Inhibition of NADPH supply by 6-aminonicotinamide: effect on glutathione, nitric oxide and superoxide in J774 cells

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Abstract We have examined the integrity of J774 cell nitric oxide (NO) production and glutathione maintenance, whilst NADPH supply was compromised by inhibition of the pentose pathway with 6-aminonicotinamide. In resting cells 6-phosphogluconate accumulation began after 4 h and glutathione depletion after 24 h of 6-aminonicotinamide treatment. Cellular activation by lipopolysaccharide/interferon-λ decreased glutathione by ~50% and synchronous 6-aminonicotinamide treatment exacerbated this to 31.2% of control ($P < 0.05$). In activated cells NO_2^- production was inhibited by 60% with 6-aminonicotinamide ($P < 0.01$), and superoxide production by 50% ($P < 0.01$) in zymosan-activated cells. NADPH production via the pentose pathway is therefore important to sustain macrophage NO production whilst maintaining protective levels of glutathione.

Key words: Nitric oxide; Glutathione; Superoxide; NADPH; 6-Aminonicotinamide; Macrophage

1. Introduction

We have previously employed the anti-metabolite 6-aminonicotinamide (6-AN) to investigate the role of the pentose pathway (PP) in providing NADPH [1]. 6-AN is taken up by cells and transformed into 6-amino-$NADP^+$ by NAD-glycohydrolase (EC 3.2.2.5). This NADP analogue inhibits 6-phosphogluconate dehydrogenase (6-PGDH) with a K_i of 0.13×10^{-6} M, approximately 400-fold more powerful than its inhibition of other NADP(H) requiring enzymes, including glutathione reductase and glucose 6-phosphate dehydrogenase (G6-PDH) [2].

Nitric oxide (NO), produced by activated inflammatory cells, plays an important role in the immune response, regulating both tumour and parasite growth [3–5]. The synthesis of NO is dependent on cofactor availability [6,7] with NADPH providing the reducing equivalents (i) directly for arginine reduction and (ii) indirectly for the formation of tetrahydrobiopterin (BH_4). It has been suggested that BH_4 is not a catalytic cofactor, but rather activates NO synthase by stabilising the enzyme in its dimeric form and maintaining key groups in a reduced state [8]. Both de novo biosynthesis and salvage of BH_4, and dihydrobiopterin reduction require NADPH. In macrophages the oxidative section of the PP, via the activity of G6-PDH and 6-PGDH, is a major source of this cofactor [9], although mitochondrial $NADP^+$ dehydrogenases linked to malate and isocitrate also contribute to NADPH supply [10]. This provision of NADPH is critical to the maintenance of reducing power within the cell and is regulated by the redox state of the NADPH/$NADP^+$ couple. We have previously demonstrated in J774 cells that this redox couple is under oxidative pressure when NO is actively being synthesised [11].

NADPH performs other essential cellular functions, including maintenance of glutathione in the reduced form. Glutathione plays a vital role as a cellular antioxidant, protecting against free radical-mediated cytotoxicity. The intracellular thiol status is maintained in a reduced state by glutathione, allowing sulphydryl-dependent enzymes, signal transduction and gene expression, to function normally [12,13]. There is evidence that glutathione performs a similar function in maintaining NO synthase activity [14,15].

NADPH consumption may also occur in the presence of high glucose concentrations via autoxidation of both free and protein-associated glucose [16,17], and by activation of the aldose reductase-mediated polyol pathway [18]. In addition, aldose reductase can act as a sepiapterin reductase [19]. Thus, in conditions of high glucose concentration, both substrate competition between glucose and sepiapterin, and restricted NADPH availability could limit vital components of the NO synthase system. Such interactions might contribute to the disruption of NO homeostasis in diabetes.

In this study we have used a mouse macrophage cell line, J774.2, to examine the competition for available NADPH when either the PP is inhibited by 6-AN, or when high glucose concentrations are present. NADPH-requiring systems examined were (i) the intracellular redox status (glutathione), (ii) NO production from inducible NO synthase and (iii) superoxide production occurring during phagocytosis.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (LPS) was supplied from Difco Laboratories, West Molesey, UK. RPMI 1640 medium, Hanks' balanced salt solution with calcium, magnesium and bicarbonate, HEPES pH 7.2, foetal calf serum (FCS), penicillin and streptomycin were purchased from Life Technologies, Paisley, UK. Glutathione, glutathione reductase, superoxide dismutase, NADPH, 6-phosphogluconate dehydrogenase, haemoglobin, tetrahydrobiopterin and 6-aminonicotinamide were all supplied from Sigma, Poole, UK. All other chemicals used were Analar or equivalent grade. The aldose reductase inhibitor sorbinil was a gift from Pfizer UK and murine interferon-λ (λ-IF) was a gift from Dr A Sergeant, NIBSC, Potters Bar, UK.

