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Immunology

Arachidonic acid metabolism in glutathione-deficient macrophages

(leukotriene/prostaglandin/buthionine sulfoximine/hydroxyicosatetraenoic acid/phagocytosis)

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ABSTRACT **Mouse resident peritoneal macrophages were treated with the glutathione (GSH) synthesis inhibitor buthionine sulfoximine to deplete intracellular GSH. The arachidonic acid metabolites released by the GSH-depleted macrophages in response to a zymosan challenge were analyzed by HPLC. Buthionine sulfoximine treatment resulted in inhibition of both prostaglandin E_2 and leukotriene C synthesis that was directly related to the degree of GSH depletion. Macrophages in which GSH levels were reduced to 3% of normal exhibited reductions to 4% and 1%, respectively, in PGE_2 and LTC formation. The total quantity of cyclooxygenase metabolites secreted by GSH-deficient macrophages was identical to that of control cells as a result of increased synthesis of prostacyclin and, to a lesser extent, 12-L-hydroxy-5,8,10-heptadecatrienoic acid. Total lipoxygenase products were decreased, however; increased formation of hydroxyicosatetraenoic acids only partially compensated for the deficit in leukotriene C production. These findings extend our earlier observations on the inhibition of leukotriene C synthesis in GSH-depleted macrophages and confirm with intact cells the previously suggested role of GSH in prostaglandin E_2 formation.**

We recently showed that depletion of intracellular glutathione (GSH) with the GSH synthesis inhibitor buthionine sulfoximine (Bso) inhibits leukotriene C [5(*S*)-hydroxy-6(*R*)-glutathionyl-7,9,11,14-icosatetraenoic acid; LTC] synthesis by mouse peritoneal macrophages (1). This finding supported a biosynthetic pathway for LTC in which GSH is a direct precursor (2).

In this report, we present further studies indicating that, although GSH-depleted macrophages secrete total cyclooxygenase products in quantities identical to those of control cells, there is a specific decrease in the synthesis of the cyclooxygenase metabolite prostaglandin (PG) E_2 and a compensatory increase in prostacyclin formation. The inhibition of PGE_2 formation is directly related to the extent of GSH depletion.

MATERIALS AND METHODS

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of specific pathogen-free female and male ICR mice (Trudeau Institute, Saranac Lake, NY) weighing 25–30 g as described (3). Peritoneal cells (6×10^6/ml) in α minimal essential medium (α-Me medium; GIBCO)/10% fetal calf serum were added to 35-mm-diameter plastic culture dishes (1 ml per dish) or to 12-mm glass coverslips (0.1 ml per coverslip). After 2 hr at 37°C in 5% CO_2/95% air, cultures were washed three times in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (P_i/NaCl) to remove nonadherent cells. Fresh medium/10% serum (1 ml per dish) containing [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid ([^{3}H]20:4; specific activity, 78.2 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) at 0.5 μCi/ml was then added, and the cells were incubated overnight. [^{3}H]20:4 was omitted from cells cultured on coverslips.

Preparation of Unopsonized Zymosan. Zymosan was purchased from ICN (Plainview, NY), and stock suspensions in α-Me medium were prepared by the method of Bonney *et al.* (4).

Incubation of Macrophages with Bso. Bso was synthesized as described (5, 6). Sterile stock solutions (P_i/NaCl, 20 mM) were diluted 1:100 in α-Me medium for addition to macrophage cultures.

