Oxidative Metabolic Responses of Rabbit Pulmonary Alveolar Macrophages

By Laurence A. Boxer, Ghazally Ismail, John M. Allen, and Robert L. Baehner

During phagocytosis of opsonized lipopolysaccharide-coated paraffin oil droplets, rabbit alveolar macrophages released hydrogen peroxide into the extracellular medium. Employment of the surface membrane stimulant phorbol myristate acetate led to activation of the hexose monophosphate shunt, which activity could be further enhanced in the presence of superoxide dismutase or attenuated in the presence of catalase. These studies demonstrate that rabbit alveolar macrophages release superoxide and hydrogen peroxide during surface membrane perturbation. In turn, hydrogen peroxide generation can stimulate the hexose monophosphate shunt.

During phagocytosis, polymorphonuclear leukocytes (PMN) evince a series of cyanide-insensitive metabolic activities that include increased production of superoxide anion (O₃) and hydrogen peroxide (H₂O₂), stimulation of glucose oxidation via the hexose monophosphate (HMP) shunt, and increased reduction of nitroblue tetrazolium (NBT) to blue formazan. In PMN the reduction of NBT is due mainly to O₃, whereas the increased HMP shunt is dependent on H₂O₂ production. Exposure of these cells to an anaerobic environment abolishes the metabolic activities. Information on similar metabolic events in alveolar macrophages (AM) is controversial. On the one hand there is evidence that phagocytizing rabbit AM show increased oxygen uptake, peroxide production, NBT reduction, and HMP shunt activity. On the other hand there are conflicting data suggesting that rabbit AM are incapable of generating O₃ and H₂O₂ but are capable of activating the HMP shunt by means independent of O₃ and H₂O₂. To resolve the conflicting data, we quantitated H₂O₂ production by AM with a variety of membrane perturbants including opsonized lipopolysaccharide paraffin oil droplets (LPS-PO), which allows for precise quantification of ingestion rates. We also quantitated HMP activities either following surface membrane activation or during phagocytosis in the presence of specific oxygen by-product scavengers.
OXIDATIVE METABOLISM OF MACROPHAGES 487

MATERIALS AND METHODS

Collection and Preparation of Rabbit AM

Suspensions of 90%-95% rabbit AM with the remainder of cells consisting of no more than 1%-2% heterophils and 4%-9% lymphocytes were obtained by tracheal lavage as previously described.18 The cells collected in 0.15-M NaCl were centrifuged at 400 g for 10 min and resuspended in Krebs-Ringer phosphate buffer (KRP), pH 7.4.

Metabolic Studies of Rabbit AM

The HMP shunt activity was determined by glucose-1-14C oxidation to 14CO2 as previously described.19 Activation of the HMP shunt was initiated by the addition of either 0.1 ml opsonized zymosan or 30 ng phorbol myristate acetate (PMA, Consolidated Midland, Brewster, N.Y.) to 2.5 × 10^6 rabbit AM (RAM) suspended in either 0.9 ml or 1.0 ml of KRP, pH 7.4. A stock solution of PMA was prepared by dissolving the drug in dimethylsulfoxide (DMSO, grade I, Sigma Chemical, St. Louis, Mo.) at a concentration of 1 mg/ml. Control cells were incubated with an equal concentration of DMSO. In some experiments, either 50 μg/ml superoxide dismutase (SOD) (grade I, Sigma) or 1400 units of catalase (grade I, Sigma) were added to the incubation mixtures.

The quantitative rate of NBT reduction over 15 min by AM during phagocytosis of opsonized LPS-PO was quantitated as previously described.20,21 Cells at a concentration of 2 × 10^6 RAM/ml were suspended in KRP and warmed to 37°C prior to initiation of the assay. A baseline sample was taken by mixing particles and cells at ice-bath temperature, and the optical density of the baseline sample was used as a blank. In some of the tubes, 50 μg/ml SOD were added.

Superoxide production by rabbit AM was measured by SOD-sensitive reduction of cytochrome C as described by Babior et al.1 Each tube contained a final volume of 1.5 ml 60-μM ferricytochrome C (grade I, Sigma) and 0.5 ml opsonized zymosan suspension or buffer. Each experimental condition was tested in the presence of 50 μg SOD. In these experiments the AM were preincubated with 5 μg/ml cytochalasin B for 15 min at 37°C prior to quantitation of superoxide release. The cytochalasin B was prepared by dissolving the drug in DMSO at a concentration of 1 mg/ml. Control cells were preincubated with equal concentration of DMSO. Solutions were monitored for superoxide release by measuring the absorbance of the supernatants obtained from samples incubated for 25 min at 350 nm. Control samples for each experimental tube were maintained at ice-bath temperature for the same length of time. In some experiments 0.24-mM NBT was added to the tubes containing ferricytochrome C, but without added SOD, and then the cells were monitored for their ability to release O_2^- . Addition of 0.24-mM NBT failed to significantly affect the optical density of 80-μM ferricytochrome C over a 20-min incubation at 37°C; conversely, ferrocytochrome C generated from a solution that previously contained phagocytizing cells and ferricytochrome C failed to reduce 0.24-mM NBT to formazan. When AM containing formazan were added to a solution of ferrocytochrome C and allowed to incubate at 37°C for 20 min and were then removed by centrifugation at 650 g for 10 min, the formazan generated within the AM did not interfere with the reading of the optical density of the ferrocytochrome C.

