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Induction of nitric oxide synthesis in J774 cells lowers intracellular glutathione: effect of modulated glutathione redox status on nitric oxide synthase induction

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Under pathological conditions, the induction of nitric oxide synthase (NOS) in macrophages is responsible for NO production to a cytotoxic concentration. We have investigated changes to, and the role of, intracellular glutathione in NO production by the activated murine macrophage cell line J774. Total glutathione concentrations (reduced, GSH, plus the disulphide, GSSG) were decreased to 45% of the control 48 h after cells were activated with bacterial lipopolysaccharide plus interferon γ. This was accompanied by a decrease in the GSH/GSSG ratio from 12:1 to 2:1. The intracellular decrease was not accounted for by either GSH or GSSG efflux; on the contrary, rapid export of glutathione in control cells was abrogated during activation. The loss of intra- and extracellular glutathione indicates either a decrease in synthesis *de novo*, or an increase in utilization, rather than competition for available NADPH. All changes in activated cells were prevented by pretreatment with the NOS inhibitor L-*N*-(1-iminoethyl)ornithine. Basal glutathione levels in J774 cells were manipulated by pretreatment with (1) buthionine sulphoximine (glutathione synthase inhibitor), (2) acivicin (γ-glutamyltranspeptidase inhibitor), (3) bromo-octane (glutathione S-transferase substrate) and (4) diamide/zinc (thiol oxidant and glutathione reductase inhibitor). All treatments significantly decreased the output of NO following activation. The degree of inhibition was dependent on (i) duration of treatment prior to activation, (ii) rate of depletion or subsequent recovery and (iii) thiol end product. The level of GSH did not significantly affect the production of NO, after induction of NOS. Thus, glutathione redox status appears to plays an important role in NOS induction during macrophage activation.

INTRODUCTION

Nitric oxide (NO) and related nitroso compounds are important physiological molecules involved in vascular regulation, neurotransmission and the immune response [1–3]. In pathological conditions, the induction of nitric oxide synthase (NOS) in macrophages is responsible for NO production, to a level at which cytotoxicity and microbial killing occur [4,5]. The molecular species responsible has been identified as NO (NO*, NO^+ and NO^-), although other molecules such as peroxynitrite ($ONOO^-$) have been implicated [6,7].

Cytotoxic effects and host defence by NO are achieved by inhibition of key regulatory enzymes, including glyceraldehyde 3-phosphate dehydrogenase, ribonucleotide reductase, glutathione peroxidase [8–10] and in particular the blocking of oxidative phosphorylation at complex IV (cytochrome *c* oxidase) [11].

Intracellular mechanisms are also present to protect host cells from cytotoxic attack by NO, but these mechanisms are not clearly defined. NO can be made less reactive by combining with thiols, in particular with glutathione to form *S*-nitrosoglutathione (GSNO) [12,13]. Glutathione is the most abundant low molecular mass thiol and is crucial for protection of cells from oxidant attack [14]. Other vital functions of GSH (reduced glutathione) include a role in membrane transport, protein and nucleic acid synthesis, gene expression and signal transduction [15–17]. Recently, in murine macrophages and rat hepatocytes, a link has been made between the intracellular glutathione concentration and the level of NOS mRNA transcript following activation [18,19]. In contrast, in hepatic macrophages there was no effect of lowering glutathione on NO production [20]. Glutathione also causes a severalfold elevation in NOS intrinsic activity and is essential to prevent depletion of NOS activity in activated macrophages [21]. Preloading of human umbilical endothelial cells with GSH also increases the level of NO production [22].

During aging, and in disease states such as diabetes and AIDS, there is a diminution of intracellular glutathione [23,24] and this is associated with impaired cellular function, including a decrease in antioxidant status, transport and biosynthetic capacity. However, the role of intracellular GSH in NO-mediated cytotoxicity, and antioxidant protection in relation to NO production, is not known.

Using a murine macrophage cell line (J774), we have investigated changes in intracellular glutathione concentrations and redox status following the induction of NO production by activation with interferon (IF)-γ plus lipopolysaccharide (LPS). We have also investigated the role of glutathione homoeostasis, both before and after NOS induction, in regulating NO synthesis.

Abbreviations used: IF, interferon; LPS, lipopolysaccharide; NOS, nitric oxide synthase; FCS, foetal calf serum; L-NIO, L-*N*-(1-iminoethyl)ornithine; GSNO, *S*-nitrosoglutathione; ETA, ethacrynic acid; BSO, buthionine sulphoximine; BrO, 1-bromo-octane; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; 2VP, 2-vinylpyridine.