2.2. Macrophage activation

J774.2 cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) as previously described [9]. Cells were grown to confluence in 96-well flat-bottomed micro-titre plates (200 μl/well), and activated with LPS plus λ-IF (20 ng/ml and 12 IU/ml respectively), followed 4 h later by treatment with 6-AN (200 μM). Medium was removed for nitrite determination 20 h after activation, and the remaining adherent cells washed in phosphate buffered saline, frozen and stored at −70°C for glutathione

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and 6-phosphogluconate analysis. In some instances medium was removed after 20 h, and the cells washed once in Hanks' balanced salt solution with 10 mM HEPES pH 7.2. Culture medium was then restored and cytokine activation re-initiated, followed by a further 20 h incubation period.

Cells grown under identical conditions, but in 35-mm tissue culture dishes, were used for superoxide measurements. Prior to activation with opsonised zymosan, medium was replaced with Hanks' balanced salt solution containing 10 mM HEPES pH 7.2 and 5% FCS.

Cells were incubated with 5 mM (control) or 50 and 100 mM glucose, which was added 15 min after cell activation. Sorbinil (30 μM) was used to inhibit aldose reductase.

2.3. Nitrite measurement

Nitrite (NO_2^-) concentration in the medium was determined by the Griess reaction [20]. Briefly, 100 μl of conditioned medium was mixed 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 then determined after 5 min using a Dynatec MR5000 plate reader. NO_2^- concentration was calculated using sodium nitrite as a standard.

2.4. Glutathione measurement

Total glutathione (GSH+GSSG) was measured in cell lysates using the Tietze method of recycling reduced glutathione with glutathione reductase and NADPH [21]. The reduced glutathione was detected by the colour change at 412 nm associated with 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) reduction. This assay was adapted for use in a microtitre plate using a Dynatec MR5000 plate reader at 405 nm and Mikrotek kinetics software [11]. Cells were lysed by adding 100 μl water containing 1 mM EDTA to each well and freezing immediately. Following thawing, plates were shaken for 30 s and then sonicated for 60 s at room temperature. Assays were carried out immediately. Validation of this procedure was carried out by processing 20–100 pmol of GSH and GSSG as above, and by including GSH and GSSG internal standards in J774 extracts. Recovery was >95% in all instances.

2.5. Superoxide measurement

Superoxide production by J774 cells was measured by superoxide dismutase inhibitable lucigenin-enhanced chemiluminescence (CL), as described previously [5]. Cells were activated with opsonised zymosan and lucigenin CL measured from time zero for 60 min at 6-min intervals.

2.6. 6-Phosphogluconate (6-PG) measurement

Frozen cell samples were extracted with 200 μl of 0.5 N perchloric acid and centrifuged at 14 000×*g* for 2 min. Supernatants were adjusted to pH 5–7.5 with 10 μl of 50% ethanolamine and 3 N potassium hydroxide and centrifuged at 14 000×*g* for 2 min. This supernatant was then assayed for 6-phosphogluconate by measuring spectrophotometrically at 340 nm, $NADP^+$ reduction catalysed by 6-PGDH, as previously described [14].

2.7. NO synthase assay

Cells were grown to confluence in 25-cm^2 flasks, and activated for 24 h with LPS/λ-IF, then harvested by scraping after treatment with calcium/magnesium-free Hanks containing 1 mM EDTA. Cells were collected by centrifugation (400×*g* for 5 min), resuspended in 250 mM sucrose containing 20 mM triethanolamine pH 7.4 and sonicated at 4°C for 30 s (1600 W and 80 μM). NO synthase was measured in activated cell sonicates (control and 6-AN treated) and the effect of the inclusion of non-activated 6-AN treated cell extracts studied. Enzyme activity was determined from the rate of met-haemoglobin formation (Δ absorbance at 401 and 419 nm) over 15 min at 37°C in a Cary 1E spectrophotometer. Activity was calculated using a $\Sigma_{401-419}$ of 76 mM^{-1} cm^{-1}. Each assay (1 ml) contained 50 mM potassium phosphate pH 7.2, 60 mM valine, 3 μM oxyhaemoglobin, 1.25 mM magnesium chloride, 0.25 mM calcium chloride, 100 μM arginine, 2 mM glutathione, 100 μM NADPH, 5 μM BH_4 and 200 units of SOD.

