The Phagocytosis-Associated Respiratory Burst in Human Monocytes Is Associated with Increased Uptake of Glutathione


*J Immunol* 2000; 165:3333-3340;
doi: 10.4049/jimmunol.165.6.3333

http://www.jimmunol.org/content/165/6/3333

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites 54 articles, 34 of which you can access for free at:

http://www.jimmunol.org/content/165/6/3333.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Phagocytosis-Associated Respiratory Burst in Human Monocytes Is Associated with Increased Uptake of Glutathione


During the phagocytic respiratory burst, oxygen is converted to potent cytotoxic oxidants. Monocytes and macrophages are potentially long-lived, and we have hypothesized that protective mechanisms against oxidant stress are varied and fully expressed in these cells. We report here that the respiratory burst in monocytes is accompanied by an increase in the uptake of $[^{35}]$S-glutathione ($[^{35}]$GSH) after 20–30 min to levels up to 10-fold greater than those at baseline. By 30 min, 49% of the cell-associated radioactivity was in the cytosol, 41% was in membrane, and 10% was associated with the nuclear fraction. GSH uptake was inhibited by catalase, which removes hydrogen peroxide ($H_2O_2$), and micromolar $H_2O_2$ stimulated GSH uptake effectively in monocytes and also lymphocytes. Oxidation of GSH to glutathione disulfide with $H_2O_2$ and glutathione peroxidase prevented uptake. Acivicin, which inhibits GSH breakdown by $\gamma$-glutamyl transpeptidase (GGT), had no effect on the enhanced uptake seen during the respiratory burst. Uptake of cysteine or cystine, possible products of GGT activity, stayed the same or decreased during the respiratory burst. These results suggest that a GGT-independent mechanism is responsible for the enhanced GSH uptake seen during the respiratory burst. We describe here a sodium-independent, methionine-inhibitable transport system with a $K_m$ (8.5 $\mu$M) for GSH approximating the plasma GSH concentration. These results suggest that monocytes have a specific GSH transporter that is triggered by the release of $H_2O_2$ during the respiratory burst and that induces the uptake of GSH into the cell. Such a mechanism has the potential to protect the phagocyte against oxidant damage.

Materials and Methods

Preparation of monocytes and lymphocytes

Mononuclear leukocytes were separated from heparinized blood of normal donors using lymphocyte separation medium (Organon Teknika, Durham, NC). Monocytes were washed and resuspended in DMEM (Life Technologies, Grand Island, NY), and 4 $\times$ 10^6 cells were added to each 35-mm diameter tissue culture dish. The cells were allowed to adhere for 2 h at 37°C in a humidified chamber containing 5% CO$_2$ (4). The adherent cell population contained at least 82–89% monocytes by microscopic examination after staining with Wright-Giemsa or esterase stains (Sigma, St. Louis, MO); other cells appeared to be lymphocytes. More than 98% of the...
cells were viable as determined by trypan blue exclusion. Lymphocyte separation medium, DMEM, and other incubation solutions were free of bacterial LPS (<0.01 ng/ml), as tested by the Limulus amebocyte lysate assay, E-toxate, Sigma.

To isolate lymphocytes, cells nonadherent following 2-h incubation in DMEM were transferred to new dishes and allowed to adhere during a second 2-h incubation. The final nonadherent cell population contained >95% lymphocytes by microscopic examination of Wright-Giemsa-stained smears. PMA-stimulated superoxide anion release by these lymphocyte preparations was negligible.

