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

Tamas Seres,* Roy G. Knickelbein,* Joseph B. Warshaw,* and Richard B. Johnston, Jr. 2*†

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 $[^{35}]$Sglutathione ($[^{35}]$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.

Elimination of invading micro-organisms by neutrophils, monocytes, and macrophages depends heavily on the generation of reactive oxygen species during the phagocytosis-associated respiratory burst. The NADPH oxidase that mediates this process assembles in the plasma membrane, and toxic oxidants are released to the inside and outside of the cell (1). Thus, the oxidants created in this process, particularly superoxide anion ($O_2'^-$), hydrogen peroxide ($H_2O_2$), hypochlorous acid, and hydroxyl radical, carry the potential to damage the phagocytes themselves as well as other cells at sites of inflammation (2).

The intracellular reducing potential supplied by the thiol tripeptide glutathione (L-$\gamma$-glutamyl-L-cysteinylglycine [GSH]) is believed to play an essential role in protecting cells against oxidant damage (3). We have published data indicating that GSH also serves as the key component in the respiratory burst-driven formation of cellular protein-mixed disulfides, a process termed $S$-thiolation (4–7). This reversible process can modify enzyme function and could protect protein sulfhydryl groups from irreversible oxidative damage (4, 5). The importance of GSH to cell integrity is emphasized by experiments in which inhibition of GSH synthesis by L-buthionine-(S,R)-sulfoximine in adult mice or newborn rats led to mitochondrial degeneration, multiorgan failure, and death (3, 8).

Most cell types can metabolize extracellular GSH by an ectoenzyme, $\gamma$-glutamyl transpeptidase (GGT), which transfers glutamate to an amino acid acceptor. Cysteinylglycine is then hydrolyzed, and the amino acids are internalized independently (9, 10). The rate of transport and the intracellular availability of cysteine/cystine appear to control the rate of GSH synthesis (9–12). Transport of these amino acids is accomplished by well-characterized amino acid transporters (13, 14). More recently, distinct transport systems for GSH have been described on the canalicular and sinusoidal membranes of rat and human liver, and analysis of cell lines and tissues suggests that a transport mechanism also exists on certain other cell types (15–18).

The studies reported here were framed on the hypothesis that stimulation of the phagocytic respiratory burst induces increased uptake of cysteine, cystine, or GSH, individually or in combination. The results indicate that activation of the respiratory burst in human monocytes or exposure of the cells to $H_2O_2$ decreases the uptake of cysteine, does not affect cystine uptake, and induces a prominent increase in uptake of GSH.

Materials and Methods

Preparation of mononocytes 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 supernoxide 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, 1.2 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 l-[35S]cysteine (0.8 nM) with 1 μM cold cysteine in the presence of 1 nM GSH as a reducing agent, or 0.9 μCi l-[35S]GSH (21 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 and subtracted from the cell-associated radioactivity measured at 130 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.0 mM MgSO4, 1.2 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. For Na+-free conditions, NaCl was replaced with an equal concentration of choline chloride.

The radioactivity of the mixture of radioactive and cold thiols was measured by liquid scintillation counting. The specific activity of the thiol (counts per minute per picomoles of thiol), and the protein content of the reaction mixture (milligrams) were determined.

The cell-associated radioactivity measured in these experiments might be a combination of uptake, extracellular binding, and association with any nonwashed 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 uptake, except when classic transport methodology was used. 

Acivicin (500 μM; 10-min preincubation) was used for estimating the extent of radiolabeled thiol uptake measured by GGT, which cleaves GSH to glutamate and cysteinylglycine (19). Experiments were performed in Na+-HEPES and Na+-free HEPES buffers; results were equivalent.

Cellular transport of cysteine and cystine uptake was performed using inhibitors selective for each transporter system. ASC, L, Xc, and χ were inhibited using 1 mM serine, 1 mM 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid, 400 μM l-aspartic acid-β-hydroxamate, or 200 μM quisqualate, respectively (14). All chemicals were obtained from Sigma.

Michaelis 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), bromsulphthalein (1–100 μM), and GSH disulfide (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 l-[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 μl to a final concentration of 0.1–2 μM. 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% in buffer and 98% 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 l-[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 × g for 10 min. The supernatant was saved, and the pellet was further fractionated using Opti Prep, as described in the manufacturer’s booklet (Nycowood Pharma, Oslo, Norway). To isolate nuclei, the 1,000 × g pellet was homogenized in dilaurein 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 × g for 10 min. The pellet was layered on the 25/30/35% (w/v) discontinuous gradient of Opti Prep and centrifuged at 20,000 × g for 20 min. The nuclear fraction was collected from a distinct band at the 30/35% interface. The initial supernatant, saved after the first centrifugation at 1,000 × g, was centrifuged at 100,000 × 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 membrane-bound cystolic thiol. This supernatant was treated with 10% (final concentration) TCA, then centrifuged at 14,000 × g for 10 min to precipitate soluble proteins and any protein-bound thiol. Radioactivity in the TCA supernatant represented free thiol.

