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Production of Hydrogen Peroxide by Peritoneal Macrophages from Rats Exposed to Subacute and Chronic Hypoxia

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Summary

Hydrogen peroxide production was measured in non-elicited rat peritoneal macrophages using luminol-dependent chemiluminescence (LDCL). Isolated cells were activated by a chemotactic peptide (FMLP) or by a phorbol ester (PMA) or by the combination of both. A hundred-fold higher LDCL intensity was achieved with PMA relative to FMLP. However, when FMLP was added subsequently to PMA it produced approximately the same response as did PMA. These measurements were carried out with cells isolated from controls and from animals exposed to normobaric hypoxia (10 % O_2) for 3 hours, 3 days, or 21 days. Hypoxia had a dual effect. Acutely (within 3 hours) it attenuated the production of hydrogen peroxide triggered by PMA, whilst during longer exposure (3 or 21 days) it increased the response induced by FMLP. Hypoxia can thus modulate the capacity of respiratory burst in peritoneal macrophages.

Key words
Chemiluminescence – Luminol – Respiratory burst – Peritoneal macrophages – Hydrogen peroxide

Introduction

Macrophages play an important role in the immune system with respect to their involvement in antigen presentation, in stimulation of lymphocytes, and in phagocytosis and production of reactive oxygen species (ROS). The latter substances comprising mainly superoxide, hydrogen peroxide and the hydroxyl radical originate from a metabolic pathway called respiratory burst. Central to this pathway is the activity of an enzyme NADPH oxidase which is activated during phagocytosis (Maly and Schürer-Maly 1995), and is responsible for the respiratory burst – extramitochondrial oxygen reduction. Besides activation of receptors that mediate phagocytosis such as F_c and C_{3b} receptors, the respiratory burst can be initiated by a wide range of stimuli. These stimuli include N-formyl-methionyl-leucyl-phenylalanine (FMLP) which binds to the receptor for the microbial protein located on the cell surface, and phorbol myristate acetate (PMA) activating cytoplasmic protein kinase C (Alvarez *et al.* 1993).

Since oxygen represents the vital substrate for production of ROS by NADPH oxidase, hypoxia might directly influence the extent of their production. Furthermore, metabolic adjustments to hypoxia resulting in changes in the concentration of several sensitive cellular factors may affect character of the respiratory burst.

As hypoxia is an accompanying factor in several pathological states (Bulkley 1987, Vízek *et al.* 1993), we have studied the effects of short (3 hours and 3 days) and prolonged hypoxia (21 d) on the production of hydrogen peroxide by rat peritoneal macrophages stimulated by FMLP, PMA, or by a combination of both. In the preceding study (Wilhelm *et al.* 1996) we presented the results obtained in rat alveolar macrophages. Hydrogen peroxide was assayed by luminol-dependent chemiluminescence (LDCL) produced by the reaction between luminol and H_2O_2 catalyzed by horseradish peroxidase. The results indicate that hypoxia can specifically influence different signalling pathways leading to H_2O_2 production.

Material and Methods

Induction of hypoxia

Male Wistar rats weighing 250 g were divided into five groups of 8 animals each. The first group served as the control for acute hypoxia and was sacrificed together with the group placed in a hypoxic chamber for 3 days. Another group (Control 2) stayed in the animal facility for three weeks breathing air and allowed free access to food and water. This group was sacrificed together with animals placed in the hypoxic chamber for 21 days. The other groups were exposed to hypoxia in the isobaric hypoxic chamber (F_{iO2}= 0.1) (Hunter *et al.* 1974) and used after 3 hours, 3 days and 21 days.

Preparation of peritoneal macrophages

Non-elicited peritoneal macrophages were isolated from animals killed by cervical dislocation. Sixty millilitres of Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) were injected into the peritoneal cavity and the peritoneum was vigorously massaged for 5 min. About two thirds of this volume was then recovered from an incision into the abdominal wall. The cells were collected by centrifugation (400 x *g*, 10 min), then washed in HBSS and sedimented under the same conditions. Cells were resuspended in approximately 2 ml of HBSS and counted under a light microscope.

