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MODULATION OF THE ACTIVITY OF THE NADPH OXIDASE SYSTEM BY REACTIVE OXYGEN SPECIES: INFLUENCE OF CATALASE

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The nicotinamide adenine dinucleotide phosphate oxidase complex (Nox) is a major source of non-mitochondrial reactive oxygen species in cells. Nox contains both membrane (Cytb₅₅₈) and cytosolic (p40^{phox}, p47^{phox}, p67^{phox} and Rac) components. Nox has been submitted to a combination of oxygen free radicals produced by irradiation and to hydrogen peroxide. Irradiation of a single component with high doses led to partial inactivation; however, the irradiation of the whole system during its assembly phase with lower doses (2–10 Gy) led either to activation (2.7 Gy) or to strong inactivation if irradiation took place during the first minute of the assembly. Incubation of the membrane fractions or of p67^{phox} with H₂O₂ led to fast inactivation. Catalase protected weakly p67^{phox} from H₂O₂. Conversely, incubation of the membrane fractions with catalase led to over-activation of the system.

INTRODUCTION

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Nox) is well known as a major source of non-mitochondrial reactive oxygen species (ROS) in many cells, including phagocytes, renal cells and vascular tissues [for review, see Brown and Griendling⁽¹⁾ and references therein]. In phagocytes, ROS are essential for host defence by destroying the microbial pathogens. This destruction is related to a strong increase in the oxygen consumption therefore being termed the 'respiratory burst'. The production of superoxide radical (O₂^{•-}) comes from a single electron reduction in the molecular oxygen using NADPH as an electron donor. The superoxide is subsequently transformed into other ROS. The importance of a functional NADPH oxidase enzyme is underlined by a human genetic disorder, the chronic granulomatous disease, in which a dysfunctional oxidase is associated with recurrent and severe bacterial and fungal infections.

Due to the high toxicity of ROS under physiological conditions, the oxidase activity is highly regulated. Its deregulation is associated with several pathologies involving inflammatory processes like in traumatic events⁽²⁾ and various cardiovascular diseases (atherosclerosis, vascular inflammation, angiogenesis etc.)⁽³⁾. NADPH oxidase also plays a prominent role in cancer radiotherapy. The system seems to be dose-dependently up-regulated by ionising radiation and contributes to X-ray-induced cell death⁽⁴⁾. Moreover, it induces apoptosis in cells having undergone ionising radiation^(5, 6) and especially by low doses⁽⁷⁾.

The structure and the function of NADPH oxidase have been reviewed extensively⁽¹⁾. It contains both membrane and cytosolic components. The membrane component, the flavocytochrome b₅₅₈ (Cytb₅₅₈) consists of two subunits gp91^{phox} (also known as Nox2) and p22^{phox}. Nox2 holds the redox co-factors (one flavin adenine dinucleotide and two hemes) and the NADPH-binding site. The cytosolic component includes p40^{phox}, p47^{phox}, p67^{phox} and Rac proteins. In resting phagocytes, membrane and cytosolic components are believed not to be assembled. Upon cell activation by pro-inflammatory mediators, the cytosolic proteins undergo post-translational modifications and migrate to their membrane partners to form the activated NADPH oxidase complex, allowing electron flow through the complex⁽⁸⁾.

The activation of NADPH oxidase seems to be due to over-expression of the cytosolic fractions⁽⁹⁾; however, a feed-forward mechanism of activation by hydrogen peroxide has been shown in vascular cells⁽¹⁰⁾. Indeed, it is shown that some proteins might also be activated by free radical oxidation⁽¹¹⁾. In order to provide information about the ROS influences on the oxidase activity itself, a cell-free system in which the superoxide production could be measured as a function of the whole system or individual components^(12, 13) was produced and optimised. In whole cells, the activation of the system involves multiple post-translational modifications (like phosphorylations at serine residues). In cell-free systems, these steps were bypassed by the addition of small amphiphilic molecules [detergent, phosphatidic

acid or arachidonic acid (AA)], which activated the system in similar manners⁽¹⁴⁾.