Each cell fraction was studied to verify the success of the separation procedure. We found 93% (mean; n = 3) of the total activity of GGT in the membrane fraction, consistent with the known localization of this enzyme (19, 23). In contrast, 99% (mean; n = 3) of the total activity of glucose-6-phosphate dehydrogenase (19) was located in the cytosolic fraction.

Release of radioactivity from monocytes

Monocytes were incubated with l-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 l-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 nM 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).
of the marked decrease in cysteine uptake. Total thiol uptake decreased during the respiratory burst because Na⁺-dependent (Figs. 1 and 3). This transport was inhibited 43% by DPI (21, 22). Release of O₂⁻ and rate of GSH uptake as a function of the time after addition of PMA. C, Effect of preincubation for 10 min with varying concentrations of DPI on PMA-stimulated release of O₂⁻ and uptake of GSH. Data points in A, B, and C represent the mean ± SEM (n = 3).

Data presentation
All data shown are the mean ± SEM of n experiments, each performed in duplicate or triplicate. Statistical significance was determined using Student’s t test unless otherwise specified.

Results
Thiol uptake in human monocytes during the respiratory burst
Under basal culture conditions, in Na⁺-HEPES buffer were stimulated with PMA, and the uptake of [³⁵S]-labeled Cys₂, 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 Cys₂ uptake was not significant.

The relationship between the PMA-induced 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 O₂⁻ and uptake of GSH were inhibited to an equivalent extent over a range of DPI concentrations of 0.2–2 μM (Fig. 2C). Preincubation for 10 min with 1 μM DPI had no effect on the increased GSH uptake stimulated by 0.5 mM H₂O₂ (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 that 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%) of 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 xₐ, a glutamate/cystine exchanger. Sodium-dependent cystine
transport was inhibited 83 ± 12% (n = 4) by L-aspartic acid-β-hydroxamate, a specific inhibitor of system X_{AG}, which also transports aspartate and glutamate.

Cysteine transport was only 24% Na\textsuperscript{+} independent (Figs. 1 and 3). Most (82 ± 2%; n = 4) of the Na\textsuperscript{+}-dependent transport was inhibited by serine, implicating system ASC. The Na\textsuperscript{+}-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\textsuperscript{+}-independent GSH uptake

The effect of the respiratory burst on uptake of GSH in Na\textsuperscript{+}-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\textsuperscript{+}-HEPES buffer (Fig. 2A). The characteristically slower rate of O\textsubscript{2}\textsuperscript{2} 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, H\textsubscript{2}O\textsubscript{2}. Uptake of GSH during the PMA-induced respiratory burst was consistently (but not statistically significantly) further enhanced by SOD, which accelerates H\textsubscript{2}O\textsubscript{2} production (Fig. 5). In contrast, catalase, which removes H\textsubscript{2}O\textsubscript{2}, 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, H\textsubscript{2}O\textsubscript{2} plays a particularly important role in inducing GSH uptake. In support of this conclusion, H\textsubscript{2}O\textsubscript{2} 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 H\textsubscript{2}O\textsubscript{2} 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 H\textsubscript{2}O\textsubscript{2}, 214 ± 28 pmol/mg; control, 27.5 ± 6.6 pmol/mg (n = 3).

Because the GSH used for uptake studies was radiolabeled on the cysteinyl moiety, it was necessary to demonstrate that we were measuring the uptake of intact GSH and not cysteinylglycine or cysteinylglycine.
cysteine released by the action of GGT, then dipeptidase. Therefore, cells were preincubated for 10 min with acivicin, 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 H$_2$O$_2$ 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 H$_2$O$_2$ (10 $\mu$M; 30 min) to convert GSH to GSSG, uptake of [35S]GSH was less than uptake at baseline (no stimulus or H$_2$O$_2$ added; 9 ± 1 pmol/mg protein vs baseline, 20 ± 3 pmol/mg; mean ± SEM; n = 3). In these experiments, H$_2$O$_2$ 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 GSH was preincubated with the strong oxidant diamide (50 $\mu$M).

