting CL values also proved to be higher for cells preincubated at 38°C. It is known from comparative studies of neutrophil isolation procedures that techniques employing room temperatures or higher enhance the density of fMLP receptors on neutrophils³². Hence it is possible that the priming phenomenon exerted by

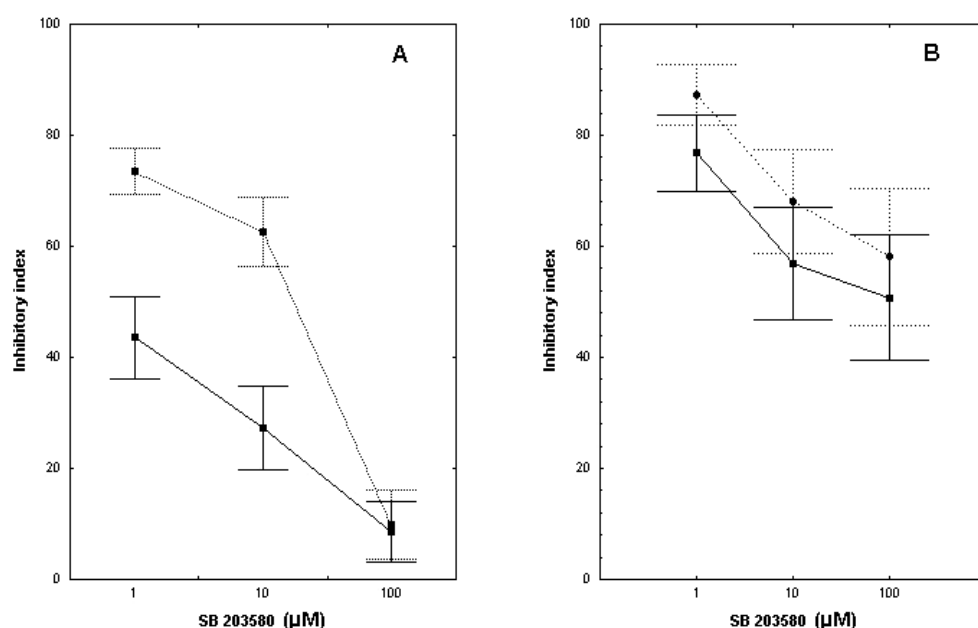


Figure 2. Influence of SB 203580 on chemiluminescence after stimulation with 10^{-5} M fMLP (A) and 37 ng/ml PMA (B); mean $\pm$ SE, $n=6$; dotted line: 37°C, solid line: 38°C. Inhibitory index = 100% ($CL_{\max+inh}/CL_{\max}$), where $CL_{\max+inh}$ is peak chemiluminescence in the presence of inhibitor, $CL_{\max}$ the peak chemiluminescence without inhibitor.

38°C in regard to fMLP stimulation is dependent on upregulation of membrane fMLP-receptors. However, this cannot explain the priming of PMA stimulation, a ligand for conventional protein kinase C. Therefore, another mechanism must account for the priming phenomenon. Because priming is considered to result mainly from changes in intracellular signal transduction pathway activity⁶, and probably all structural features of primed cells, including altered receptor expression, are secondary to these, we decided to explore some elements of the signal-transducing pathways in neutrophils subjected to temperature. One of them was cytosolic calcium level ($[Ca^{2+}]_{\text{cyt}}$). Increase in $[Ca^{2+}]_{\text{cyt}}$ is one of the earliest events transducing signal from fMLP plasma membrane receptors into the cell interior. Ca^{2+} is released in response to cell stimulation mainly from intracellular stores and, to a minor degree, originates from extracellular Ca^{2+} sources, especially at the beginning of the calcium increase¹. Altered $[Ca^{2+}]_{\text{cyt}}$ seems, based on previous studies by others, to be involved in the priming of neutrophils. It was shown that the LPS- and IL-8-induced primed state of neutrophils is related with increased resting $[Ca^{2+}]_{\text{cyt}}$ as well as increased calcium response to stimulation³⁹.

In our experiments, measurements of $[Ca^{2+}]_{\text{cyt}}$ were performed parallel to fMLP-stimulated CL examination. They showed changes in neither Ca^{2+} mobilization to the cytosol after stimulation with fMLP nor resting cytosolic Ca^{2+} concentration in neutrophils preincubated at 38°C. Hence, the priming effect of 38°C cannot be attributed to the increase in $[Ca^{2+}]_{\text{cyt}}$. Instead, we hypothesize that the possible mechanism of the observed temperature-enhanced oxidative

response to stimulation is the increased background activity of p38MAPK α , also called p38 stress-activated protein kinase 2a. p38MAPK α is one of the cascade kinases activated following binding of fMLP to its plasma membrane receptors^{20, 30, 31, 40}. p38MAPK α activity leads finally to phosphorylation of the crucial NADPH oxidase components which, in turn, leads to activation of the enzyme and results in a rapid increase in ROS generation¹¹. The kinase, in accord with its other name, can be activated by an array of environmental stresses, probably including the increase in the temperature of the environment which is the case in the present study (examined temperature compared with the control of 37°C). This activation differs in kinetics from that caused by fMLP. The increase in activity of p38MAPK α under stress is slow and peaks after 30–60 min of exposure to the stress factor. Moreover, it lasts longer (at least 120 min) and vanishes slowly following stress withdrawal³¹. This pattern of increased activity resembles that induced by TNF- α , which is known to facilitate response to fMLP in a p38MAPK-dependent manner^{12, 36}. Hence, temperature-induced changes in p38MAPK α activity may be a basis for priming toward ROS generation by neutrophils.

