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HEAT SHOCK INHIBITS NADPH OXIDASE IN HUMAN NEUTROPHILS

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SUMMARY: The heat shock response is a conserved, physiological, transient cellular response to injury. Several studies have suggested a link between the heat shock response and oxidative injury. We have investigated the effects of heat shock on superoxide anion generation by human neutrophils stimulated with opsonized zymosan or phorbol myristate acetate. Human neutrophils exposed to elevated temperatures or to the heavy metal cadmium synthetized a variety of heat shock proteins. In parallel to this protein synthesis, we observed a selective, reversible and temperature-dependent inhibition of NADPH oxidase activation, which was independent from variations of cytosolic pH or thiol group oxidation. Inhibition of NADPH oxidase by heat shock appeared related to the synthesis of heat shock proteins and may represent an intrinsic cellular mechanism to down regulate superoxide production.

INTRODUCTION: Exposure to elevated temperatures induces in cells and tissues a physiological response called the heat shock (HS) response which consists in transient induction of transcription and synthesis of a specific set of proteins called the heat shock proteins (HSPs), together with inhibition of normal protein synthesis (for review: 1,2). Although the precise functions of HSPs are not yet fully understood, their presumed role is to protect cells from injury. Several lines of evidence suggest a close link between the HS response and oxidative injury: hydrogen peroxide induces a HS response in human monocytes *in vitro* (3) and reperfusion injury induces the synthesis of HSPs *in vivo* (4); bacteria resistant to oxidative injury spontaneously overproduce HSPs (5) and HS protects the human monocytic line U937 from oxidative injury (6).

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Abbreviations: AA: arachidonic acid; DTT: dithiotreitol; HS: heat shock; HSPs: heat shock proteins; OZ: opsonized zymosan; PAGE: polyacrylamide gel electrophoresis; PGE2: prostaglandin E2; PMA: phorbol myristate acetate; SOD: superoxide dismutase, SDS: sodium dodecyl sulfate.

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Stimulation of human neutrophils induces generation of superoxide anions (O_2^-) and other reactive oxygen species. This respiratory burst involves the activation of the complex electron transport system, NADPH oxidase, which reduces molecular oxygen to O_2^- at the expense of NADPH. Much effort is being directed toward defining the complex mechanisms and regulatory steps of activation and inactivation of NADPH oxidase (7,8) since those represent a potential target for pharmacological control of the enzyme.

Cytoplasts and cytokineplasts are membrane-bound, anucleate, granule-poor cytoplasmic fragments generated from neutrophils (9). Cytoplasts are formed by high-speed centrifugation of cytochalasin B-treated neutrophils through Ficoll gradients and are able to generate O_2^- when stimulated. In contrast, cytokineplasts, which are prepared by brief exposure of adherent neutrophils to heat, do not generate O_2^-. The effects of HS on the production of reactive oxygen species by whole phagocytes have however not yet been studied. We investigated the effects of HS on the generation of O_2^- by human neutrophils. A soluble and a particulate stimulus, respectively phorbol myristate acetate (PMA) and opsonized zymosan (OZ), have been described to induce the activation of NADPH oxidase through distinct pathways (7). We report here that preexposure to temperatures inducing the synthesis of HSPs inhibited the induction of O_2^- generation by both PMA and OZ, whereas other neutrophil functions were unaffected by HS. Cadmium, at concentrations inducing the synthesis of HSPs, also inhibited NADPH oxidase. The experiments presented suggest that the inhibition of NADPH oxidase by HS is a specific event, independent from thiol group oxidation or variations in cytosolic pH and related to the synthesis of HSPs.

MATERIALS AND METHODS

Cells and HS: Neutrophils prepared from venous blood of healthy donors as described (7) were resuspended in MEM (Eurobio, Paris) supplemented with 25 mM Hepes and 0.5 mM $MgCl_2$, pH 7.4 and were incubated for 20 min at temperatures ranging from 37 to 47°C as previously described in detail for human monocytes (3). After HS the cells were allowed to recover at 37°C for 15 or 150 min, then stimulated with PMA (100 ng/ml) or OZ (3 mg/ml) (7). In some experiments, the K^+/H^+ ionophore nigericin (Sigma Chemical Co., St Louis, MO), the thiol protecting agent dithiotreitol (DTT) (Sigma), or cycloheximide (Sigma), were added to the cell suspensions at 37°C, 5 to 15 min before the beginning of the experiment (none of these molecules modified O_2^- generation in control experiments). In other experiments, neutrophils were exposed for 30 to 180 min to cadmium (1 to 200 µM) (Sigma) before measurement of O_2^- production.

Measurement of superoxide production: Generation of O_2^- by human neutrophils was measured by the SOD-inhibitable reduction of ferricytochrome C (7).