Assay of hydrogen peroxide

Hydrogen peroxide production was measured by luminol-dependent chemiluminescence (LDCL) originating in the reaction between hydrogen peroxide and luminol catalyzed by horseradish peroxidase as in our previous report (Wilhelm *et al.* 1995). Hydrogen peroxide was assayed after stimulation of the cells with N-formyl-methionyl-leucyl-phenylalanine (FMLP), or with phorbol-myristate-acetate (PMA), which were added at the beginning of the measurement. Alternatively, FMLP was added subsequently to PMA, exactly 500 s from the beginning of the measurement. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) was purchased from Sigma (St. Louis, MO). A stock solution of 1 mM luminol was prepared by dissolution in 5 mM NaOH, the pH was adjusted to 7.4 with HCl. Horseradish peroxidase (HRP) from Boehringer (Mannheim, Germany) was diluted to 250 U/ml with Ca^{2+}/Mg^{2+}-free HBSS (pH = 7.4). FMLP (Sigma) was made to a 0.1 mM stock solution by dissolving it in 0.5 mM NaOH. A 160 μM PMA stock solution was prepared by dissolution in dimethylsulfoxide (Sigma). LDCL was measured on a Luminometer 1250 (LKB-Wallac Oy, Finland), equipped with the thermostated cell holder. The instrument was coupled through a custom-made analog-digital converter to a computer, where the data was collected in 1 s periods. The intensity of LDCL was expressed in mV of photomultiplier response. The measurements were carried out at 37 °C in the plastic measuring cuvette. The reaction mixture of total volume of 1 ml consisted of 100 μl of luminol (final concentration 0.1 mM), 10 μl of HRP (2.5 U/ml), 50 μl FMLP (5 μM), 10 μl PMA (1.6 μM). A cell suspension was added as the last to start the reaction. The final cell concentration was $1x10^6$ per ml.

Statistics

The results are presented as means ± S.E.M. The significance of differences between the control and hypoxic groups were tested using ANOVA. The values of $P<0.05$ were considered significant.

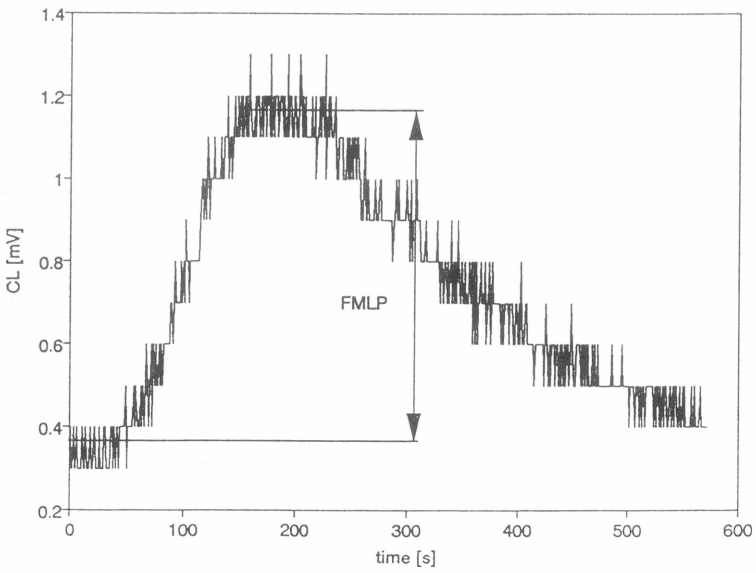

Fig. 1. *The time-course of chemiluminescence production triggered by FMLP added at time 0 s.*

Results

The presence of HRP in the reaction mixture created a prerequisite for the detection of light production by peritoneal macrophages as no LDCL was observed in its absence. Addition of FMLP to the cell suspension containing luminol plus HRP initiated chemiluminescence response with a maximum around 200 s (Fig. 1). About a hundred-fold higher LDCL intensity was triggered by the addition of PMA. When FMLP was added subsequently to PMA, 500 s from the start of the measurement, the net response due to the addition of FMLP was immediate and approximately the same as that induced by PMA, i.e. about two orders of magnitude higher than the response elicited by FMLP alone (Fig. 2).