Assay for Release of 20:4 Metabolites. Macrophages labeled overnight with [^{3}H]20:4 were washed twice with P_i/NaCl, and zymosan (160 μg/ml) in serum-free α-Me medium was added. After 2 hr at 37°C in 5% CO_2/95% air, the medium was removed, and the 20:4 metabolites were extracted by a modification of the method of Unger *et al.* (7). Briefly, to 1 ml of medium was added 1 ml of ethanol and 10 μl of 88% (wt/wt) formic acid. The resulting solution was then extracted with two 1-ml portions of chloroform/0.005% butylated hydroxytoluene (Sigma). The chloroform phases were combined and concentrated to dryness under nitrogen. The sides of the tubes were rinsed during the drying procedure with chloroform/methanol, 2:1 (vol/vol) and ethanol/water, 4:1 (vol/vol) to improve the recovery of 20:4 metabolites. The residues were dissolved in 400 μl of solvent 1, [methanol/water/acetic acid, 65:34.9:0.1 (vol/vol/vol), pH 5.4], and subjected to HPLC on a column (4.6 mm $\times$ 25 cm) of 5-μm Ultrasphere ODS (Altex, Rainin Instruments, Brighton, MA). The column was eluted with 60 ml of solvent 1 followed by 40 ml of solvent 2 [methanol/water/acetic acid, 75:25:0.01 (vol/vol/vol)] and 40 ml of solvent 3 [methanol/acetic acid, 100:0.01 (vol/vol)] at a flow rate of 1 ml/min (system 1). In some experiments, the elution with solvent 2 was omitted (system 2). Fractions of 1 ml were collected, and the radioactivity of whole fractions or aliquots was measured by liquid scintillation counting in 2 ml of Hydrofluor (National Diagnostics, Somerville, NJ) using an LKB Ultrobeta scintillation counter. Corrections were made for counting efficiency (40%).

For separation of individual cyclooxygenase products, fractions 4–15 from HPLC systems 1 or 2 were combined, evaporated at reduced pressure, and dissolved in 400 μl of solvent 4 [water/acetonitrile/benzene/acetic acid, 76.7:23:0.2:0.1 (vol/vol/vol/vol)] for further HPLC on a Waters fatty acid analysis column (system 3). PGs and thromboxane (TX) were eluted with 100 ml of solvent 4 at a flow rate of 2 ml/min, and the column was then washed with 40 ml of solvent 3. Fractions of 2 ml were collected, and their radioactivity was measured in 3 ml of Hydrofluor (counting efficiency, 30%).

Assay of GSH and Cell Protein. Cell monolayers were washed twice with P_i/NaCl and scraped into 500 μl of 0.05%

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Abbreviations: LTC, leukotriene C; 20:4, arachidonic acid; Bso, buthionine sulfoximine; α-Me medium, α minimal essential medium; P_i/NaCl, Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline; PG, prostaglandin; TX, thromboxane; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HETE, hydroxyicosatetraenoic acid; GSH, glutathione.

Triton X-100 (Rohm and Haas, Philadelphia, PA). Samples of the cell lysates (190 μl) were acidified with 10 μl of 0.1 M HCl, and protein was precipitated by addition of 10 μl of 50% (wt/vol) sulfosalicylic acid. The protein was removed by centrifugation, and the supernatants were assayed for total GSH (GSH and oxidized GSH) by a modified 5,5′-dithiobis(2-nitrobenzoic acid)–GSH reductase procedure (8, 9). GSH standards contained Triton X-100, sulfosalicylic acid, and HCl in quantities identical to the samples.

The protein content of Triton X-100 macrophage cell lysates was determined by the method of Lowry, *et al.* (10) using bovine serum albumin as a standard.

Cell Viability and Phagocytic Index. Cell viability was assessed by trypan blue exclusion using cells cultured on glass coverslips under conditions identical to those used in the experiments. The extent of phagocytosis of zymosan was measured by direct phase-contrast microscopy of macrophages incubated on glass coverslips in the presence of zymosan and then fixed in 2.5% (wt/vol) glutaraldehyde. Data are expressed as phagocytic index, which is defined as percent of cells that ingested zymosan times the average number of particles per cell (11).