**Amino acid and GSH uptake**

Immediately before uptake studies, the plates were removed from the CO2 incubator, and DMEM was replaced with Na+-HEPES buffer containing 130 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 0.2 mM KH2PO4, 20 mM HEPES, and 1.0 mM glucose, pH 7.4. For Na+-free conditions, NaCl was replaced with an equal concentration of choline chloride. (In this paper we refer to the choline buffer as Na+-free HEPES buffer.) Uptake was initiated by replacing the above solution with 1.0 ml of HEPES buffer containing 0.9 µCi of [35S]cysteine (0.8 nM) with 1 mM cold cysteine, 0.9 µCi [35S]cysteine (0.8 nM) with 1 µM cold cysteine in the presence of 1 mM GSH as a reducing agent, or 0.9 µCi [35S]GSH (2.1 nM) in the presence of 1 µM cold GSH, as we previously described (14). The radioactivity of the mixture of radioactive and cold thiols was measured by liquid scintillation counting, and the specific activity of the thiols was calculated as counts per minute per pico-gram. PMA (500 ng/ml) or opsonized zymosan (OZ; 1 mg/ml) to stimulate the respiratory burst, 130 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.0 mM MgSO4, 1.2 mM K2HPO4, 20 mM HEPES, and 1.0 mM glucose, pH 7.4. For Na+-free conditions, NaCl was replaced with an equal concentration of choline chloride. (In this paper we refer to the choline buffer as Na+-free HEPES buffer.) Uptake was initiated by replacing the above solution with 1.0 ml of HEPES buffer containing 0.9 µCi of [35S]cysteine (0.8 nM) with 1 mM cold cysteine, 0.9 µCi [35S]cysteine (0.8 nM) with 1 µM cold cysteine in the presence of 1 mM GSH as a reducing agent, or 0.9 µCi [35S]GSH (2.1 nM) in the presence of 1 µM cold GSH, as we previously described (14). The radioactivity of the mixture of radioactive and cold thiols was measured by liquid scintillation counting, and the specific activity of the thiols was calculated as counts per minute per pico-gram. PMA (500 ng/ml) or opsonized zymosan (OZ; 1 mg/ml) to stimulate the respiratory burst, 130 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.0 mM MgSO4, 1.2 mM K2HPO4, 20 mM HEPES, and 1.0 mM glucose, pH 7.4. For Na+-free conditions, NaCl was replaced with an equal concentration of choline chloride. (In this paper we refer to the choline buffer as Na+-free HEPES buffer.) Uptake was initiated by replacing the above solution with 1.0 ml of HEPES buffer containing 0.9 µCi of [35S]cysteine (0.8 nM) with 1 mM cold cysteine, 0.9 µCi [35S]cysteine (0.8 nM) with 1 µM cold cysteine in the presence of 1 mM GSH as a reducing agent, or 0.9 µCi [35S]GSH (2.1 nM) in the presence of 1 µM cold GSH, as we previously described (14).

The reaction was allowed to proceed at 37°C, then was terminated by removing medium with suction and washing four times with ice-cold unlabeled HEPES buffer. Cells were lysed by adding 1 ml of a solution of 0.1 N NaOH, 2% NaCO3, and 0.02% sodium-potassium tartrate. After incubation for 3 h, aliquots were taken for protein analysis (Lowry method) and determination of radioactivity (counts per minute) in the cells, the specific activity of the thiol (counts per minute per pico-gram thiols, and the protein content of the reaction mixture (milligrams).

The cell-associated radioactivity measured in these experiments might be a combination of uptake, extracellular binding, and association with any nonwashable extracellular fluid volume. Therefore, control experiments were performed at 4°C to inhibit uptake. Cell-associated radioactivity at 4°C, consisting of extracellular binding and trapping, which was negligible, was subtracted from the cell-associated radioactivity measured at 37°C. In this paper we refer to the accumulation of cell-associated thiol as the uptake and release of GSH was presented as picomoles per milligram of protein, as described above.

Michaels constants (Km) were calculated from the Lineweaver-Burk plot of GSH uptake determined at five different concentrations (5–500 µM). Thirty- and 5-min GSH uptakes were determined in unstimulated or PMA-stimulated cells, respectively, and the maximum velocity (Vmax; picomoles per milligram per minute) was calculated. Methionine (1–100 µM), bromosulfophthalein (1–100 µM), and GSH disul-fide (GSSG) (10–500 µM) were studied as expected inhibitors of GSH uptake. The Km, which is the concentration of inhibitor that doubles the apparent Km (or decreases the affinity) of the substrate, was determined using the Dixon plot (14).

**Respiratory burst**

Release of O2·− from monocytes was measured as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c, corrected for the protein content (4, 20). The cells were stimulated by PMA (500 ng/ml) or OZ (1 mg/ml). Values in the absence of stimulus were subtracted from those obtained after stimulation. Study of the respiratory burst and of amino acid transport were performed in parallel using cells from the same preparation. In other experiments PMA-stimulated release of O2·− and uptake of [35S]GSH were studied in the presence of 50 µg/ml SOD or 2000 U/ml catalase.