In this paper, NADPH oxidase has been submitted to a combination of free radicals produced by gamma irradiation and to hydrogen peroxide. In order to unravel the important features of the effect of ROS on this system, catalase has been added, which actually revealed unexpected effects.

MATERIALS AND METHODS

Products

Chemicals [NADPH, AA, cytochrome *c*, catalase bovine, affinity resin Nickel, phosphate buffer saline (PBS)] and anti-histidine antibody came from Sigma Aldrich (L'Isle d'Abeau, France). Resins SP-Sepharose HP, Glutathione-Sepharose 4B and Q-Sepharose were provided by GE-Healthcare-Bioscience. The plasmids expressing the human cytosolic factors were a generous gift from Dr M. C. Dagher (Université Joseph-Fourier, Grenoble, France) and the plasmid expressing the bovine cytosol factor p47^{phox} from Dr M. Quinn (University of Montana, USA).

Protein purification

Cytosolic proteins

Recombinant bovine His-tagged p47^{phox} (p47^{phox}), human His-tagged p67^{phox} (p67^{phox}) and human GST-tagged Rac1-Q61L (Rac1-Q61L) were expressed and purified as described previously⁽¹²⁾.

Membrane fractions

Neutrophils were extracted from 8–10 l of bovine blood by two successive hemolysis followed by the Percoll sedimentation^(13, 15). The neutrophils were disrupted by brief sonication, and membrane fractions were separated from the cytosolic fraction and other cell contents on a sucrose gradient. The membrane fractions were collected by centrifugation at 150 000×*g* for 90 min at 4°C and resuspended in buffer containing PBS pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.5 mM leupeptine and stored at –80°C for further experiments.

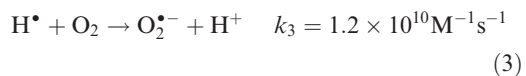
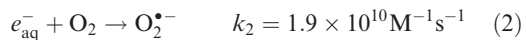
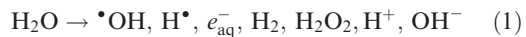
Measurements of the NADPH oxidase activity in the cell-free system

The superoxide production was measured as the rate of cytochrome *c* reduction. Briefly, in our cell-free system, the optimal conditions required a reaction mixture containing p67^{phox} (10 μg ml⁻¹, 150 nM), p47^{phox} (10 μg ml⁻¹, 200 nM) and Rac1Q61L (20 μg ml⁻¹, 400 nM), 18 μg of bovine membrane fractions containing gp91^{phox} and p22^{phox}. The

activation of the system was performed by the addition of 60 μg ml⁻¹ (200 μM) AA (starting point of the assembly). The final volume was 1 ml in PBS buffer, 10 mM MgSO₄. The mixture was incubated for 5 min at 25°C. Then, 50 μM cytochrome *c* was added and the reaction was initiated by the addition of 200 μM NADPH (*t*₀). The superoxide dismutase (SOD)-inhibitable reduction in cytochrome *c* was followed spectrophotometrically at 550 nm on an Uvikon 943 double-beam spectrophotometer (Kontron Instruments). Parallel control experiments were performed in the presence of 350 U ml⁻¹ of SOD enzyme. An extinction coefficient (Δε of the cytochrome *c* reduced minus oxidised forms) of 24 755 mM⁻¹ cm⁻¹ was measured using known quantities of superoxide ions produced by gamma radiolysis and was used to calculate the rate of the reaction. This value is in agreement with that from the literature⁽¹⁶⁾.

Free-radical production

The quantity and the rate of production of free radicals were chosen with either gamma stationary or pulse radiolysis. In both cases, the selection of •OH and superoxide free radicals was obtained through the following reactions^(17, 18).