3. Results

Resting confluent J774 cells treated with 6-AN began to accumulate 6-PG after 4 h, and reached a plateau by 24 h (Fig. 1). This evidence of 6-PGDH inhibition preceded, by approximately 20 h, a decrease in total glutathione. The rapid and irreversible decrease in glutathione obtained after treatment with a combination of diamide and BCNU is shown for comparison. In pre-confluent cell populations, 6-AN treatment began lowering glutathione within 1 h of the start of 6-PG accumulation (data not shown).

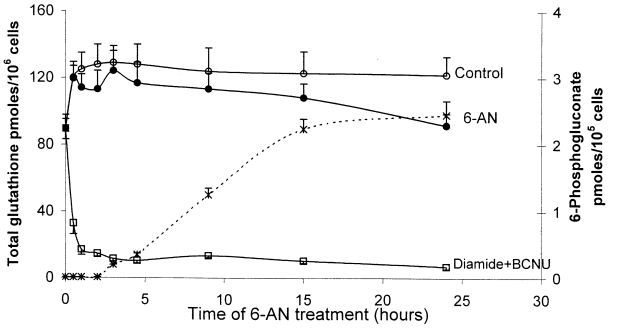

Fig. 1. Effect of 6-aminonicotinamide on total glutathione and 6-phosphogluconate concentrations in J774 cells. Each value is the mean ± S.E.M. of six observations. Solid lines are glutathione concentrations and dashed line is 6-phosphogluconate concentration.

In view of this time course, all further studies were carried out on fully confluent cells treated with 6-AN for 16 h. During macrophage activation experiments addition of LPS plus λ-IF was made 4 h before the start of the 6-AN treatment period. This strategy avoided (1) interference caused by either a decreased cellular glutathione, or a direct effect of nicotinamide on LPS/λ-IF NOS induction [9,22], and (2) any possibility that glutathione reductase inhibition might mimic NADPH competition.

In cells activated with LPS plus λ-IF, as previously described a decrease in glutathione was observed [11], which was compounded by 6-AN treatment (Fig. 2a). When these cells were washed and subjected to a change of medium, glutathione levels increased in all treatments after 20 h. However, at this time, glutathione decreased in resting cells treated with 6-AN. In the LPS/λ-IF treated cells the loss of glutathione due to cell activation was reversed, but in 6-AN treated activated cells, some glutathione recovery occurred but failed to achieve the level to that of the control (Fig. 2b).

NO production (medium nitrite) resulting from cell activation was also severely inhibited in 6-AN treated cells, and this inhibition persisted through washing and medium replenishment (Fig. 2a,b). We have used nitrite levels to evaluate NO production in this study. We have previously shown [5] that cytokine activated J774.2 cells produce 50% nitrite and 50% nitrate, however, the ratio of these two products can change in the presence of elevated superoxide due to peroxynitrite

Table 1
Effect of 6-aminonicotinamide on J774 cell NO synthase activity

Treatment	μmol/min/10^6 cells	
A. Non-activated+6-AN	N.D.	
B. Activated	0.78 ± 0.10	N.S.
C. Activated+6-AN	0.66 ± 0.74	N.S.
A+B	0.83 ± 0.91	N.S.
A+C	0.75 ± 0.52	N.S.

Each value is the mean ± S.E.M. of four experiments. N.D.: not detected; N.S.: not significant.

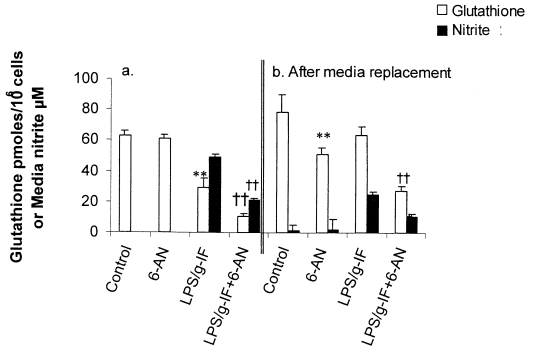

Fig. 2. Effect of 6-aminonicotinamide on J774 cell glutathione and nitrite production, following activation with LPS and IF-λ. Each value is the mean ± S.E.M. of six observations. $^{**}P < 0.01$ vs control, $^{\dagger}P < 0.05$ vs LPS/λ-IF and $^{\dagger\dagger}P < 0.01$ vs LPS/λ-IF.

formation. Activation of these cells using LPS/λ-IF does not result in superoxide production, as determined by lucigenin CL (data not shown), and during activation with zymosan superoxide production is complete after 1.5 h, well in advance of any evidence of iNOS activity.