**In control experiments we studied the effect on GSH uptake of inhibiting the PMA-stimulated respiratory burst with diphenyleneiodonium (DPI) (21, 22), DPI (ICN, Costa Mesa, CA) dissolved in DMSO (21) was added in a volume of 2% to a final concentration of 0.1–2 mM. After preincubation with the cells in Na+-HEPES buffer for 10 min, PMA (500 ng/ml) was added for an additional 10 min (O2·− release) or 30 min (GSH uptake). DMSO alone (2 µl) had no effect on GSH uptake or O2·− release. Monocyte viability (methylene blue exclusion) was 99% after incubation for 30 min in 2 µM DPI.

**Subcellular localization of cell-associated radioactivity**

We studied the subcellular localization of the respiratory burst-stimulated cell-associated [35S]labeled thiol by separating the cells into membrane, nuclear, and cytosolic fractions. After incubation of the cells with [35S]GSH and stimulation with PMA as described, the monocytes were scraped from the dishes and sonicated for 60 s (six 10-s bursts) on ice. An aliquot was taken to determine total counts per minute. Cell debris and nuclear material were pelleted from the remaining homogenate, using an Eppendorf centrifuge at 1,000 x g for 10 min. The supernatant was saved, and the pellet was further fractionated using Opti Prep, as described in the manufacturer’s booklet (Nycodem Pharma, Oslo, Norway). To isolate nuclei, the 1,000 x g pellet was homogenized in diluent A (8% (w/v) sucrose, 25 mM KCl, 5 mM MgCl2, and 20 mM Tris, pH 7.8), and a second centrifugation was performed at 1000 x g for 10 min. The pellet was layered on the 25/30/35% (w/v) discontinuous gradient of Opti Prep and centrifuged at 100,000 x g for 30 min. The interphase material formed a distinct band at the 30/35% interface. The supernatant, saved after the first centrifugation at 1,000 x g, was centrifuged at 100,000 x g for 30 min, and the radioactivity of the membrane fraction (pellet) was measured by directly resuspending it in scintillation fluid. The radioactivity remaining in the supernatant represented the nonmembrane-bound cytosolic thiol. This supernatant was treated with 10% (final concentration) TCA and cytosolic thiol was measured by direct scintillation counting.

**Release of radioactivity from monocytes**

Monocytes were incubated with [35S]labeled GSH with or without stimulation with PMA for 30 min as described above. The reaction was stopped by removing the medium and washing the cells four times. The cells were then centrifuged at 37°C in HEPES buffer with or without 1 mM dithioerythritol (DTE) to release plasma membrane protein-bound radioactivity by reducing disulfide bonds. Radioactivity released into the extracellular medium and that which remained associated with the washed adherent cells was determined. The uptake and release of GSH was presented as picomoles per milligram of protein, as described above.

**Release of radioactivity from monocytes**

Monocytes were incubated with [35S]labeled GSH with or without stimulation with PMA for 30 min as described above. The reaction was stopped by removing the medium and washing the cells four times. The cells were then incubated for 10 min at 37°C in HEPES buffer with or without 1 mM dithioerythritol (DTE) to release plasma membrane protein-bound radioactivity by reducing disulfide bonds. Radioactivity released into the extracellular medium and that which remained associated with the washed adherent cells was determined. The uptake and release of GSH was presented as picomoles per milligram of protein, as described above.

**FIGURE 1.** Cystine (Cys2), cysteine (Cys) and GSH uptake in human monocytes during the respiratory burst. Uptake of [35S]labeled Cys2, Cys, or GSH was determined over 30 min in the presence of Na+-HEPES buffer with and without PMA as a stimulus for the respiratory burst. When Cys uptake was studied, 1 mM GSH was added to the reaction mixture to prevent auto-oxidation. Data points represent the mean ± SEM of duplicate measurements in each of three separate cell preparations (n = 3). PMA induced a significant reduction in uptake of Cys (p < 0.01) and an increase in uptake of GSH (p < 0.002).
Thiol uptake in human monocytes during the respiratory burst

Results

Thiol uptake in human monocytes during the respiratory burst

Under basal culture conditions, in Na+-HEPES buffer cystine uptake was 93% of the total thiol taken up in these experiments. In the presence of PMA cysteine uptake decreased by 70%, whereas cystine uptake was not significantly altered. In contrast, GSH uptake was increased by 3.1-fold (Fig. 1). The combined cystine and cysteine uptake represented 93% of the total thiol taken up in these experiments. In the presence of PMA cysteine uptake decreased by 70%, whereas cystine uptake was not significantly altered. In contrast, GSH uptake was increased by 3.1-fold (Fig. 1).