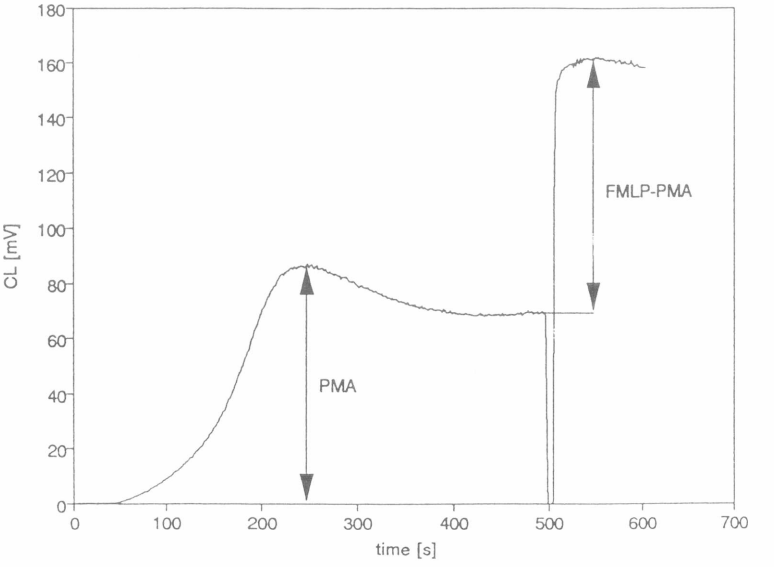

Fig. 2. *Chemiluminescence production after consecutive stimulation of macrophages with PMA and FMLP. At time 0 s PMA was added, and the addition of FMLP followed exactly 500 s from the beginning of the measurement. The arrows indicate the values taken for the calculations.*

The chemiluminescence induced by FMLP, PMA, and by FMLP subsequently to PMA (FMLP-PMA) was investigated in cells isolated from rats exposed to hypoxia. In controls, the peak LDCL values induced by PMA (84.5±11.9 mV) and by FMLP added subsequently to PMA (85.0±12.4 mV) were approximately the same. Hypoxia produced a similar decrease in both of these parameters after 3 hours (33.8±3.4 mV for PMA and 32.9±3.9 mV for FMLP-PMA). After 3 days of hypoxia, both of these parameters did not differ significantly from the control values. The value for PMA was 62.0±5.8 mV, and for FMLP-PMA it was 53.5±9.3 mV. This situation is illustrated in Figure 3.