In air atmosphere and without the help of any other scavenger, reactions (2) and (3) take place, thus OH and O₂^{•-}, radicals are present simultaneously. Those were the conditions that have been used in this paper. The radiation chemical yields of O₂^{•-} and •OH were evaluated by simulation to be 0.31 and 0.28 μmol J⁻¹, respectively (O. Mozziconacci, unpublished).

Gamma radiolysis

Gamma irradiations were carried out using the panoramic ⁶⁰Co gamma-source IL60PL Cis-Bio International (France) at the University Paris-Sud (Orsay, France). The dose rate was between 1 and 37 Gy min⁻¹ determined by Fricke dosimetry⁽¹⁷⁾. All irradiations were performed at room temperature and in aerobic conditions. The solutions to be irradiated (typically 300 μl) contained 1 μg μl⁻¹ of the chosen protein in phosphate buffer (30 mM sodium phosphate, 50 mM NaCl, pH 7.5).

RESULTS

Irradiation of cytosolic subunits in aqueous solution

Aqueous solutions of p67^{phox}, p47^{phox} or Rac (0.3–1 mg ml⁻¹, 4–20 μM) were irradiated by gamma rays in aerobic condition. After irradiation, the system was reconstituted in a cell-free assay containing the non-irradiated other oxidase partners and the production rate of superoxide was measured. To minimise the eventual degradations of the protein due to long-time irradiations realised at room temperature, the irradiation was fixed at constant time (12 min) and thus the dose rate was variable. Non-irradiated samples were kept also at room temperature for identical times. Each irradiation was done in triplicate and the results are the average of the three independent measurements.

The results obtained after 12 min irradiation are shown in Table 1.

The activity was always lowered upon the irradiation of one component; however, the sensitivity varied. The most sensitive were the membrane fractions, probably because of the chain peroxidation that slowed down and/or inhibited the electron transfer in the Cytb₅₅₈. The irradiation of p67^{phox} with 220 Gy led to a more drastic decrease in the activity than that of the other cytosolic units.

Effect of free radicals on the oxidase activity during the phase assembly

The oxidase activity of the cell-free system irradiated by pulse radiolysis at different times after the beginning of the assembly process was measured, which started by the addition of the activator, AA (Table 2). This period was increased from 0 up to 5 min corresponding to the standard incubation time for the control cell-free system. The irradiation pulse duration (maximum 1 μs) was always negligible compared with the assembly duration of 5 min. After irradiation, the system was then allowed to finish assembling until the end of the 5 min, and then NADPH was added to trigger the reaction.

Table 1. Variation in the activity of the NADPH oxidase after gamma irradiation under aerobic conditions of one component, expressed as the per cents of the activity with the non-irradiated component.

Irradiated Component	Dose (Gy)	
	70	220
p67 ^{phox}	66 ± 5	35 ± 5
p47 ^{phox}	75 ± 10	50 ± 10
Rac	78 ± 10	68 ± 10
Membrane fractions	22 ± 10	28 ± 10

Table 2. Effect of gamma irradiation during the assembly process of the NADPH oxidase complex.

Dose (Gy)\time after addition of AA	30 s	60 s	240 s
2.7	100 ± 5	110 ± 5	115 ± 5
5.4	60 ± 5	45 ± 5	100 ± 5
11	65 ± 5	58 ± 5	85 ± 5

NADPH oxidase was pulse irradiated at various times after activation with AA. Results are given as the per cents of the activity of non-irradiated complex. The points represented the mean ± SEM of three independent experiments.

The oxidase activity varied as a function of the dose and the delay time after mixing. For the lowest dose (2.7 Gy), the activity increased slightly up to 110 % and remained constant for delays higher than 1 min. For an irradiation dose of 5.4 Gy or higher, the oxidase activity underwent first drastic lowering to ca. 50 % of the control when the system was irradiated 1 min after the mixing of all the NADPH oxidase components. However, if irradiation took place 3–4 min after the mixing, the oxidase activity remained to a value comparable with that of the non-irradiated complex.