Total thiol uptake decreased during the respiratory burst because of their substrate and inhibitor specificity and whether Na\(^+\)-independent mechanisms have been described for cystine, cysteine, and GSH uptake. To explore these mechanisms in monocytes, uptake was measured in HEPES buffer with and without PMA. When Cys uptake was studied, 1 mM GSH was added to the reaction mixture to prevent auto-oxidation. Data points represent the mean ± SEM (n = 3). PMA induced a significant reduction in the uptake of Cys (p < 0.04) and a significant increase in the uptake of GSH (p < 0.001); the reduction in Cys\(_2\) uptake was not significant.

The relationship between PMA-induced respiratory burst and GSH uptake was studied as a function of time after addition of PMA (Fig. 2A). During the first 20 min total superoxide release and total GSH uptake increased together; after 20 min GSH uptake gradually declined. In these experiments GSH uptake increased ~12-fold over baseline during the respiratory burst. When the data were expressed as rates (Fig. 2B), a close relationship was shown between superoxide release and GSH uptake in the presence of PMA. Both rates peaked by 5 min after addition of PMA and declined rapidly thereafter.

The relationship between the PMA-stimulated respiratory burst and GSH uptake was also studied after inhibition of the respiratory burst by preincubation of the monocytes for 10 min with DPI (21, 22). Release of O\(_2^-\) and uptake of GSH were inhibited to an equivalent extent over a range of DPI concentrations of 0.2–2 \(\mu\)M (Fig. 2C). Preincubation for 10 min with 1 \(\mu\)M DPI had no effect on the increased GSH uptake stimulated by 0.5 mM H\(_2\)O\(_2\) (without DPI 58 ± 6 pmol/mg; with DPI 59 ± 11 pmol/mg; mean ± SEM; n = 3). Sodium-independent uptake of thiols during the respiratory burst and in unstimulated monocytes

Both Na\(^+\)-dependent and Na\(^+\)-independent mechanisms have been described for cystine, cysteine, and GSH uptake. To explore these mechanisms in monocytes, uptake was measured in HEPES buffer in which sodium chloride was replaced with choline chloride. The effect of stimulating the respiratory burst with PMA was similar to the effect in Na\(^+\)-HEPES buffer; there was a decrease in uptake of cysteine (44%), a minimal effect on cystine uptake, and a stimulation of GSH uptake by 4.9-fold during the respiratory burst. Almost all (93%) the GSH uptake was sodium independent (Fig. 3, compared with Fig. 1). Under basal (unstimulated) conditions, about 60% of GSH uptake was Na\(^+\)-independent.

Transporters for cysteine, cystine, or both can be differentiated by their substrate and inhibitor specificity and whether Na\(^+\) is necessary for transport activity. We explored cystine and cysteine uptake (5 min, 37°C) in monocytes under basal culture conditions by determining transport in the presence of these selective inhibitors (14). Cystine transport was predominantly (72%) Na\(^+\)-independent (Figs. 1 and 3). This transport was inhibited 43 ± 13% (mean ± SEM; n = 4) by quisqualate, a specific inhibitor of system \(x_c\), a glutamate/cystine exchanger. Sodium-dependent cystine

FIGURE 2. Relationship between the release of superoxide anion and uptake of GSH during the respiratory burst. A, Monocytes cultured in Na\(^+\)-HEPES buffer were stimulated with PMA, and the uptake of \(^{35}\)S-labeled Cys\(_2\), Cys, or GSH was determined over 30 min in Na\(^+\)-free HEPES buffer with and without PMA. In the presence of PMA cysteine uptake decreased by 70%, whereas cystine uptake was not significantly altered. In contrast, GSH uptake was increased by 3.1-fold (Fig. 1). Total thiol uptake decreased during the respiratory burst because of the marked decrease in cysteine uptake.

FIGURE 3. Cystine (Cys\(_2\)), cysteine (Cys), and GSH uptake in human monocytes during the respiratory burst in sodium-free buffer. Uptake of \(^{35}\)S-labeled Cys\(_2\), Cys, or GSH was determined over 30 min in Na\(^+\)-free HEPES buffer with and without PMA. When Cys uptake was studied, 1 mM GSH was added to the reaction mixture to prevent auto-oxidation. Data points represent the mean ± SEM (n = 3). PMA induced a significant reduction in the uptake of Cys (p < 0.04) and a significant increase in the uptake of GSH (p < 0.001); the reduction in Cys\(_2\) uptake was not significant.
transport was inhibited 83 ± 12% (n = 4) by L-aspartic acid-β-hydroxamate, a specific inhibitor of system XAG, which also transports aspartate and glutamate. Cysteine transport was only 24% Na⁺ independent (Figs. 1 and 3). Most (82 ± 2%; n = 4) of the Na⁺-dependent transport was inhibited by serine, implicating system ASC. The Na⁺-independent cysteine transport was inhibited by 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid (46 ± 3%), indicating the participation of system L, a transporter that carries a number of amino acids, including cysteine (14).