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EXPERIMENTAL

Materials

Murine IF-γ was a gift from Professor F. Liew, Glasgow University, U.K. Bacterial LPS was supplied from Difco Laboratories, West Molesey, U.K.; RPMI medium, foetal calf serum (FCS), penicillin and streptomycin from Gibco, Paisley, U.K. Ethacrynic acid (ETA), buthionine sulphoximine (BSO), 1-bromo-octane (BrO), acivicin, diamide, GSH and glutathione reductase were all supplied from Sigma Chemical Co., Poole, Dorset, U.K., and 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) from Bristol Meyers, Hereford, U.K. All other chemicals used were Analar or equivalent grade.

Macrophage activation

J774.2 cells were grown in culture in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) as previously described [25]. Cells were harvested from culture flasks by scraping and plated out at a density of 5×10^5/ml in 96-well flat bottomed micro-titre plates (200 μl/well). After adherence (6–18 h), cells were treated with glutathione-lowering drugs for 0 to 12 h before, and 5 h post-activation with LPS plus IF-γ (20 ng/ml and 12 IU/ml, respectively). The drugs used were BSO (200 μM), acivicin (100 μM), BrO (60 μM), diamide (100 μM) with zinc sulphate (50 μM) and ethacrynic acid (60 μM). In order to block NO production by activated macrophages, L-*N*-(1-iminoethyl)ornithine (L-NIO) (100 μM), a specific NOS inhibitor, was added 10 min before the activation of the macrophages. Medium was removed for nitrite determination 24 and 48 h after activation, and the remaining adherent cells washed in phosphate-buffered saline and frozen for glutathione analysis.

Murine peritoneal macrophages were harvested and prepared as previously described using CBA mice treated with 3% sterile thioglycollate [6].

Nitrite measurement

Nitrite (NO_2^-) concentration in the supernatants was determined by the Griess reaction [26]. Briefly, 100 μl of the supernatants were incubated with an equal volume of Griess reagent [naphthylethylenediamine dihydrochloride (0.1% w/v) plus sulphanilamide (1% w/v in H_3PO_4 5% v/v)] at room temperature. The absorbance at 540 nm was determined in a Dynatec MR500 plate reader. NO_2^- concentration was determined using sodium nitrite as a standard. GSNO levels were measured as total *S*-nitrosothiol by cleavage of the RS–NO bond with mercuric chloride (30 μM), followed by determination of nitrite by the Griess reaction. GSNO over a range of 0–30 μM was used as standard and the levels in samples calculated from the difference of the nitrite levels with and without mercuric chloride.

Glutathione measurement

Total glutathione (GSH + GSSG) was measured in cell lysates, using the Tietze method [27] of recycling GSH with glutathione reductase and NADPH. The GSH was detected by the colour change at 412 nm associated with 5,5-dithiobis-(2-nitrobenzoic acid) reduction. This assay was adapted for use in a micro-titre plate using a Dynatec MR500 plate reader at 405 nm and Mikrotec kinetics software. Cells were lysed by adding 100 μl of water containing 1 mM EDTA to each well and storing immediately at -70 °C. Following thawing, plates were shaken for 30 s and then sonicated for 60 s at room temperature. Assays were carried out immediately.

GSSG was measured, using the above procedure, after cell lysates had been treated with 2-vinylpyridine (2VP, 2 μl/100 μl sample for 60 min) [28]. Glutathione levels were quantified using standards of GSSG with and without 2VP, and expressed as GSSG equivalents. GSH levels were calculated from the difference between total glutathione and GSSG. Validation of this procedure was carried out by processing 20–100 pmol of GSH and GSSG as above, and by including 66 pmol of GSH and GSSG standard in J774 extracts. Samples were then assayed with or without 2VP derivatization. There was no effect on GSH and GSSG measurements resulting from sample processing, when compared with standards prepared and assayed immediately. The recovery of the internal standards in J774 extracts was found to be 66.8 ± 5.4 pmol for GSSG and 65.2 ± 7.1 pmol for GSH.

ATP measurement

ATP was measured using a chemiluminescent method linked to firefly luciferase and luciferin [29]. Immediately before the assay, frozen cells were treated with 0.5 M perchloric acid at 4 °C, centrifuged at 10000 ***g*** for 2 min at 4 °C and supernatants neutralized using 1 M triethanolamine and KOH. The potassium perchlorate precipitate was removed by centrifugation at 10000 ***g*** for 2 min at 4 °C and the supernatant was assayed immediately for ATP.