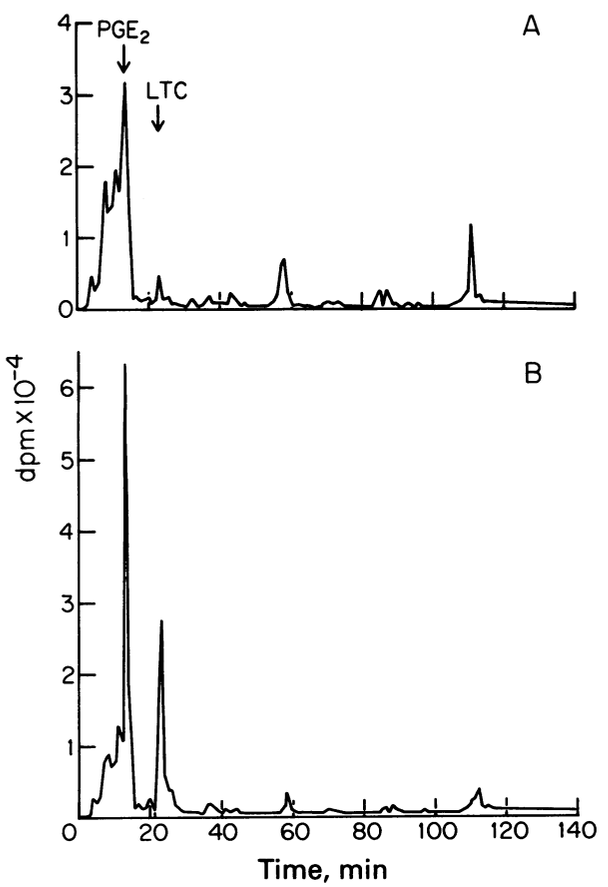

FIG. 1. HPLC of 20:4 metabolites from Bso-treated macrophages. (*A*) Macrophages were labeled for 16 hr with [^{3}H]20:4; Bso (200 μM) was present in the culture medium for 12 hr of the labeling period. The monolayers were then washed and placed in serum-free α-Me medium/200 μM Bso containing unopsonized zymosan at 160 μg/ml. After 2 hr at 37°C, the medium was removed and the [^{3}H]20:4 metabolites were extracted. The extracts were concentrated under nitrogen and subjected to HPLC using system 1. The ^{3}H elution profile obtained from the combined medium of three 35-mm cultures is shown. (*B*) As in *A* except that Bso was omitted. Elution times: cyclooxygenase products, 4–15 min; LTC, 23 min; HHT, 58 min; diHETEs and other polar lipoxygenase products, 30–50 min; HETEs, 60–100 min; 20:4, 111 min. Recovery of 20:4 metabolites through extraction and HPLC was ≈70%.

Calculations. The radiolabel content of HPLC column fractions containing 20:4 metabolites was converted to pmol/μg of cell protein by the following formula:

$$\text{pmol of 20:4 metabolite}/\mu\text{g of cell protein} = \frac{\text{dpm}_1}{\text{dpm}_2} \times 123,$$

in which dpm_1 is the ^{3}H content of the isolated metabolite, dpm_2 is the total ^{3}H incorporated by the cells, and 123 corrects for the distribution of 70% of total incorporated radiolabel in cell phospholipid and a macrophage phospholipid 20:4 content of 86 pmol/μg of cell protein (12, 13). The values thus obtained were then corrected for recovery of metabolites through the extraction and purification procedure.

Radiolabeled Standards. The elution characteristics and recoveries of 20:4 metabolites during extraction and HPLC were monitored with the aid of radiolabeled standards. Each standard was purified by HPLC and then dissolved in α-Me medium from unlabeled macrophage cultures that had been exposed to