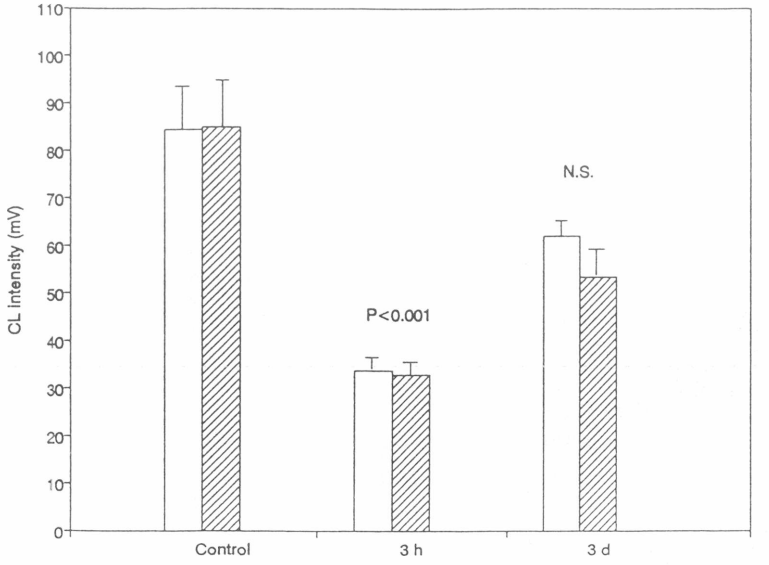

Fig. 3. *The effect of 3 hours or 3 days of hypoxia on the LDCL of macrophages stimulated with PMA (open columns) or with FMLP subsequently to PMA (FMLP-PMA) – hatched columns. N.S. – non-significant.*

Ageing of the animals for 3 weeks, which was accompanied by a weight gain of about 100 g, led to a decrease in the observed parameters in control animals. Thus, the PMA-induced chemiluminescence decreased to 69.0±12.9 mV and the parameter FMLP-PMA we reduced to 28.7±4.9 mV after 3 weeks. Therefore, the effects of 3 weeks' of hypoxia were related to values labelled as Control 2 in Figure 4. In both cases, hypoxia caused an increase which, was however, not statistically significant. The observed value of PMA-induced chemiluminescence after 3 weeks of hypoxia was 119.6±22.7 mV, for FMLP-PMA 40.9±6.9 mV.

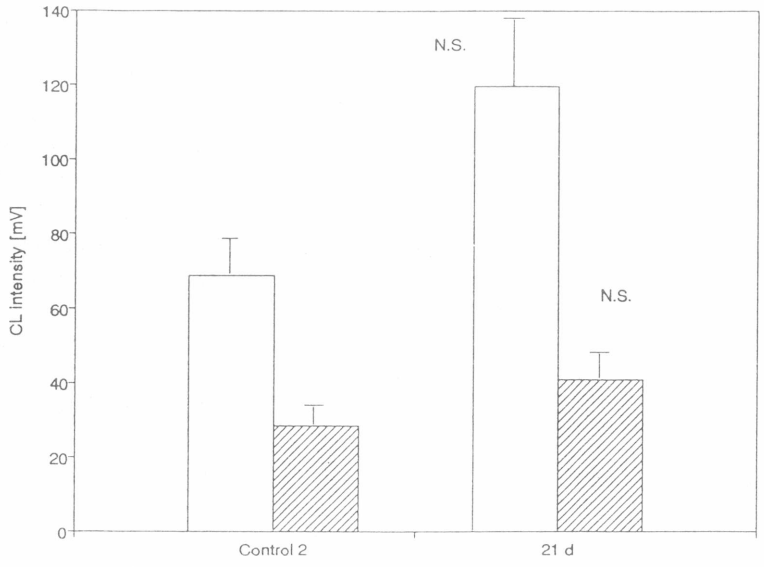

Fig. 4. *The effect of 21 days pf of hypoxia on LDCL stimulated with PMA (open columns) or with FMLP added subsequently to PMA (FMLP-PMA) – hatched columns.*

The effect of FMLP added at the beginning of LDCL measurements was much smaller than when it was subsequently added to PMA. The value elicited by FMLP alone in the controls was 0.47±0.03 mV, and it did not change after 3 weeks when a value of 0.48±0.06 was obtained. Exposure to 3 hours of hypoxia had no effect on this parameter (0.43±0.09 mV), while a statistically significant increase was found after 3 days (0.63±0.06 mV). After 3 weeks of hypoxia, the FMLP-induced LDCL doubled (1.19±0.30 mV) (Fig. 5).