Superoxide measurement

The absence of superoxide production by J774 cells was demonstrated by measuring lucigenin-induced chemiluminescence, as described previously [6].

Statistical analysis

Results are expressed as means ± S.E.M. and statistical significance was calculated by analysis of variance, followed by a Dunnett's test for multiple comparisons using Sigmastat (Jandel Scientific). In the Figures, error bars represent the S.E.M. for experiments which all involved six or more samples. Error bars are omitted when covered by a second symbol for that time point.

RESULTS

Intracellular ATP concentrations in J774 cells activated by LPS plus IF-γ

ATP concentrations were measured to assess continuing biochemical integrity following activation with LPS plus IF-γ. After 24 h of activation, ATP levels decreased by 15% (not significant) compared with the control value (control 22.2 ± 1.51 versus LPS plus IF-γ 19.5 ± 0.59 nmol/10^5 cells). Simultaneous treatment of the activated cells with 100 μM L-NIO prevented the small decrease in ATP (22.2 ± 2.6 nmol/10^5 cells).

Nitrite production and intracellular glutathione concentrations in activated J774 cells

The concentration of total glutathione in confluent J774 cells in RPMI with 10% FCS declined by 30% ($P > 0.05$) over a 48 h period (time zero: 11.2 ± 0.9, after 24 h 8.93 ± 0.61 and after 48 h 7.86 ± 0.45 nmol/10^6 cells). These levels could be restored to baseline values after incubating cells with fresh media for 12 h.

When stimulated with LPS plus IF-γ, the cells produced NO, measured as nitrite in the medium. There was a concomitant fall in total glutathione (45% of the time-matched control, $P < 0.01$)

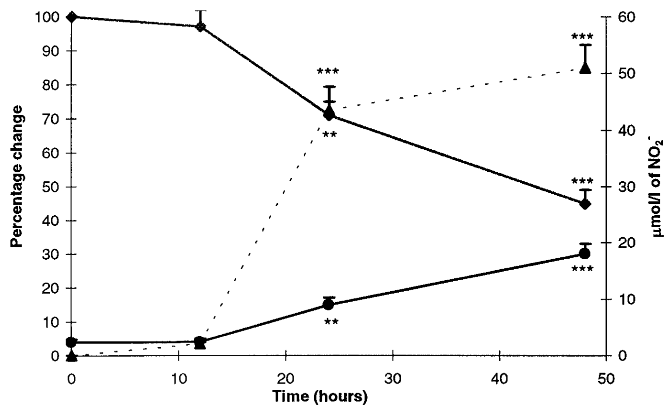

Figure 1 Changes in total and oxidized intracellular glutathione and nitrite output in J774 cells following induction of NOS with LPS/IF-γ

J774 cells were exposed to LPS/IF-γ (20 ng/ml and 12 IU/ml) and the intracellular total (GSH + GSSG) (◆) or disulphide only (GSSG) (●) measured. Nitrite (NO_2^-) (▲) was measured in the media taken from the same cells. Glutathione values are expressed as the percentage change over the corresponding timed control ± S.E.M. Nitrite values are μmol/l ± S.E.M. Values are taken from at least four separate experiments run in quadruplicate. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$ against baseline value (Dunnett's test).

48 h after activation (Figure 1). The level of glutathione disulphide increased over this time period, to give a GSH/GSSG ratio, 48 h after activation, of 2:1 rather than 12:1 found in controls. The fall in total glutathione was linear over a period from 12 to 48 h, whilst the formation of nitrite approached a peak by 24 h.

Exposure of J774 cells to LPS alone did not lower intracellular GSH and the depletion in GSH, with a LPS plus IF-γ challenge, was totally abrogated by the NO synthase inhibitor, L-NIO (100 μM) (Table 1). An increase in the concentration of GSSG inside activated cells was also prevented by L-NIO, which maintained a GSH/GSSG ratio the same as that of the control.

Parallel experiments with mouse peritoneal macrophages showed a similar but smaller depletion of intracellular GSH when activated with LPS plus IF-γ (5.31 ± 0.26 versus 4.11 ± 0.09 nmol/10^6 cells, $p > 0.01$). This occurred within 24 h and was again completely blocked using 100 μM L-NIO (5.02 ± 0.22).

Considerable quantities of glutathione (41 pmol/10^4 cells$\cdot$h^{-1}) were exported from non-activated J774 cells over the first 24 h (Table 1), and this efflux was inhibited following activation with LPS plus IF-γ. L-NIO overcame this inhibition and the resultant glutathione levels in the media were close to those of the controls.