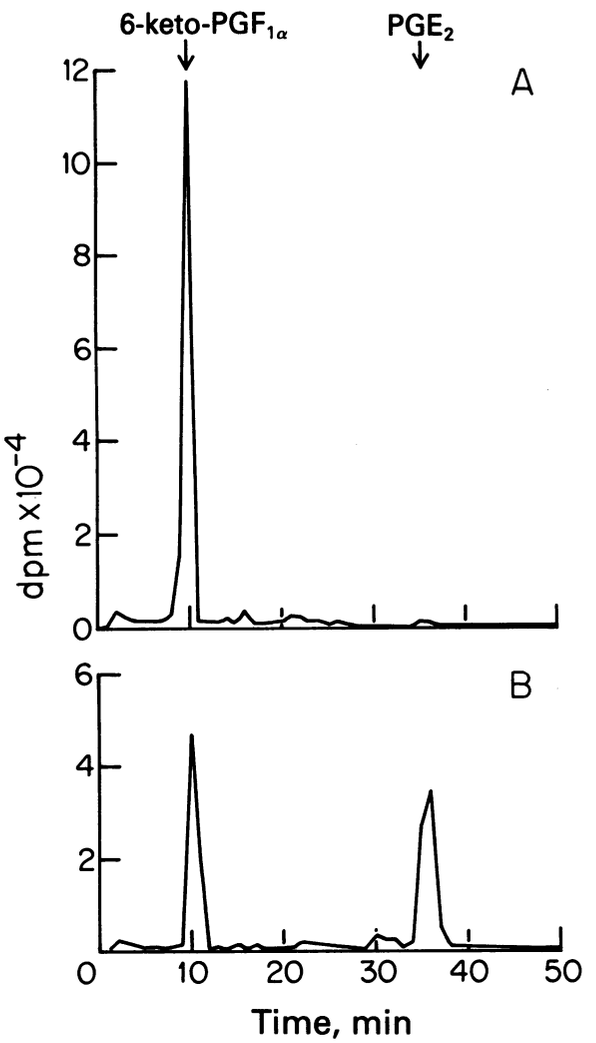

FIG. 2. HPLC of cyclooxygenase products synthesized by GSH-depleted and control macrophages. (*A*) Conditions for macrophage culture are described in the legend to Fig. 1*A*. Concentrated medium extracts were subjected to HPLC by system 1. Fractions eluting at 4–15 min were combined, concentrated, and subjected to HPLC using system 3. ^{3}H contents of peaks eluting at 10 and 36 min were 134,400 and 2900 dpm, respectively. (*B*) As in *A* except that Bso was omitted. ^{3}H contents of peaks eluting at 10 and 36 min were 67,100 and 70,600 dpm, respectively. Elution times: 6-keto-$PGF_{1\alpha}$, 10 min; TXB_2, 22 min; $PGF_{2\alpha}$, 27 min; PGE_2, 36 min. Recovery of PGs was 60–65% through extraction and two HPLC systems.

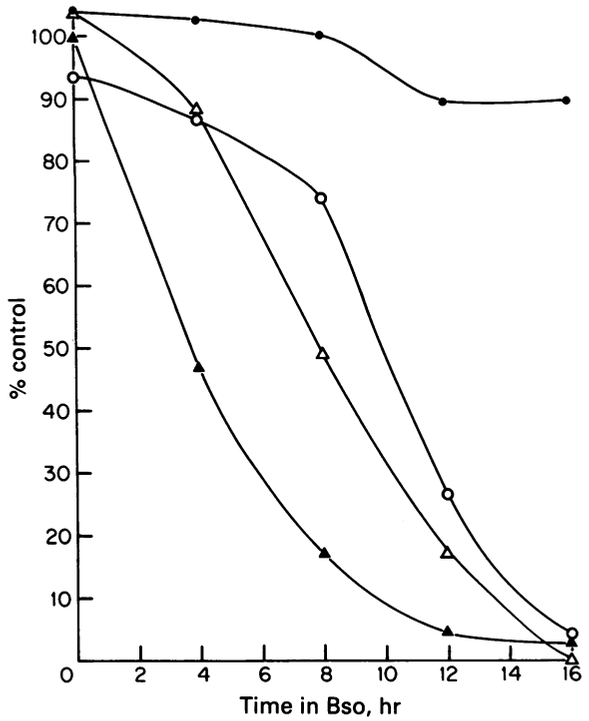

FIG. 3. Effect of GSH depletion on PGE_2 (○), LTC (△), and total 20:4 (●). Macrophage monolayers were exposed to 200 μM Bso for the indicated times during the [^{3}H]20:4-labeling period. The cells were then washed, placed in serum-free α-Me medium/200 μM Bso containing zymosan at 160 μg/ml, and incubated for 2 hr at 37°C. 20:4 metabolites were extracted from the combined medium of triplicate cultures, and crude extracts were subjected to HPLC using system 1. Material eluting at 4–15 min was combined and chromatographed on HPLC system 3. LTC was quantitated from the ^{3}H content of fractions eluting at 20–25 min on HPLC system 1. PGE_2 was quantitated from the radioactivity of fractions eluting at 35–37 min on HPLC system 3. Total 20:4 release was calculated from the radioactivity of the culture medium. GSH levels (▲) were determined from parallel triplicate cultures incubated with Bso for the given times in the absence of [^{3}H]20:4. These cultures were not exposed to zymosan. Data are expressed as percent of values obtained from control cultures that received no exposure to Bso. These cultures contained 38 pmol of GSH/μg of cell protein and released 24 pmol of 20:4/μg of cell protein, 5.0 pmol of PGE_2/μg of cell protein, and 4.4 pmol of LTC/μg of cell protein.