NAPDH oxidase activity was assessed in a membranous preparation as previously described (10). Briefly, the cells were stimulated in MEM by 1 µg/ml PMA for 5 min at 37°C, sonicated and the membrane fraction pelleted by centrifugation. The enriched NADPH oxidase preparation was resuspended adjusting the volume to obtain 10^6 cell equivalent/ml and the O_2^- generating capacity assayed.

Labeling and electrophoresis: After HS or exposure to cadmium, neutrophils were washed twice with phosphate buffered saline (Gibco, Paisley, Scotland), then resuspended in RPMI 1640 (Gibco) without methionine and without serum and labeled at 37°C for 90 min with 6 µCi (^{35}S)-methionine/ml (Amersham, Buckinghamshire, England). Cells were centrifuged, precipitated in 30% TCA at 4°C for 1 hour, washed three times, lysed in sodium dodecylsulfate (SDS) sample buffer, and proteins resolved using SDS polyacrylamide gel electrophoresis (PAGE) in slab gels with 10% polyacrylamide as described in ref. 3.

Measurement of prostaglandin E2 (PGE2) synthesis: Radioimmunoassay of PGE2 was performed according to Sors et al. (11) in supernatants collected after stimulation of neutrophils ($2x10^6$ cells/ml) with OZ for 20 min at 37°C. The cross-reactivity of the anti-serum with other prostaglandins is below 0.2%.

RESULTS AND DISCUSSION

When neutrophils were exposed to elevated temperatures for 20 min, then allowed to reequilibrate at 37°C for 15 min, generation of O_2^- was inhibited in a temperature-dependent manner (Fig 1). Inhibition of NADPH oxidase by HS required exposure for at least 10 min which was not explained by the time necessary for equilibration of temperature since, in these experiments, neutrophils were resuspended in prewarmed media. HS did not, by itself, induce O_2^- generation by neutrophils (data not shown). The inhibition of O_2^- production by HS was identical in cells washed immediatly after HS indicating that it was not related to a soluble factor(s).

Inhibition of NADPH oxidase by HS was not the result of non specific cellular damage, since i) more than 98% of neutrophils excluded trypan blue under all experimental conditions; ii) incorporation of (^{35}S)-methionine into proteins was not decreased at temperatures inducing a 50-75% inhibition of NADPH oxidase (Fig 2); iii) preexposure of neutrophils to 43°C did not alter the rapid and reversible rise in cytosolic free Ca^{2+} induced upon stimulation with the chemoattractant peptide f-met-leu-phe, in both Ca^{2+}-containing and Ca^{2+}-free medium (as assessed by Quin-2

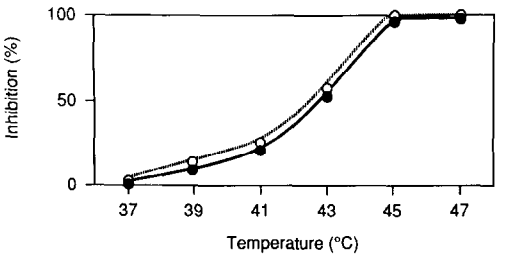

Figure 1
Temperature-dependance of NADPH oxidase inhibition. Neutrophils were exposed to the indicated temperatures for 20 min, allowed to recover at 37°C for 15 min and stimulated with PMA (o---o) (100 ng/ml) or OZ (●——●) (3 mg/ml) for 5 min. Values are expressed as % inhibition of controls (neutrophils maintained at 37°C), and represent means of duplicates from two separate experiments.

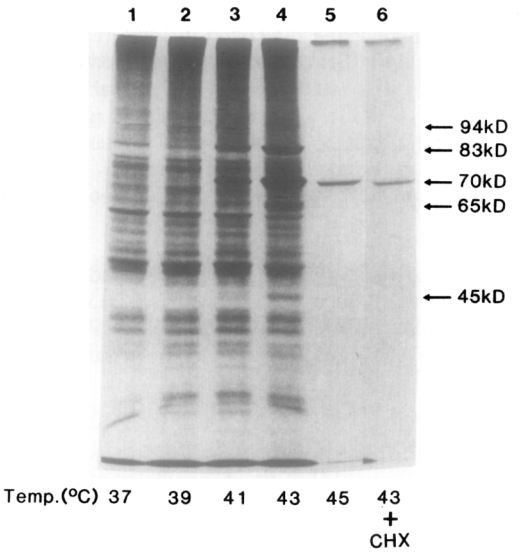

Figure 2
Effects of elevated temperatures on protein synthesis by neutrophils. Human neutrophils ($2x10^7$ cells/ml in 10 ml serum-free and methionine-free DMEM) were incubated for 20 min at 37°C (control cells, lane 1), 39°C (lane 2), 41°C (lane 3), 43°C (lane 4), 45°C (lane 5), then labeled with (^{35}S)-methionine as described in "Methods". Lane 6: cells incubated with cycloheximide (CHX), 10 µg/ml, for 15 min before incubation at 43°C, compare to lane 4. SDS-PAGE with 10% polyacrylamide and autoradiography (2 day exposure). Aliquots corresponding to equal numbers of cells were loaded in each lane.