Characterization of Na⁺-independent GSH uptake

The effect of the respiratory burst on uptake of GSH in Na⁺-free buffer was studied during phagocytosis of a serum-opsonized particle (OZ) (20). The relationship between the OZ-stimulated respiratory burst and GSH uptake as a function of time (Fig. 4) was similar to that shown with PMA as stimulus in Na⁺-HEPES buffer (Fig. 2A). The characteristically slower rate of O₂ release with OZ compared with that using PMA (20) was associated with a longer interval before decline in GSH uptake (30 min with OZ, Fig. 4; 20 min with PMA, Fig. 2A). In these experiments GSH uptake during the respiratory burst was 10.2 times higher than uptake under basal conditions (control).

During the respiratory burst, superoxide anion is converted to the more stable and membrane-permeable oxidant, H₂O₂. Uptake of GSH during the PMA-induced respiratory burst was consistently (but not statistically significantly) further enhanced by SOD, which accelerates H₂O₂ production (Fig. 5). In contrast, catalase, which removes H₂O₂, reduced GSH transport during the respiratory burst almost to the level of the control (no PMA).

These results suggested that among the oxygen derivatives of the respiratory burst, H₂O₂ plays a particularly important role in inducing GSH uptake. In support of this conclusion, H₂O₂ in micromolar concentrations induced a concentration-dependent enhancement of GSH transport to levels over 7 times greater than those at baseline (Fig. 6). In experiments not shown the time course of this effect was similar to that seen with the PMA-induced respiratory burst, with GSH uptake reaching a maximum after 15 min of exposure, then decreasing slowly (by ~15%) by 30 min (n = 3). The highly reactive H₂O₂ derivative sodium hypochlorite (NaOCl; Sigma; 0.05 mM) did not enhance GSH uptake significantly during a 30-min incubation: NaOCl, 35.1 ± 6.5 pmol/mg; 0.05 mM H₂O₂, 214 ± 28 pmol/mg; control, 27.5 ± 6.6 pmol/mg (n = 3).

Because the GSH used for uptake studies was radiolabeled on the cysteinyi moiety, it was necessary to demonstrate that we were measuring the uptake of intact GSH and not cysteinylglycine or cysteinylcysteine.
cysteine released by the action of GGT, then dipeptidase. Therefore, cells were preincubated for 10 min in NaCl, an irreversible inhibitor of GGT, before exposure to PMA. As shown in Fig. 7, acivicin preincubation resulted in a modest (14%) decrease in GSH uptake in unstimulated cells, but no change in the enhancement associated with the respiratory burst, suggesting that GSH is being transported in intact form, and that GGT is not involved in the increased GSH uptake seen during the respiratory burst.

We explored the possibility that H₂O₂ stimulated GSH uptake by oxidizing GSH to GSSG, which, in turn, was taken up preferentially. When uptake was studied in the presence of glutathione peroxidase (1 U/ml) and H₂O₂ (10 μM; 30 min) to convert GSH to GSSG, uptake of [³⁵S]GSH was less than uptake at baseline (no stimulus or H₂O₂ added; 9 ± 1 pmol/mg protein vs baseline, 20 ± 3 pmol/mg; mean ± SEM; n = 3). In these experiments, H₂O₂ alone enhanced uptake of GSH, as shown in Fig. 6, and glutathione peroxidase alone had no appreciable effect. A similar effect (mean 70% reduction below baseline; n = 3) was noted when the cells were preincubated with the strong oxidant diiodomethane (50 μM).

Distribution of GSH taken up by human monocytes during oxidant stress

We explored the cellular distribution of radioactivity in monocytes after a 30-min incubation in Na⁺-free HEPES buffer with [³⁵S]GSH under basal conditions or stimulated with PMA (Fig. 8).