The rate of H_2O_2 production by 1 × 10^6 AM was measured by the method of Root et al.22 The release of H_2O_2 by 0.9 ml of AM exposed to either 0.1 ml of opsonized zymosan or 10 μg/ml final concentration of digitonin was quantitated in the extracellular medium by observing the decrease in fluorescence intensity of scopoletin during its oxidation by horseradish peroxidase. The AM were preincubated at 37°C in KRP containing 5-mM glucose and 1-mM sodium azide for 15 min. The cells were then pelletted at 250 g for 10 min, resuspended in 1 ml of KRP, and exposed to a membrane perturbation agent for 5 min at 37°C. At the end of the incubation the cells were removed by centrifugation at 4°C and 200 g for 10 min. The supernatant was then examined for its ability to oxidize scopoletin in the presence of horseradish peroxidase (HRP, type II, Sigma) as previously described.

The statistical significance of each experiment was determined by the paired t test.

RESULTS

Rabbit AM at rest demonstrated modest activity of the HMP shunt that could be slightly attenuated (p < 0.05) by the addition of catalase (Table I). Following challenge with opsonized zymosan, there was a 2.85-fold increase in HMP shunt...
activity that represented an increase in counts per minute (cpm) of 787. The stimulated activity could be further enhanced by addition of SOD to 4.25-fold above the corresponding basal value, which reflected an increase of 28% above the corresponding increment in cpm ($p < 0.01$). In contrast, addition of catalase led to a 3.63-fold increase in glucose-1-14C oxidation above the corresponding basal value, but the absolute increase in cpm was similar to that in the control. With addition of the nonparticulate membrane perturbant PMA, shunt activity increased 3.03-fold, which reflected an increment of 867 cpm. In the presence of SOD, PMA stimulation was further enhanced up to 4.88-fold, representing an absolute increase in cpm of 38% above the control ($p < 0.001$). In contrast to the findings observed with the phagocytic challenge, addition of catalase to the PMA-stimulated cells resulted in a 3-fold increase similar to the control values, but the absolute shunt increase in cpm was noticeably decreased by 27% as compared with the control value ($p < 0.005$).

Following phagocytic challenge with C3-opsonized LPS-PO, NBT reduction increased by 2.1-fold over the value observed with unopsonized particles (Table 2). Addition of SOD resulted in a significant decrease in NBT reduction ($p < 0.025$) only in the presence of opsonized active complement-coated particles.

When cytochalasin-B-treated AM were exposed to opsonized zymosan, they released appreciable amounts of superoxide anion into the extracellular medium. Following addition of NBT, the reduction of ferricytochrome C was largely abrogated (Table 3).

Hydrogen peroxide release from AM was increased 3.35-fold following addition of opsonized zymosan. Similarly, the surface membrane perturbant digitonin enhanced the release of H$_2$O$_2$ by 2.59-fold over basal values (Table 4).

**DISCUSSION**

There is considerable evidence that rabbit AM exhibit bursts in metabolic activities accompanying phagocytosis of particles or membrane perturbation by soluble surface agents. In PMN the stimulation of HMP shunt, iodination

**Table 1. Oxidation of Glucose-1-14C by Rabbit AM**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Resting</th>
<th>Zymosan</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (2)</td>
<td>427 ± 118</td>
<td>1214 ± 56</td>
<td>1294 ± 46</td>
</tr>
<tr>
<td>SOD (2)</td>
<td>309 ± 67</td>
<td>1314 ± 73</td>
<td>1508 ± 36</td>
</tr>
<tr>
<td>Catalase (2)</td>
<td>290 ± 54</td>
<td>1052 ± 52</td>
<td>919 ± 17</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SEM in counts per minute/2.5 X $10^6$ AM/30 min incubation. The numbers in parentheses indicate the numbers of separate experiments performed in triplicate.*

**Table 2. Phagocytosis and NBT Reduction by Rabbit AM**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Rate of Phagocytosis</th>
<th>Rate of NBT Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM alone (3)</td>
<td>0</td>
<td>0.054 ± 0.004</td>
</tr>
<tr>
<td>AM + C3-LPS-PO (3)</td>
<td>66 ± 12</td>
<td>0.076 ± 0.011</td>
</tr>
<tr>
<td>AM + C3-LPS-PO + SOD (3)</td>
<td>74 ± 8</td>
<td>0.153 ± 0.013</td>
</tr>
<tr>
<td>AM + LPS-PO (3)</td>
<td>0</td>
<td>0.084 ± 0.012</td>
</tr>
<tr>
<td>AM + LPS-PO + SOD (3)</td>
<td>0</td>
<td>0.081 ± 0.001</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SEM. The numbers in parentheses indicate the numbers of separate experiments performed in triplicate.*
Table 3. Reduction of Ferricytochrome C by AM