fluorescence, B.S. Polla, D. Pittet, unpublished data); iv) we tested the activity of another membrane associated enzyme, phospholipase A2 by measuring the release of (^{3}H)-AA previously incorporated in cell membranes as described elsewhere (7). Even after exposure to 45°C, a temperature which completely inhibited O_2^- generation, the release of (^{3}H)-AA induced by OZ was still 73% of control. Moreover, PGE2 production by neutrophils stimulated with OZ was not affected by HS : 597.3 ± 335.1 pg PGE2/ml in control cells vs 596.3 ± 298.2 in cells exposed to 43°C (means ± SD, n=3). Finally, the lack of cellular damage was also reflected by the recovery of NADPH oxidase activity 150 min after HS (Table 1). Neutrophils recovered the ability to generate O_2^- even in presence of the protein synthesis inhibitor cycloheximide which abolished the translation of normal protein synthesis (see Fig 2, lane 6). O_2^- generation upon PMA stimulation 15 min after HS (43°C) was inhibited by 44.1%; 150 min after HS, generation of O_2^- was inhibited by 6.8% in absence or by 11.3% in presence of 10 µg/ml cycloheximide added to the cell suspension before HS (n=3). This suggests that the recovery of NADPH oxidase activity after HS did not depend on protein synthesis.

Stimulation of intact neutrophils by either PMA or OZ renders the "silent" NADPH oxidase active, probably through the assembly in the membrane of the different constituents of this respiratory burst enzyme (10). To determine the effects of heat shock on the direct expression of NADPH oxidase, the cells

Table 1

Inhibition and recovery of superoxide generation after HS

	O_2^- (nmol./min/10^6 cells)		
	37°C (control)	43°C	p
A- Recovery 15 min (n=12)			
OZ	4.59 ± 0.44	1.78 ± 0.35	< 0.001
PMA	8.98 ± 1.32	3.90 ± 1.02	< 0.001
B- Recovery 150 min (n=6)			
OZ	4.40 ± 0.63	2.48 ± 0.75*	< 0.02
PMA	9.06 ± 0.88	6.44 ± 1.56**	NS

Neutrophils were exposed to 43°C for 20 min as described in "Methods" and allowed to recover at 37°C for 15 (A) or 150 (B) min before stimulation with OZ (3 mg/ml) or PMA (100 ng/ml). The results are expressed as means ± SD of 12 or 6 experiments. The paired Student's t test was used for statistical analysis.
* $p<0.05$ and ** $p<0.001$ when compared to cells allowed to recover for only 15 min after HS in the same experiment.

were heated, stimulated with PMA and the O_2^--generating capacity was assessed in membrane preparation (10). O_2^- generation by membranes isolated from control cells was 4.88 ± 1.99 nmol./min/10^7 cell equivalent and 1.28 ± 1.63 in membranes isolated from cells exposed to 43°C (means ± SD, n=3). These results indicated that NADPH oxidase was affected by HS; we could however not distinguish direct inhibition of the enzyme (by a factor which was not removed during membrane preparation) from inhibition of a step of the complex NADPH oxidase assembly and activation cascade. Since inhibition of NADPH oxidase was also observed in isolated membranes, it is unlikely that HS acted by increasing SOD activity in intact cells.

Since HS inhibited O_2^- generation initiated by both PMA and OZ, HS may act on an activation step of NADPH oxidase common to these agonists. The Na^+/H^+ antiporter has been described as a regulator of intracellular pH that controls signal transduction proximal to NADPH oxidase expression: when the Na^+/H^+ antiporter is inhibited, O_2^- generation induced by PMA or OZ is decreased (12,13). Under these conditions, addition of the K^+/H^+ ionophore nigericin, by restoring proton secretion can restore the full expression of the oxidase (12,13). Nigericin, used at a concentration which totally equilibrates intracellular to extracellular pH (1 µg/ml), did not reverse the inhibition of O_2^- generation after HS, indicating that regulatory processes for proton secretion were not affected by HS. Another possible transducing mechanism for the HS signal is oxidation

stress (1,2) and oxidation of thiol groups has been shown to decrease the generation of O_2^- (14). Membrane oxidation could therefore provide a link between HS and inhibition of O_2^- generation. In order to address this issue, neutrophils were preincubated with 0.25 mM DTT for 10 min before HS, a concentration reported to restore cell function altered by oxidative stress (15). After HS, cells were washed twice at 37°C in order to remove DTT which would interfere with cytochrome c reduction. DTT however did not modify the inhibition caused by HS, indicating, together with the conservation of PGE_2 production, that thiol group oxidation was not involved in the inhibition of NADPH oxidase by HS.