In unstimulated cells, most (78%) of the cell-associated radioactivity was in the cytosol, with 18% being in the membrane and 4% in the nuclear fraction (n = 3). After stimulation with PMA, there was an almost 6-fold increase in total radioactivity. The cellular distribution of radioactivity was different from that under basal conditions, with equivalent levels being achieved in the cytosol and membrane fractions (49 and 41% of the total, respectively) and a smaller amount localizing in the nuclear fraction (10%; Fig. 8). Thiol in the cytosol under basal conditions and after PMA stimulation was further separated into TCA-soluble and -precipitable fractions; the respiratory burst increased the levels of cytosolic free GSH by 2.1-fold and cysteine protein-bound GSH by 3.4-fold (mean; n = 3), in agreement with our previous studies showing avid binding of GSH to cysteine proteins during the respiratory burst (4–7).

In an attempt to probe further the differentiation of internalized thiol from extracellular binding, we allowed GSH to associate with the cell for 30 min in basal or PMA-stimulated conditions. After washing the cells as usual, the ability of 1 mM DTE to reduce disulfide bonds and release radioactivity into the supernatant during a 10-min incubation was taken as a measure of extracellularly bound GSH (Table I). The total cell-associated radioactivity before the incubation with buffer or DTE was increased about 7-fold during the respiratory burst. There was only a minor (3%) difference in the amount of radioactivity released into the supernatant of stimulated cells by buffer compared with DTE, in agreement with the concept that cell-associated radioactivity represents internalized thiol.

Table I as well as Figs. 2A and 4 show that there was gradual efflux of radiolabel after peak uptake had occurred. Thus, uptake (accumulation of radiolabel) is a net value, although efflux appears to be small during the initial 30–40 min.

Kinetics of GSH uptake

If activation of a transport protein were responsible for the increase in GSH entering the cell during the respiratory burst or exposure to H₂O₂, then uptake might be expected to follow Michaelis-Menten kinetics. Consistent with this premise, uptake was found to be a saturable event, with a similar high affinity (Kₘ) for GSH in both the presence and the absence of added H₂O₂ (Table II). The Kₘ demonstrated was in the range of the concentration of GSH in plasma under physiologic conditions (15, 24, 25). In contrast, the Vₘₐₓ was increased 11-fold in the presence of H₂O₂.

Table II. Effect of H₂O₂ and transport inhibitors on GSH uptake in human monocytes

<table>
<thead>
<tr>
<th></th>
<th>H₂O₂ (-)</th>
<th>H₂O₂ (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ (μM)</td>
<td>Vₘₐₓ (pmol/mg/min)</td>
</tr>
<tr>
<td>GSH</td>
<td>6.1 ± 0.3</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>GSSG</td>
<td>111.8 ± 45.5</td>
<td>53.6 ± 12.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>–</td>
<td>18.5 ± 4.5</td>
</tr>
<tr>
<td>BSP</td>
<td>–</td>
<td>77.5 ± 1.5</td>
</tr>
</tbody>
</table>

* Results are means ± SEM for transport studied in at least three different monocyte preparations. Uptake of GSH was studied in the absence of Na⁺. Uptake was determined for at least five different substrate concentrations. Kₘ was calculated from Lineweaver-Burk plots, and Vₘₐₓ was calculated by using the Dixon plot. BSP, Bromsulfophthalein.

Materials and Methods

The cells were exposed to HEPES buffer with or without PMA for 30 min. The reaction was stopped by scraping the monocytes from the dishes and sonicating them in Eppendorf tubes for 60 s (six 10-s bursts) on ice. An aliquot was taken to determine total counts per minute. The cell debris and the nuclear material were centrifuged at 1,000 × g for 10 min. The supernatant was separated into the membrane fraction, obtained as the pellet after centrifugation at 100,000 × g for 30 min, and the supernatant, which represented the cytosolic fraction. Nucleus-associated GSH was separated from the cell debris as described in Materials and Methods. Results represent the mean ± SEM of three experiments performed in duplicate. Measured radioactivity was significantly higher in each of the three fractions from cells exposed to PMA (p < 0.005 to p < 0.05).