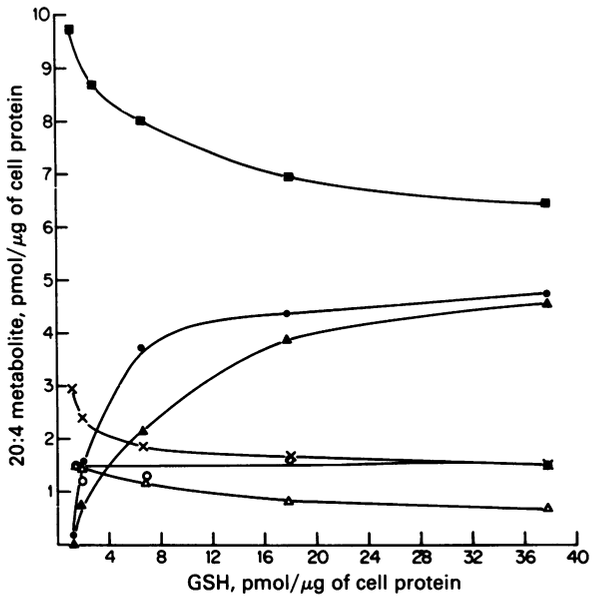

FIG. 4. 20:4 metabolites released by GSH-depleted macrophages. Experimental details are identical to those in Fig. 3 except that HPLC system 2 was used instead of system 1. PGE_2 (●), 6-keto-$PGF_{1\alpha}$ (■), and LTC (▲) were measured as described in Fig. 3. HHT (△), diHETEs (and other polar lipoxygenase products) (○), and HETEs/unreacted 20:4 (×) were determined from radioactivity eluting from HPLC system 2 at 58 min, 30–50 min, and 60–80 min, respectively.

zymosan. Conditions for extraction and HPLC of these solutions were identical to those used for experimental samples. Sources of standards were as follows: 6-keto-[5,8,9,11,12,14,15-^{3}H]$PGF_{1\alpha}$ (80–120 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]TXB_2 (100–150 Ci/mmol), [9-^{3}H]$PGF_{2\alpha}$ (5–15 Ci/mmol), and [5,6,8,11,12,14,15-^{3}H]PGE_2 (100–200 Ci/mmol) were purchased from New England Nuclear. 12-[^{3}H]Hydroxy-5,8,10,14-icosatetraenoic acid (12-HETE) and 5-[^{3}H]hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE) were obtained from calcium ionophore A23187-stimulated human blood platelets (14) and polymorphonuclear leukocytes (15), respectively, and were the gift of Nicholas Pawlowski of The Rockefeller University. [^{3}H]LTC was obtained from [^{3}H]20:4-labeled mouse peritoneal macrophages and was purified as described (13). The elution characteristics of dihydroxyicosatetraenoic acids (diHETEs) and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) were determined from those of the corresponding 20:4 metabolites of polymorphonuclear leukocytes (16) and platelets (14), respectively. In addition, the elution times of diHETEs on reverse-phase HPLC (9 to 10 min) using solvent 2 as the only eluting solvent were identical to those reported in the literature (16). The synthesis of HHT by both platelets and macrophages was inhibited (20% of control) by indomethacin.