Effect of H₂O₂ addition on the oxidase activity

Hydrogen peroxide should be produced in high concentration in the vicinity of the NADPH complex by superoxide disproportionation. Therefore, it was of special interest to analyse directly the effect of H₂O₂ addition on the oxidase activity, principally at the level of the most sensitive membrane component. Thus, the neutrophil membrane fractions were incubated with two concentrations of H₂O₂: a high one considered as lethal in cell cultures (900 μM) but close to concentration raised locally during the respiratory burst, and a low, non-toxic one (9 μM)⁽¹⁹⁾. Incubations were performed for various times (maximum 18 min) on ice to minimise the degradations due to heat. Catalase (25 units ml⁻¹) was then added to remove excess H₂O₂, the system was reconstituted and the activity was measured. Figure 1 shows that the incubation of a membrane fraction with H₂O₂ induced a dose- and time-dependent inhibition of the NADPH oxidase activity as was recently demonstrated by our group⁽¹³⁾. The activity fell down to ca. 75 and 35 % of its initial value for an incubation time of 6.5 min with 9 and 900 μM H₂O₂, respectively. When incubation time was increased up to 18 min, the activity fell down to ca. 58 and 26 %. The incubation in the presence of tested H₂O₂ concentrations did not modify the absorption spectra of the membrane protein (data not shown)

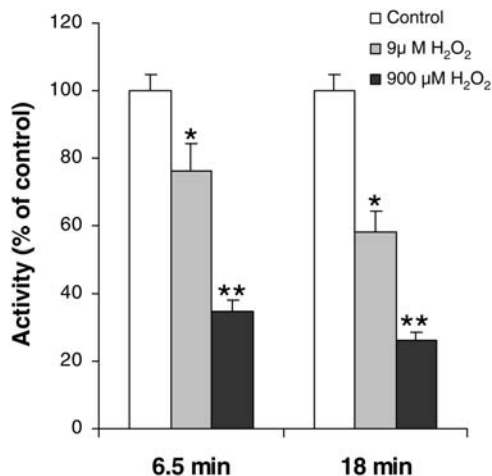


Figure 1. Sensitivity of the membrane fractions to hydrogen peroxide. The membrane fractions were incubated with 900 or 9 μM H_2O_2 for 6.5 or 18 min as indicated. Catalase (25 units ml^{-1}) was then added and the system was reconstituted (see the text). Results are expressed as mean \pm SEM from at least three independent experiments. * p < 0.05; ** p < 0.01.

In another series of experiments, the membrane fractions were incubated with catalase for the same times (6.5–18 min), also on ice, and similarly the system was reconstituted and the activity was measured (Figure 2).

It appeared that catalase had a strong protective effect when incubated with the membrane fractions since the activity reached ca. 150 % of the control value.

Similar experiments were done with the cytosolic fraction p67^{phox} (Figure 3, 'control+cat' vs. 'control'). Conversely to membrane fractions, the activity of the system was markedly diminished in the presence of catalase.

Finally, in order to unravel the effects of free radicals from those of hydrogen peroxide, p67^{phox} had been irradiated in the absence and in the presence of catalase (25 units ml^{-1}) and reconstituted the system as explained in Materials and Methods. The results are shown in Figure 3. Catalase protected weakly p67^{phox} protein from irradiation ('96 Gy+cat irradi' vs. '96 Gy irradi'), indicating that H_2O_2 played a minor role in the inhibition of the activity by the irradiation of this cytosolic component. However, the most surprising was that the incubation of p67^{phox} with catalase lowered by more than 40 % the NADPH oxidase activity ('control+cat' vs. 'control'). This effect is opposite to that on membrane fractions.