FIGURE 8. Uptake and distribution of GSH in monocytes during the respiratory burst. Monocytes were preincubated with [³⁵S]GSH in Na⁺-free HEPES buffer with or without PMA for 30 min. The reaction was stopped by scraping the monocytes from the dishes and sonicating them in Eppendorf tubes for 60 s (six 10-s bursts) on ice. An aliquot was taken to determine total counts per minute. The cell debris and the nuclear material were centrifuged at 1,000 × g for 10 min. The supernatant was separated into the membrane fraction, obtained as the pellet after centrifugation at 100,000 × g for 30 min, and the supernatant, which represented the cytosolic fraction. Nucleus-associated GSH was separated from the cell debris as described in Materials and Methods. Results represent the mean ± SEM of three experiments performed in duplicate. Measured radioactivity was significantly higher in each of the three fractions from cells exposed to PMA (p < 0.005 to p < 0.05).
The effects of compounds known to inhibit various GSH transporters in hepatocytes (15, 16) were also investigated, and \( K_m \) was determined by analyzing the data using a Dixon plot. Methionine inhibited hydrogen peroxide-stimulated GSH transport at a concentration close to the \( K_m \); concentrations severalfold higher were required for inhibition of GSH transport by GSSG or bromsulfophthalein (Table II).

**GSH uptake in lymphocytes**

The role of \( H_2 O_2 \) or the respiratory burst induced by PMA in GSH uptake was compared in human monocytes and lymphocytes (Fig. 9). PMA did not induce superoxide anion release in our lymphocyte preparations; therefore, the direct effect of PMA on GSH uptake could be evaluated. Hydrogen peroxide caused a significant increase in GSH uptake in both purified monocytes (\( p < 0.002 \)) and purified lymphocytes (\( p < 0.02 \)). In contrast, the respiratory burst induced by PMA caused a 4-fold increase in GSH uptake in monocytes, but PMA had no effect on GSH uptake by lymphocytes.

**Discussion**

During the process of phagocytosis professional phagocytes undergo a vigorous respiratory burst in which they consume oxygen and convert it enzymatically to \( O_2^- \), which interacts to form \( H_2 O_2 \), then hypochlorous acid, hydroxyl radical, and other potent oxidants. It has been recognized that this process is accompanied by activation of the hexose monophosphate shunt and oxidation of GSH to GSSG through the action of GSH peroxidase and \( H_2 O_2 \) (26). GSSG is cycled back to GSH by glutathione reductase. Data from a variety of experimental systems indicate that the redox cycling of GSH plays an important role in protecting the cell against oxidant damage (26, 27). We report here that the respiratory burst-induced cell-associated radioactivity is due primarily to uptake, not oxidant-stimulated mixed disulfide formation with plasma membrane ectoenzymes. In addition, incubation of the PMA-stimulated cells with DTE, which inhibits GSH breakdown catalyzed by \( \gamma \)-glutamyl transpeptidase (19), had no effect on the \( H_2 O_2 \)-induced GSH uptake (Fig. 7). Uptake of Cys and Cys\(_2\), possible thiol products of GSH breakdown, was decreased or did not appreciably change during the respiratory burst (Figs. 1 and 3). Oxidation of \([^{35}S]GSH\) to \([^{35}S]GSSG\) by preincubation with \( H_2 O_2 \) \( / \) GSH peroxidase or diadme reduced uptake of the thiol to 30–50% of the baseline (no \( H_2 O_2 \)) value, in agreement with the higher \( K_m \) for GSSG than for GSH found on kinetic analysis (Table II). In these experiments we cannot rule out a direct effect of the diadme on the cell, but the facts that \([^{35}S]GSH\) uptake was inhibited by the strong oxidant diadme and was not increased by hypochlorous acid support the conclusion that the \( H_2 O_2 \)-induced cell-associated radioactivity is due primarily to uptake, not oxidant-stimulated mixed disulfide formation with plasma membrane ectoenzymes. In addition, incubation of the PMA-stimulated cells with DTE, which would release externally bound thiols, elicited only a few more counts per minute than did buffer alone (Table I).

Uptake of GSH against a concentration gradient would be expected to require active transport. The data reported here support the existence of a \( Na^+ \)-independent GSH transporter on human monocytes. A transport mechanism for GSH has been described in several cell types, including human platelets and canalicular and sinusoidal membranes of rat and human liver (15–18, 32–35). GSH transport in various mammalian cell lines can be bidirectional, at least under certain conditions (33, 35). The \( Na^+ \)-independent activity, redox regulation, and inhibition by methionine and bromsulfophthalein that we found with monocyte GSH transport are characteristic of the liver sinusoidal GSH transporter (32, 35, 36), but the \( K_m \) of this hepatic transporter is in the millimolar range, as would be appropriate for its mediating GSH efflux from the liver into the bloodstream. We found monocytes to have a transport mechanism with a considerably higher affinity for GSH (\( K_m = 6–9 \) \( \mu M \)), which was close to the GSH concentration in plasma. A high affinity transporter for GSH has also been described in human platelets (34).