Measurements of cellular and media GSNO were also made using mercuric chloride-mediated cleavage of the S–NO bond. No increase in the total nitrite produced during LPS plus IF-γ activation (42.1 ± 3.1 μM NO_2^-) was evident following $HgCl_2$ treatment (40.9 ± 4.2 μM NO_2^-), indicating an absence of GSNO formation.

NO production in J774 cells with altered glutathione levels

J774 cells were exposed to glutathione-lowering agents 12, 2 and 0 h before, and 5 h after, activation with LPS plus IF-γ. Glutathione was measured for each treatment at the time of activation (zero time), and then 24 and 48 h after activation. Nitrite was also measured in conditioned medium from these cells 24 and 48 h after LPS plus IF-γ activation.

As before, ATP levels (24 h) were monitored in cells, and remained within 10 % of the control for all these treatments, with the exception of ETA (50 μM) which had greatly reduced ATP levels ($< 0.23 \pm 0.01$ nmol/10^5 cells). For this reason, data for this treatment are not presented or discussed.

The time course for changes in intracellular glutathione concentration are shown in Figure 2(A). BSO caused a decline in glutathione levels over 24 h. After 2, 6, 12, 24 and 48 h, the glutathione level was 50, 40, 30, 0 and 0 % of the control. Diamide in association with Zn^{2+} caused a rapid fall over the first 6 h to 20 % of the control value; this level recovered slowly to 50 % of the control by 48 h. BrO lowered glutathione over the first 2 h (40 % of control), followed by a full recovery and then an increase to 160 % of the control at 24 h. Acivicin produced a small (20 %) fall in glutathione over the first 2 h, which recovered to control levels within 6 h.

Nitrite production in activated J774 cells during these treatments is shown in Figure 2(B). The decline in total glutathione to 30 % of the control, caused by 12 h BSO pretreatment, was associated with a significant (40 %) inhibition of nitrite production. The smaller fall in glutathione with shorter BSO treatments (−2, 0 and +5 h) caused no significant decrease in NO production over the 24 h period. Acivicin, which had no effect on total intracellular glutathione levels, showed an unexpected and large inhibition of nitrite production. This was most evident when cells were treated prior to activation. By contrast, BrO, which caused a rapid drop in glutathione followed by a recovery, inhibited nitrite production when treatment occurred at the time of activation. The thiol oxidant diamide, in the presence of zinc, promoted a striking inhibition of NO production at all times of treatment.

Table 1 Effect of LPS/IF-γ activation on J774 intra- and extracellular total and glutathione disulphide

For details see the Materials and methods section. All values are the means and S.E.M.s of at least four experiments run in quadruplicate. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$ against control value (Dunnett's test).

Treatment	Total glutathione (pmol of GSH + GSS)		Glutathione disulphide (% of total glutathione)		Extracellular glutathione (nmol of GSH + GSS/200 μl of media)	
	24 h	48 h	24 h	48 h	24 h	48 h
Control	95.4 ± 9.1	65.3 ± 6.2	4.82 ± 0.62	6.20 ± 0.81	1.05 ± 0.11	1.19 ± 0.08
L-NIO (100 nM)	105 ± 8.3	66.5 ± 6.1	4.19 ± 0.60	4.23 ± 0.52	0.99 ± 0.10	1.11 ± 0.10
LPS (20 ng/ml)	90.2 ± 9.6	59.6 ± 5.6	5.61 ± 0.95	9.51 ± 0.69	0.89 ± 0.09	0.79 ± 0.09
LPS/IF-γ-(12 IU/ml)	71.5 ± 6.6*	31.6 ± 2.9**	12.8 ± 1.5**	33.5 ± 4.2***	0.11 ± 0.01***	0.08 ± 0.01***
LPS/IF-γ + L-NIO (100 μm)	90.2 ± 8.7	71.1 ± 8.1	7.21 ± 0.79*	11.5 ± 1.3*	0.91 ± 0.11	0.93 ± 0.12