RESULTS

Inhibition of PGE_2 Synthesis with GSH Depletion. Previous work indicated that treatment of macrophages with 200 μM Bso results in 80–90% inhibition of GSH synthesis and exponential loss of intracellular GSH (1). Exposure of macrophages to 200 μM Bso for time periods of 4–16 hr resulted in progressive reduction in zymosan-induced LTC release concomitant with depletion of intracellular GSH. We have now extended these studies of GSH depletion and 20:4 metabolism using HPLC analysis (systems 1 and 2) that allows separation of the cyclooxygenase and lipoxygenase products secreted by macrophages. [^{3}H]20:4-labeled macrophages were challenged with zymosan after 0 or 12 hr of exposure to Bso and analyzed by HPLC. Chromatograms of crude medium extracts from these cultures are shown in Fig. 1. The samples from Bso-treated cultures show sharp reductions in the radiolabeled compounds eluting at 13 and 23 min, when compared with those from control cultures (no Bso); PGE_2 (in addition to some 6-keto-$PGF_{1\alpha}$) and LTC, respectively, elute in these fractions. The material eluting from 4 to 15 min was combined and rechromatographed using HPLC system 3. The resulting chromatograms (Fig. 2) indicated that major radiolabeled peaks corresponding to 6-keto-$PGF_{1\alpha}$ (10 min) and PGE_2 (36 min) were obtained from control cultures. In comparison, the quantity of 6-keto-$PGF_{1\alpha}$ was increased and that of PGE_2 was decreased in the medium from Bso-treated cultures.

The relationship between intracellular GSH content and 20:4 metabolism is shown in Fig. 3. The zymosan-induced release of total 20:4, PGE_2, and LTC by Bso-treated cultures in addition to their GSH content are presented as percent of control (no Bso). Release of both LTC and PGE_2 decreased as cellular GSH levels decreased, although total 20:4 release was only minimally affected. Lower GSH levels (3.6 pmol/μg of cell

Table 1. Effect of GSH depletion on 20:4 metabolism

Time in Bso before zymosan exposure, hr	GSH	20:4 metabolite, pmol/μg cell of protein			Total 20:4, pmol/μg of cell protein
		Total cyclooxygenase*	Total lipoxygenase†	Unidentified‡	
Control	37.7 ± 2.3	10.96	7.03	5.51	23.5 ± 2.5
0	37.7 ± 2.3	11.81	7.46	5.73	25.0 ± 1.6
4	17.8 ± 0.6	12.13	7.15	5.42	24.7 ± 0.5
8	6.6 ± 0.6	12.91	5.30	5.19	23.4 ± 0.6
12	1.9 ± 1.1	11.45	4.33	5.02	20.8 ± 0.3
16	1.2 ± 1.0	11.40	4.49	5.01	20.9 ± 0.9

Experimental details are identical to those in Fig. 4. GSH and total 20:4 data are mean ± SD of triplicate cultures.
* Sum of PGE_2, 6-keto-$PGF_{1\alpha}$, and HHT.
† Sum of polar HETEs, LTC, HETEs, and unreacted 20:4.
‡ Polar metabolites that coelute with PGs in HPLC system 1 but not in HPLC system 3.

protein) were required to obtain 50% inhibition of PGE_2 synthesis than were required for similar inhibition of LTC synthesis (6.7 pmol of GSH/μg of cell protein).

Effect of GSH Depletion on Other 20:4 Metabolites. In our earlier studies, silicic acid column chromatography was used to characterize the 20:4 metabolites synthesized by Bso-treated macrophages. These experiments indicated that GSH depletion resulted in an increase in synthesis of 20:4 metabolites having the elution characteristics of HETEs. These findings were explored further by using HPLC systems 1 and 2. As indicated in Fig. 1, no single radiolabeled peak was produced in markedly increased quantity by Bso-treated macrophages. Instead, moderate increases were observed in the multiple minor peaks eluting at 50–120 min (system 1). These compounds include HETEs, HHT, and unreacted 20:4. The changes in total 20:4 metabolites resulting from GSH depletion are shown in Fig. 4. As indicated in Table 1, the total quantity of cyclooxygenase products released by GSH-deficient macrophages was identical to that of control cells. The net total 20:4 metabolites isolated from the medium of drug-treated cultures was decreased up to 15%, depending on the degree of GSH depletion. This could be accounted for by deficits in lipoxygenase metabolites (up to 35%). Therefore cyclooxygenase products constituted a somewhat larger proportion (55%) of total 20:4 metabolites released by Bso-treated cells than by control cells (47%).

Effect of GSH Depletion on the Phagocytosis of Zymosan. In some experiments, it was noted that an inhibition of phagocytosis occurred with decreased intracellular GSH. Data from such an experiment are presented in Table 2. It is evident that the decrease in phagocytosis was not due to loss of cell viability and did not result in similar inhibition of 20:4 release.