<table>
<thead>
<tr>
<th></th>
<th>nmoles O₂⁻ (SOD)</th>
<th>nmoles O₂⁻ (NBT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting (3)</td>
<td>0.60 ± 0.14</td>
<td>1.23 ± 0.57</td>
</tr>
<tr>
<td>Stimulated (3)</td>
<td>9.15 ± 3.92</td>
<td>3.03 ± 1.76</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SEM. The AM were preexposed to 5 μg/ml cytochalasin B for 15 min prior to initiation of the assay. The difference between absorbance of a supernatant from a tube incubated in the presence of SOD and absorbance of a supernatant not incubated with SOD corresponds to nanomoles/2.5 × 10⁶ AM/25 min (second column). The difference between absorbance of a supernatant from a tube incubated in the presence of NBT and absorbance of a supernatant not incubated with NBT as measured in nanomoles O₂ available to reduce ferricytochrome C/2.5 × 10⁶ AM/25 min was quantitated (third column). The numbers in parentheses indicate the numbers of separate experiments performed in duplicate.

NBT reduction, and O₂⁻ generation during phagocytosis requires oxygen, whereas the increased rate of NBT reduction is primarily due to the O₂⁻ produced during phagocytosis. Several studies have indicated that phagocytizing rabbit AM fail to elaborate increased amounts of H₂O₂, whereas stimulation of glucose-1-¹⁴C oxidation has been observed to increase (but not utilization of Krebs-cycle intermediates). This has led one author to conclude that the stimulation of HMP shunt during phagocytosis by rabbit AM involves mechanisms independent of O₂⁻ and H₂O₂.

In this study we found that H₂O₂ was involved, in part, in stimulating the oxidation of glucose-1-¹⁴C, based on the observation that addition of SOD (which enhances the generation of H₂O₂ from O₂⁻ ) to both phagocytizing and membrane-perturbant AM further increases HMP shunt activity. Furthermore, the activation of the shunt can be attenuated by catalase during stimulation with the surface-active agent PMA. Although there is some abrogation of PMA stimulation by catalase, there is still substantial glucose oxidation. It may be that part of the HMP shunt stimulation follows production of NADP by the oxidase responsible for the initial step in production of H₂O₂ by the stimulated leukocyte. Failure to observe any appreciable effect of catalase with zymosan may relate to the inaccessibility of the H₂O₂ generating source within the phagocytizing cell.

Under conditions in which cytochalasin B (which prevents phagocytosis) was employed, O₂⁻ generation was readily observed. Others have found that the membrane-active agent digitonin induces release of O₂⁻ from AM, as determined by ferricytochrome C reduction. These findings support the hypothesis of De Chatelet et al. that rabbit AM actually generate O₂⁻ but that during phagocytosis appreciable amounts of O₂⁻ do not escape into the extracellular medium.

Superoxide generation can also be detected from cytochalasin-B-treated cells by employing NBT as a competitive scavenger of O₂⁻ in the presence of ferricytochrome C. However, on phagocytic challenge, only a small proportion of NBT

Table 4. Rate of H₂O₂ Release by AM

<table>
<thead>
<tr>
<th></th>
<th>H₂O₂ Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM alone (6)</td>
<td>0.092 ± 0.017</td>
</tr>
<tr>
<td>AM + zymosan (6)</td>
<td>0.308 ± 0.052</td>
</tr>
<tr>
<td>AM + digitonin (10 μg/ml) (6)</td>
<td>0.238 ± 0.048</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SEM (nmoles H₂O₂/min/10⁶ AM). H₂O₂ was determined by the disappearance of scopoletin fluorescence in the presence of horseradish peroxidase. The numbers in parentheses indicate the numbers of separate experiments performed in duplicate.
reduction can be attributed to $O_2^\cdot$. These findings are reminiscent of our previous observations in PMN, where $O_2^\cdot$ contributed to the majority of NBT reduction.\textsuperscript{5,6} Under conditions where larger quantities of AM were used than previously employed by others,\textsuperscript{14} increased $H_2O_2$ release was detected during both phagocytosis and surface membrane perturbation. Thus, previous discrepancies in attempts to detect $H_2O_2$ and $O_2^\cdot$ may relate to the nature of the stimulus used to activate the reduced pyridine-nucleotide-oxidase-dependent enzyme. Phagocytic challenge leads to a larger proportion of intracellularly generated oxygen by-product, which appears capable of stimulating the metabolism of glucose-1-\textsuperscript{14}C, despite failure to detect these products beyond the confines of the cell. On the other hand, conditions that evoke primarily surface membrane activation lead to both shunt stimulation and their release to the extracellular medium.

REFERENCES


OXIDATIVE METABOLISM OF MACROPHAGES


Oxidative metabolic responses of rabbit pulmonary alveolar macrophages

LA Boxer, G Ismail, JM Allen and RL Baehner