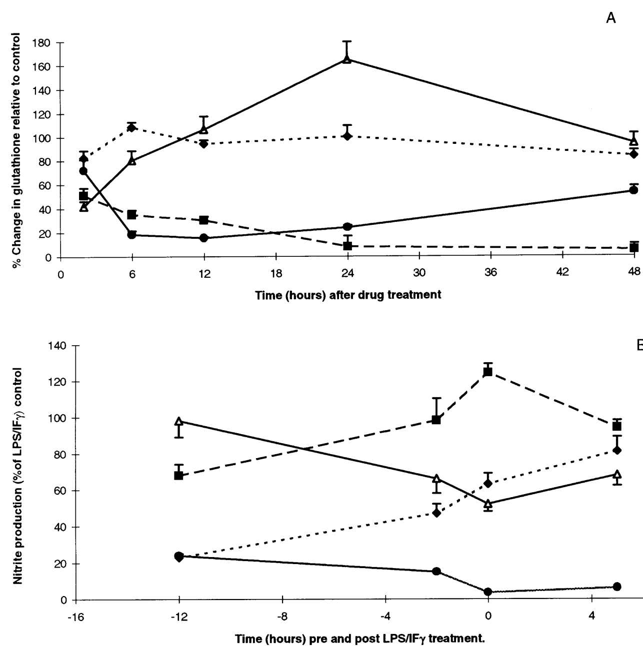

Figure 2 Effect of glutathione modulatory agents in J774 cells

(**A**) Total glutathione at different times of treatment and (**B**) 24 h LPS/IF-γ activated nitrite production following pre- and post-activation treatments. (**A**) The intracellular total glutathione was measured in J774 cells treated with acivicin (100 μM; ◇), BSO (200 μM; ■), BrO (60 μM; △) and diamide (100 μM) with zinc (50 μM) (●) for 2, 6, 12, 24 and 48 h. (**B**) Nitrite was measured in the incubation media of J774 cells activated with LPS/IF-γ (20 ng/ml and 12 IU/ml) for 24 h. The time of treatment with glutathione effectors was 12, 2, 0 h before and 4 h after LPS/IF-γ treatment. Values are means $\pm$ S.E.M. of at least four experiments run in quadruplicate and expressed as a percentage of the time matched control (no additions).

DISCUSSION

We have demonstrated a striking depletion of intracellular glutathione in J774 cells, when they are activated with LPS and IF-γ to produce NO. This is a direct consequence of NO production, since these changes are prevented with the inhibitor of NOS, L-NIO. There was no evidence of glutathione conversion to GSNO. This contrasts with the results of Clancy et al. [30], who reported that addition of NO to human neutrophils depleted glutathione by forming GSNO. However, in their study, GSNO production was not significant in the presence of 50 μM NO, the overall level of nitrite produced by activated cells in our experiments.

The fall in total intracellular glutathione, which follows NOS induction, was not accompanied by a rise in glutathione in the medium. Therefore, this fall does not depend on export of glutathione (reduced or disulphide), but upon either a decrease in synthesis *de novo* or an increase in glutathione consumption. The rate-limiting enzyme of glutathione synthesis, γ-glutamylcysteine synthase, contains an active thiol site [31] and, thus, is subject to regulation by NO mediated S-nitrosylation. γ-Glutamyl transpeptidase and certain glutathione S-transferases are also induced in macrophages [32,33], and intracellular formation of protein mixed disulphides is raised in oxidatively stressed macrophages [34]. Taken together, these events would lead to a situation in which free glutathione is eliminated from cells. The increase in protein synthesis during macrophage activation is another way in which glutathione concentrations could be changed, either by consumption of glutathione, or its precursor cystine.

J774 cells have a high intracellular content of glutathione. This is a common characteristic of macrophages and reflects their functional exposure to high levels of oxidants, such as superoxide and peroxide produced by infiltrating neutrophils, during an inflammatory response. Glutathione turnover, both re-reduction of GSSG and resynthesis of GSH from imported cystine, is very rapid, as demonstrated by the rates of recovery after our various depletion strategies. Murine macrophages, when activated, increase their rate of glutathione turnover from 2 to 0.2 h [8], and by providing an extracellular source of GSH are believed to have a function *in vivo* of creating an antioxidant environment for adjacent lymphocytes [35].

The raised intracellular ratio of GSSG/GSH, on exposure of J774 to LPS and IF-γ, indicates that these cells are subjected to an oxidative insult, although the decrease in extracellular glutathione, following activation, is contradictory. This we attribute to the disturbance in glutathione turnover discussed above. Since we found no evidence of superoxide production during LPS and IF-γ activation, the most likely explanation for the change in glutathione redox is NO itself.