The rate of the respiratory burst-associated uptake of GSH closely approximated the rate of \( O_2^- \) release; both peaked at 5 min (Fig. 2B); accumulated GSH uptake after addition of \( H_2 O_2 \) peaked at 15 min. SOD, which removes \( O_2^- \) by catalyzing its dismutation into \( H_2 O_2 \), increased GSH uptake slightly, whereas catalase reduced the stimulatory effect of PMA by \( >80\% \) (Fig. 5). \( H_2 O_2 \), which can cross cell plasma membranes relatively efficiently (37),...
stimulated rapid and vigorous GSH uptake when added to the outside of the cells in micromolar concentrations. Added hypochlorous acid had a negligible stimulatory effect. Thus, respiratory burst-associated GSH uptake appears to be driven primarily by H2O2.

Small amounts of radiolabeled GSH probably entered in PMA-induced pinocytic vesicles and OZ-induced phagocytic vacuoles, but most of the GSH uptake induced by PMA was inhibited by catalase, which removes H2O2 (Fig. 5), and by DPI, which inhibits the respiratory burst (Fig. 2 C). DPI did not inhibit the increased GSH uptake induced by H2O2. In addition, PMA stimulated selective uptake of GSH, not cysteine, cystine, or GSSG.

The mechanism by which H2O2 drives such a profound and rapid uptake of GSH remains to be defined. Rapid modulation of signal transduction is a likely explanation. The phagocytic respiratory burst and other systems of oxidant stress have been related to modification of signaling in a variety of systems (38–44). H2O2 is in particular has been reported to inhibit tyrosine phosphatase activity (39–41), to stimulate tyrosine phosphorylation (42, 43), and to stimulate the activity of mitogen-activated protein kinase (42) and protein kinase C (44). H2O2 has also been reported to influence the activation of transcription factors, including NF-κB and AP-1, in mammalian cells (42, 45) and OxyR in Escherichia coli (46), and to activate RBC KCl cotransport (47).

It is not clear what survival advantage might be offered by this rapid uptake of GSH and by the shift from uptake primarily of cysteine/cystine to uptake of GSH. We reported previously that stimulation of the respiratory burst in human monocytes induced a rapid decrease in intracellular GSH, with a nadir at 10 min of PMA labeling by 2.7-fold in the cytosol, 10-fold in the membrane fraction, and 10-fold in the nuclear fraction (Fig. 8). The GSH taken up (42 pmol/mg/min; Table II) suggests that about 20% of the GSH decline in the first 10 min could be offset by rapid GSH uptake.

Stimulation of the respiratory burst for 30 min increased thiol labeling by 2.7-fold in the cytosol, 10-fold in the membrane fraction, and 10-fold in the nuclear fraction (Fig. 8). The GSH taken up during the respiratory burst might interact in these cell compartments to form mixed disulfides (the process of S-thiolation) between a variety of cellular proteins and GSH, a process that we have demonstrated with human neutrophils and monocytes and mouse macrophages (4–7). S-thiolation of a protein can significantly modify its function (5), and the covalent disulfide bonds formed in this process are reversed enzymatically as the respiratory burst subsides (4). Thus, S-thiolation represents a form of redox buffering that could protect proteins against oxidant denaturation and modulate cellular metabolic events during phagocytosis. For example, GSH has been shown to play a protective role in maintaining the functional integrity of nuclear and mitochondrial DNA (48) and in control of apoptosis (49–51). It seems possible that the amount of GSH taken up during the respiratory burst, although small relative to the total intracellular GSH concentration, could appear at the right place in the cell at the right time to protect specific monocyte proteins against oxidant damage.

In addition to protecting against oxidant damage, GSH has been reported to play a central role in regulating a large number of biologic systems that are fundamental to the immune response, inflammation, and host defense. These include activation in T cells of NF-κB, IL-2-dependent functions including cell proliferation, and cellular cytotoxicity (52); synthesis of PGE2 and leukotriene C4 by macrophages (53); detoxification of xenobiotics through glutathione S-transferase reactions (9); inhibition of apoptosis in monocytes, lymphocytes, and neutrophils (49–51); and replication of HIV (54). It seems reasonable to speculate that the location and timing of increased GSH concentration might also be important to protecting and maintaining these regulatory systems, and thus cell function.

Acknowledgments

We thank Dr. Nazzareno Ballatori, University of Rochester School of Medicine, for constructive criticism.

References