We then investigated the possibility that inhibition of NADPH oxidase by HS was related to the synthesis of HSPs, and protein synthesis was assessed in neutrophils after exposure to a range of temperatures (Fig 2). Cells briefly exposed to 41°C synthetized the major 70 kD and 83 kD HSPs (Fig 2, lane 3) (1,2,16). After exposure to 43°C, neutrophils also synthetized other HSPs (Fig 2, lane 4), among which a so-called minor 48 kD HSP (1) and a 60-65 kD HSP which probably corresponds to the recently characterized 60 kD human HSP (17). The post transcriptional inhibition of normal protein synthesis associated with the HS response was obvious only at 45°C (Fig 2, lane 5). Only temperatures inducing the synthesis of HSPs (41°C) (Fig 2) inhibited O_2^- generation (Fig 1) by 20% or more upon stimulation with either PMA or OZ. Since cycloheximide did not inhibit the synthesis of HSP 70 by neutrophils (Fig 2, lane 6) unless it was toxic, we could not use this experimental approach to establish a closer relationship between HSPs and NADPH oxidase inhibition. We therefore used another classical inducer of HSPs, the heavy metal cadmium (1,2). Preincubation with cadmium also inhibited O_2^- generation by human neutrophils. In five separate experiments, we observed a parallelism between the synthesis of HSPs and inhibition of NADPH oxidase: only concentrations of cadmium inducing the synthesis of HSPs inhibited NADPH oxidase (Fig 3). A relationship between synthesis of HSPs and inhibition of NADPH oxidase was further supported by comparing neutrophils and monocytes. In the human monocytic line U937 differentiated with 1,25-dihydroxyvitamin D3, synthesis of HSPs is not detected at temperatures below 45°C (3), which also was, in these cells, the minimal temperature required to inhibit O_2^- generation by 20% or more (unpublished data).

Our results suggest a close link between induction of a HS response and the transient inhibition of NADPH oxidase. They also provide a possible explanation for the lack of O_2^- generation by cytokineplasts. Additional experiments are in progress in order to distinguish a direct interaction of HSPs with the oxidase from inhibition of a step of the complex NADPH oxidase activation cascade common to PMA and OZ.
The function of HSPs presumably is to protect cells from injury (3,6).

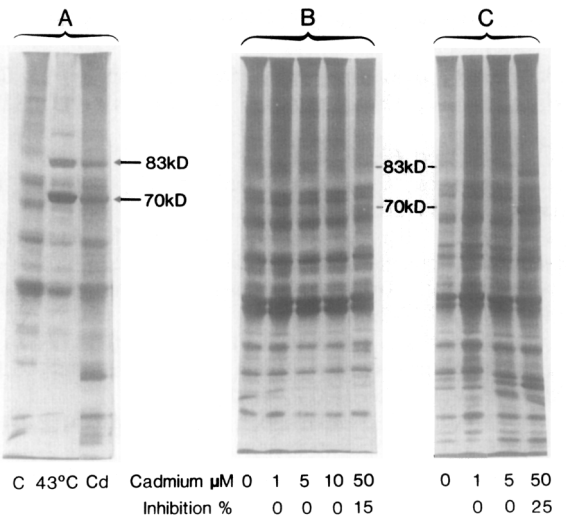

Figure 3
Effects of cadmium on protein synthesis and superoxide generation by neutrophils.
A- Neutrophils were maintained at 37°C (control, C), or exposed for 20 min at 43°C (43°C), or for 1 hour to 100 µM cadmium (Cd), then labeled as described in "Methods". Exposure to 100 uM cadmium induced a similar synthesis of HSPs as did exposure to 43°C, and inhibited generation of O_2^- as well, from 50 to 80%. At this concentration, there also was a decrease in normal protein synthesis.
B and C- Neutrophils were exposed for 1 hour (B) or for 3 hours (C) to increasing concentrations of cadmium. Up to 10 µM, there was neither induction of HSPs nor inhibition of O_2^- generation. Synthesis of the 70 kD and 83 kD HSPs were detectable after 1 hour and obvious after 3 hour exposure to 50 µM cadmium. At these concentrations there was no decrease in incorporation of (^{35}S)-methionine into proteins. Synthesis of HSPs was associated with inhibition of PMA-induced O_2^- generation (expressed as % of control incubated without cadmium). Similar results were obtained upon OZ stimulation.

Inhibition of NADPH oxidase by HS may represent an intrinsic cellular mechanism to down regulate oxygen free radical generation by phagocytes.

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