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 level of cytosolic free GSH by 2.1-fold and cytosolic protein-bound GSH by 3.4-fold (mean; n = 3), in agreement with our previous studies showing avid binding of GSH to cytosolic 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 H$_2$O$_2$, 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 ($K_m$) for GSH in both the presence and the absence of added H$_2$O$_2$ (Table II). The $K_m$ demonstrated was in the range of the concentration of GSH in plasma under physiologic conditions (15, 24, 25). In contrast, the $V_{max}$ was increased 11-fold in the presence of H$_2$O$_2$. 

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**Table II. Effect of H$_2$O$_2$ and transport inhibitors on GSH uptake in human monocytes**

<table>
<thead>
<tr>
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<th>H$_2$O$_2$ (–)</th>
<th>H$_2$O$_2$ (+)</th>
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<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>6.1 ± 0.3</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>$V_{max}$ (pmol/mg/min)</td>
<td>3.8 ± 1.8</td>
<td>42 ± 9.0</td>
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*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. $K_m$ was calculated from Lineweaver-Burk plots, and $K_i$ was calculated by using the Dixon plot. BSP, Bromsulphthalein.

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*Fig. 8. Uptake and distribution of GSH in monocytes during the respiratory burst. Monocytes were preincubated with 1-[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 $\times$ g for 10 min. The supernatant was separated into the membrane fraction, obtained as the pellet after centrifugation at 100,000 $\times$ 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 several-fold higher were required for inhibition of GSH transport by GSSG or bromsulfophthalein (Table II).

**GSH uptake in lymphocytes**

The role of $H_2O_2$ 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 ($p < 0.002$). 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 $H_2O_2$, 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 $H_2O_2$ (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 in human monocytes is also accompanied by increased uptake of GSH into the cell, up to 10-fold over baseline. Virtually all animal cells synthesize GSH (3). Most of the body’s GSH is intracellular, >99% remaining in the reduced form in the absence of oxidant stress, in concentrations of 0.5–10 mM (3, 28). Total GSH in plasma has been reported to be 6–33 $\mu$M (15, 24, 25), 60% as reduced GSH (28). Synthesis is achieved using the constituent amino acids (Glu, Cys, and Gly) supplied in part by the breakdown of plasma GSH by the ectoenzymes GGT and dipeptidase (9, 19). The key sulfhydryl amino acid cysteine and, in greater concentration, its oxidized form cystine also appear in plasma and are available for transport (24, 25). Cysteine and cystine transport has been shown to be essential for GSH synthesis in lymphocytes, macrophages, and other cells (29, 30).

We report here that under basal conditions human monocytes take up cysteine primarily by a $Na^+$-dependent system (Fig. 1 and Fig. 3). This system appears to be system ASC, based on the defining inhibition studies (9, 14) as used by us previously with alveolar type II cells (14). System L appeared to play a lesser role in monocyte uptake of cysteine. In contrast, transport of cystine was predominantly (72%) $Na^+$ independent and mediated at least in part by system $X_A[G]$. In the presence of $Na^+$, cystine was transported primarily by system $X_{AG}$. This finding supports the conclusion of Rimanion et al. (31) that human mononuclear phagocytes possess a system $X_{AG}$ cystine/glutamate transporter. They have proposed that this transporter might clear neuroexcitatory glutamate from the brain. The changes induced by the respiratory burst in uptake of all three thiols were similar in the presence and the absence of $Na^+$ (Figs. 1 and 3).

Our data suggest that the respiratory burst- and $H_2O_2$-induced increase seen in uptake of GSH, which was $^{35}$S-radio labeled in the Cys moiety, was due primarily to transport of intact GSH. Preincubation of the cells with acivicin, which inhibits GSH breakdown by $\gamma$-glutamyl transpeptidase (19), had no effect on the $H_2O_2$-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 $H_2O_2$ GSH peroxidase or diadime reduced uptake of the thiol to 30–50% of the baseline (no $H_2O_2$) 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 diadime on the cell, but the facts that $[^{35}S]$GSH uptake was inhibited by the strong oxidant diadime and was not increased by hypochlorous acid support the conclusion that the $H_2O_2$-induced cell-associated radioactivity is due primarily to uptake, not oxidant-stimulated mixed disulfide formation with plasma membrane ectoproteins. 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 $H_2O_2$ peaked at 15 min. SOD, which removes $O_2^-$ by catalyzing its dismutation into $H_2O_2$, increased GSH uptake slightly, whereas catalase reduced the stimulatory effect of PMA by >80% (Fig. 5). $H_2O_2$, 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. 2C). 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 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 and to activate RBC KCl cotransport (47).

References
16. Ballatori, N., and G. Rebbeor. 1998. Roles of MRP2 and oatp1 in hepatocellular protection and maintaining these regulatory systems, and thus cell timing of increased GSH concentration might also be important to protecting and maintaining these regulatory systems, and thus cell function.

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