Table 2. Effect of GSH depletion on phagocytosis and cell viability

GSH, pmol/μg of cell protein	Phagocytic index	Cell viability, % of control
35.2 ± 1.4	515 ± 47	96 ± 1
5.0 ± 0.3	352 ± 40	97 ± 1
2.1 ± 1.2	332 ± 18	95 ± 1
1.0 ± 0.2	284 ± 8	96 ± 1
<1	212 ± 32	97 ± 2

Macrophage cultures on glass coverslips and in 35-mm dishes were depleted of GSH by exposure to Bso. GSH was determined on cultures in 35-mm dishes that had not been exposed to zymosan. Macrophages on glass coverslips were exposed to zymosan at 160 μg/ml for 2 hr at 37°C. Cell viability was assessed by trypan blue exclusion, and phagocytic index was determined by direct phase-contrast microscopy after fixation with 2.5% glutaraldehyde. Results are mean ± range of duplicate determinations.

DISCUSSION

The data presented here indicate that macrophages depleted of intracellular GSH synthesize reduced quantities of PGE_2 and LTC when challenged with a phagocytic stimulus. The degree of inhibition of both PGE_2 and LTC synthesis increases as the levels of intracellular GSH fall and approaches 100% in severely depleted cells (<3 pmol of GSH/μg of cell protein). These findings provide evidence that GSH is required for PGE_2 synthesis by intact macrophages and are in agreement with studies using cell-free enzyme preparations (17). Those earlier studies indicate that GSH is a cofactor for the enzyme prostaglandin endoperoxide E isomerase, which converts PGH_2 to PGE_2. Thus, the cofactor role of GSH in PGE_2 synthesis is distinct from its precursor role in LTC formation. It is of interest, therefore, that the sensitivities of these two biosynthetic pathways to GSH depletion are so similar.

Macrophages exhibiting decreasing levels of GSH and 0–100% inhibition of PGE_2 synthesis released total quantities of cyclooxygenase products identical to those released by untreated control cells. The deficit in PGE_2 production was compensated for in these macrophages by large increases in prostacyclin (isolated as 6-keto-$PGF_{\alpha 1}$), together with minor increases in HHT formation. Thus, GSH depletion had no direct effect on cyclooxygenase activity, and a deficit in the synthesis of the cyclic endoperoxide precursors of PGE_2 is probably not the reason for the diminished PGE_2 release.

In our initial studies, the results indicated that 20:4 that was not used for LTC synthesis was diverted to production of other lipoxygenase products (1). Data obtained by HPLC analysis, however, showed that, although the release of HETEs by GSH-deficient macrophages was somewhat elevated over that of control cells, this increase did not completely compensate for the deficit in LTC formation. This may result in part from a 10–15% inhibition of total 20:4 release by GSH-depleted macrophages. Alternatively, reesterification of unmetabolized 20:4 or uptake of HETEs, which is reported to occur in polymorphonuclear leukocytes (18), would also influence the apparent recovery of total 20:4 metabolites and lipoxygenase products.

During the course of these studies, we observed that GSH depletion was associated with inhibition of phagocytosis in some, but not all, experiments. Although it is possible that this was due to a nonspecific toxic effect of GSH deficiency or of Bso, cell viability was not reduced in the cultures. Possibly the proposed role for GSH in microtubule assembly (19) could account for the decrease in zymosan uptake by GSH-deficient macrophages. The inhibition of phagocytosis was not associated with a similar decrease in 20:4 release. Results of earlier studies showed that the necessary step for initiation of 20:4 metabolism

by macrophages is the interaction of particle-bound ligands with specific plasma membrane receptors, indicating that particle interiorization is not required (20).

It should be noted that these studies required the use of specific pathogen-free mice as the source of cells. GSH-depleted macrophages from animals that were not pathogen free exhibited a pronounced (40–50%) reduction in total release of 20:4 metabolites. It is possible that subclinical infections in these mice resulted in alterations in the physiologic state of the macrophages that were accompanied by increased susceptibility to the toxic effects of GSH depletion.

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