Glutathione is known to be important in modulating macrophage activation [36–38]. We have examined changes in glutathione brought about by a range of agents, which cause transient or permanent depletion of glutathione via oxidation, conjugation, transport and inhibition of synthesis. Depletion of glutathione with BSO, 5 h after cell activation, did not lower the rate of nitrite production over the subsequent 43 h. This indicates that the lowered glutathione level does not alter the intrinsic activity of the enzyme, in this cell system at least, as over this period nitrite production was most prominent. The use of BSO 5 h after activation is unable to affect the induction of NOS, as it has been shown that inducible NOS synthesis is completed between 6–12 h. However, treatment with BSO 0, 2 and 12 h before the induction of NOS led to a reduction of nitrite output from cells. This suggests that intracellular glutathione has a direct influence on the NOS induction pathway. This was also clearly demonstrated by comparing nitrite production following BrO treatment at different times, during which rapid glutathione depletion was followed by a steady recovery. When treated with this agent 12 h before activation, no effect on nitrite production was observed (Figure 2B), because by this time glutathione levels had fully recovered. Following shorter periods of pretreatment, full recovery was not achieved and NOS induction was inhibited. The large elevation of glutathione, achieved 12–36 h after treatment, is often observed when cellular glutathione is lowered and the causative stress removed. This was the case with BrO, which is exported from the cell after being conjugated with glutathione by glutathione S-transferase. The 'overshoot' in glutathione concentrations, concurrent with maximal NO production, again illustrates the fact that NOS activity is independent of the glutathione concentration. Glutathione synthesis *de novo* from cystine is very rapid in these cells, as illustrated by the speed of recovery.

The small and slow recovery in intracellular glutathione following diamide and zinc treatment reflects an incomplete inhibition of glutathione reductase at this concentration of zinc [39]. No recovery occurred when BCNU, which causes total irreversible inhibition of glutathione reductase, was used in place of zinc (results not shown). It is important to note that the diamide/zinc association caused a decrease in nitrite production, even when added 5 h after the activation of J774 with LPS plus IF-γ. There may be several reasons for this. Diamide oxidizes GSH very rapidly and thus the decrease in GSH could overlap the period of NOS induction. GSSG, which accumulates following diamide treatment, could act by inhibiting protein synthesis at a time when the translation of the NOS message is

prominent. Although our data indicate that GSH does not influence intrinsic NOS activity, the possibility that GSSG inhibits activity cannot be discounted. Recently, it has been shown that Zn^{2+} can directly inhibit neuronal NOS with an apparent K_i of 30 μM. Zn^{2+} blocks NADPH-dependent reduction of NOS haem iron, and also the calmodulin-dependent superoxide-mediated cytochrome *c* reductase activity exhibited by NOS [40]. In addition, the y+ L-arginine transport system is modulated by thiol status, as *N*-ethylmaleimide has been shown to inhibit both the Na^+-dependent and -independent forms of arginine transport [41]. Similar effects on thiol status, caused by diamide in J774 cells, would result in effective inhibition of NO synthesis by this mechanism.

Also of interest is the lowering by acivicin of NO production in activated J774 cells, despite its lack of effect on glutathione levels. The Na^+-independent xc-amino acid transporter, rather than γ-glutamyltranspeptidase, is responsible for uptake of cystine by resident macrophages [42,43]. This would explain the ineffectiveness of acivicin at modulating glutathione in J774 cells. Acivicin, which did however block nitrite production, is also a glutamine analogue which inhibits carbamyl phosphate synthase II [44] and therefore influences RNA synthesis. It is possible that inducible NOS induction is inhibited by this mechanism.

The ability of the redox state of glutathione to regulate key cellular functions, such as protein synthesis [17], has a direct bearing on signal transduction linking macrophage activation with NO production. Inhibition of the phosphorylation of the protein synthesis initiation factor by GSSG is a possible mechanism by which changes in glutathione homoeostasis, as reported in this study, result in blunted NO production. For this reason, the use of a direct low molecular mass thiol oxidant such as diamide, coupled with zinc inhibition of glutathione reductase, executes the largest inhibition of nitrite production and the greatest shift in GSH to GSSG.

In conclusion, we have demonstrated depletion of cellular glutathione following activation of both murine peritoneal macrophages and the murine macrophage cell line J774. Cellular glutathione becomes oxidized, and depletion of total glutathione arises, either by inhibition of synthesis or by increased non-oxidative consumption. This does not occur when NO production is inhibited. In addition, depletion and oxidation of glutathione regulates the induction of NOS, but not the activity of the enzyme.

The possibility of altering macrophage glutathione concentrations, to regulate NO output during pathological conditions, could prove to be clinically important.

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