To test this hypothesis we used SB 203580. The agent inhibits p38 α and p38 β isoforms and, in concentrations exceeding 3 μ M, was also shown to downregulate c-Jun-N-terminal kinase and phosphatidylinositol-dependent kinase 1^{5, 22}. Therefore, concentrations of SB 203580 below 3 μ M specifically inhibit p38 α in human neutrophils. As we reported, the IC_{50} of SB 203580 lower than 1 μ M for fMLP-stimulated CL of neutrophils preincubated at 38°C strongly

suggests a high dependence of respiratory burst activation in the cells on p38MAPK α . In contrast, fMLP-induced CL of neutrophils preincubated at the reference temperature was less sensitive to p38MAPK α inhibition ($IC_{50} \approx 16 \mu M$; Fig. 2). The small effects of SB 203580 in the relevant concentrations on PMA-stimulated CL prove that the observed decreases in CL upon fMLP stimulation do not result from the inhibitor toxicity. On the other hand, an inhibition of PMA-induced respiratory burst with SB 203580 is not normally observed in resting (unprimed) neutrophils⁴⁰. However, the susceptibility to SB 203580 of neutrophil ROS production triggered by PMA was reported earlier by others and it seems to be a characteristic feature of primed neutrophils⁴. Thus, the priming effect of 38°C towards neutrophils seems to be dependent on p38MAPK α activation. However, direct measurement of p38MAPK α activity in neutrophils exposed to a temperature of 38°C must be performed in future to fully test the hypothesis.

The higher resting CL at 38 than at 37°C reported in the present study seems to confirm the hypothesis on elevated background p38MAPK α activity which can result in increased ROS production in resting cells. The elevated background p38MAPK α activity results in enhanced exocytosis of specific granules of the neutrophil and thus leads to increased expression of cytochrom *b*₅₅₈, a core NADPH oxidase component, in the plasma membrane³⁴. This increased plasma membrane expression of the cytochrome can be a molecular basis for enhanced resting CL. It also can be responsible for the increase in CL upon stimulation with PMA we observed, which stimulates neutrophil ROS production via pathways independent of p38MAPK α . The increased amounts of the cytochrome in plasma membrane is a molecular background for enhanced respiratory burst triggered by a variety of stimulators, regardless of their downstream signaling molecules³⁴.

Surprisingly, the experimental protocol we used did not reveal a priming effect at temperatures which seem to pose more stress on neutrophils than 38°C, i.e. the temperatures 39, 40, and 43°C. Moreover, inhibitory effects were observed in the case of 43°C, i.e. there was an even smaller response to stimulation both with fMLP and PMA. Also, lower resting CL values were observed for 43°C. The possible explanation for the lack of increase in ROS generation after exposure to temperatures higher than 38°C is that those temperatures simultaneously increase p38MAPK activity and impair the function of one or

more other signaling molecules. Another reason for the lack of increase in CL after exposure to high temperatures could be an increased fluidity of the plasma membrane, where NADPH oxidase is localized. Higher fluidity of the plasma membrane is potentially able to disturb the assembly of the oxidase components at the membrane necessary for its activation² and is thus able to diminish ROS generation by the enzyme. Indeed, a change in plasma membrane properties seems to be the case for 39 and 40°C. This is suggested by the increased area under the $[Ca^{2+}]_{cyt}$ curve for neutrophils suspended in Ca^{2+} -rich medium preincubated at those two temperatures, as shown in our study. This finding, together with the concomitant lack of change in $[Ca^{2+}]_{cyt}$ upon plasma membrane calcium channels blockade, indicates increased plasma membrane permeability. If the increase in permeability were connected to an increase in plasma fluidity, then the latter finding could explain the relatively weaker CL values at 40 than at 39°C. However, no change in plasma membrane fluidity was detected for the highest preincubation temperature tested, making it rather improbable that lower temperatures can affect the parameter.

The negative correlation reported in this study between the increase in resting $[Ca^{2+}]_{cyt}$ in neutrophils suspended in Ca^{2+} -rich medium preincubated at 40°C and the decrease in resting CL of corresponding samples indicates that the highly temperature-dependent decrease in CL can at least be partially overcome. This effect could not result from $[Ca^{2+}]_{cyt}$ changes because in our experiments the CL measurements were performed in media containing calcium at extremely low concentration (less than 100 nM). Hence, some other factors accompanying the increased plasma membrane permeability, for which the above change in $[Ca^{2+}]_{cyt}$ seems to be a marker, must be a reason.

In conclusion, we have shown in the present study that elevated temperature can prime human neutrophils in a Ca^{2+} -independent mechanism. Alternatively, our results indicate p38MAPK α activation as the most probable mechanism of temperature-induced priming.

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