DISCUSSION AND CONCLUSION

The aim of this work was to examine the feedback of free radicals on the activity of the NADPH

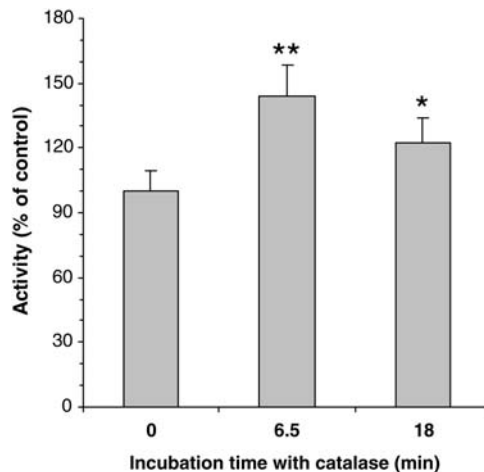


Figure 2. Effect of catalase (25 units ml^{-1}) on the NADPH oxidase. Membrane fractions were incubated with catalase in ice. The system was reconstituted afterwards. Results are expressed as per cents of the control activity. Results are expressed as mean \pm SEM from at least three independent experiments. * p < 0.05; ** p < 0.01.

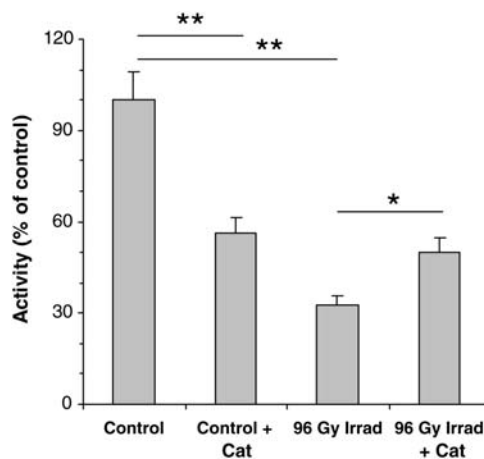


Figure 3. Variation in the activity of the NADPH oxidase when p67^{phox} was (i) incubated with catalase (control + cat); (ii) irradiated by gamma radiolysis in the absence (96 Gy irradi) or in the presence (96 Gy irradi + cat) of catalase (25 units ml^{-1}). The activity is expressed as a per cent of the activity of the control with non-irradiated p67^{phox}. Results are expressed as the per cents of the control activity and mean \pm SEM from at least three independent experiments. * p < 0.05; ** p < 0.01.

oxidase, which is the major cellular free-radical producer. A cell-free system with membrane fractions extracted from bovine neutrophils was used.

It has been shown that whereas the irradiation of a single component always lowered the activity,

irradiation during the assembly phase might either strongly alter the NADPH oxidase if irradiation took place at the beginning of the assembly or have a negligible effect if the assembled complex was irradiated (doses lower than ca. 10 Gy).

Since the disproportionation of superoxide anions produces hydrogen peroxide, the resistance of the system to it was tested. As expected, the membrane fractions were very sensitive subunits, since incubation with H₂O₂ lowered drastically the activity of the system. However, a versatile role of catalase was found: when incubated without added H₂O₂ with the cytosolic fraction p67^{phox}, it induced a strong loss of activity. Conversely, when incubated in the same manner with the membrane fractions, it increased strongly the superoxide production. One can propose that catalase might increase the oxidation of the cytosolic fractions but might be complexed by the membrane fractions resulting in a destruction of hydrogen peroxide in the place of its formation. It might be a regulation mechanism of the H₂O₂ availability which up- or down-regulates specifically the different components of the oxidase.

Very recent results indicate various effects of catalase on the ROS production by NADPH oxidase in cells and tissues. Catalase has been shown to modulate the intercellular signalling processes in tumour cells in various ways according to its local concentration⁽²⁰⁾. Also, a correlation between the antioxidant enzyme amount and the production of superoxide by NADPH oxidase in the heart has been reported⁽²¹⁾. Although our preliminary results should be more deeply investigated before asserting mechanisms, they seem to be in line with the findings reported recently.

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