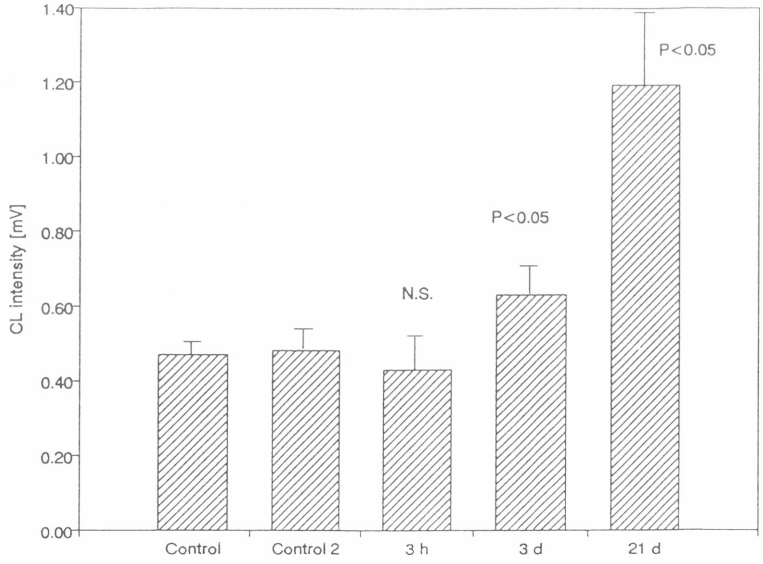

Fig. 5. *The effect of hypoxia on LDCL stimulated by FMLP added at time 0 s.*

Discussion

The present finding indicates that peritoneal macrophages exhibit luminol chemiluminescence only in the presence of added HRP and that they thus release H_2O_2 extracellularly. This is in agreement with the recently published view that NADPH oxidase of macrophages is located in the plasma membrane. Upon stimulation, it releases H_2O_2 extracellularly where it can be measured by HRP-dependent

fluorescence (Johansson *et al.* 1995). We have also observed a similar effect in rat alveolar macrophages (Wilhelm *et al.* 1995). However, the exact mechanism by which H_2O_2 is transferred extracellularly is not known. It is possible that superoxide, which is the primary product of NADPH oxidase, dismutates to H_2O_2 which can diffuse through the cytoplasmic membrane and is detected outside.

In the present study a hundred-fold difference in chemiluminescence intensity was observed in control animals between LDCL induced by FMLP and that induced by PMA. When the activation was assessed in peritoneal macrophages by oxygen consumption, only about a ten-fold higher response was observed with PMA relative to FMLP (Klegeris and McGeer 1994). But it has to be noted that the macrophages in the latter experiment were elicited by casein injections, while in the present work non-elicited cells were used. It can be speculated that macrophage eliciting involves the initiation of some signalling pathways that would change the response to the activators of respiratory bursts. This is illustrated in our experiments by the effect of FMLP added subsequently to PMA, when the response is highly accentuated.

NADPH oxidase is assembled in the membrane upon activation from several subunits located both in the membrane and in the cytoplasm (Rossi 1986). FMLP induces a GTP-binding protein-linked formation of inositolphosphates, calcium mobilization and protein phosphorylation. The activation pathway by PMA is shorter, involving activation of protein kinase C (Alvarez *et al.* 1993). It is thus possible that NADPH oxidase assembled by the action of PMA will respond to FMLP differently than the non-assembled enzyme when FMLP is present alone.

We found that hypoxia lasting for 3 hours attenuated PMA-stimulated LDCL and also the response to FMLP added subsequently to PMA. On the other hand, 3 days and 3 weeks of hypoxia increased the response induced by FMLP alone. From these results we can hypothesize that hypoxia has at least two effects. One probably concerns the activation pathway around protein kinase C and this might be rectified by short-term inhibition, observed after 3 hours and later. The other effect is produced in another part of the activation pathway started by FMLP receptor. It increases the response induced by the FMLP on a relatively long time scale and this might imply changes in the synthesis of proteins.

Several differences, both quantitative and qualitative, can be found by comparing the results of the present study with the effects of hypoxia produced in rat alveolar macrophages (Wilhelm *et al.* 1995) there. This suggests that macrophages in various tissues might respond to general hypoxia differently.

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Reprint Requests

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