The effect of 6-AN was not a result of direct NO synthase inhibition, as enzyme maximal activity measured in activated J774 lysates was not significantly different from that measured in lysates of activated and 6-AN treated cells. Further, activity was not modified when 6-AN treated lysates from resting cells were co incubated (Table 1).

In addition to this effect on the NO synthesising potential of macrophages, the capacity to produce superoxide following activation with opsonised zymosan (lucigenin CL) was also inhibited by over 50%. ($P < 0.001$) in 6-AN pre-treated cells (Fig. 3). Lucigenin CL was 98% inhibitable by superoxide dismutase (44 U/ml: data not shown).

The possibility that high glucose concentration could create competition for NADPH either by aldose reductase activity or by glucose autoxidation was examined. Treatment for 24 h with glucose (50 and 10O mM) failed to cause a significant fall in cellular glutathione concentrations (Fig. 4). The decrease in glutathione caused by cytokine activation was partially reversed when J774 cells were incubated with 100 mM glucose, and this reversal was independent of aldose reductase (sorbinil 30 μM). In activated cells, there was a small but significant decrease in nitrite production in the presence of 100 mM glucose (~20%, $P > 0.05$) and sorbinil did not reverse this fall. This effect on NO production was additive to that of 6-AN, with a further significant fall of 20% apparent in the 100 mM glucose/6-AN treated group.

At normal (5 mM) glucose concentrations, sorbinil did not affect glutathione levels or nitrite production in resting or activated cells. Culture of cells in arginine-free medium, or in the presence of the NO synthase inhibitor LNIO, prevented the activation-induced decrease in glutathione, and this was accompanied by a correspondingly lower production of nitrite (data not shown).

4. Discussion

We have previously shown that during the production of NO, glutathione concentrations in J774 cells were decreased in a NO synthase-dependent fashion [11]. We now demonstrate, using the anti-metabolite 6-AN, that macrophage NADPH provision via the PP is a key factor in NO production during cytokine-mediated activation. We also show that inhibition of this source of NADPH during activation compounds an already depleted intracellular glutathione, and that this results from competition for NADPH between glutathione reductase and NO synthase.

Addition of 6-AN to the culture medium resulted in the accumulation of 6-PG, indicating that J774 cells are able to synthesise 6-amino-$NADP^+$ via glycohydrolase. The cytosolic NADPH/$NADP^+$ redox couple in resting cells is maintained in the reduced form (NADPH to $NADP^+$ ratio of 100 to 1) by the activity of $NADP^+$ reducing enzymes. When NADPH is consumed during the reductive steps of NO and superoxide formation, or during disulphide reduction, the redox status is restored either by the oxidative segment of the PPP or by mitochondrial malate and isocitrate reductase. In neutrophils devoid of mitochondria the former is the only means of $NADP^+$ reduction, whilst in macrophages both can be deployed. As a result, glutaminolysis, a favoured route of energy metabolism in macrophages, can provide malate for $NADP^+$-dependent 'malic' enzyme and thus shift part of the burden of NADPH production from glucose to glutamine metabolism. Nevertheless, our demonstration that 6-AN, a specific PP inhibitor, can decrease NO and superoxide production during activation emphasises the important contribution towards NADPH provision, made by the PP. Despite a rapid turnover of glutathione in resting macrophages, 6-AN failed to lower this thiol in confluent cells until 24 h had elapsed. However, in

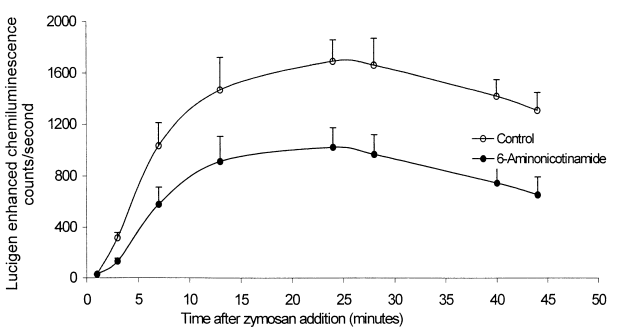

Fig. 3. Effect of 6-aminonicotinamide treatment on zymosan-induced superoxide production in J774 cells. Each value is the mean ± S.E.M. of six observations.

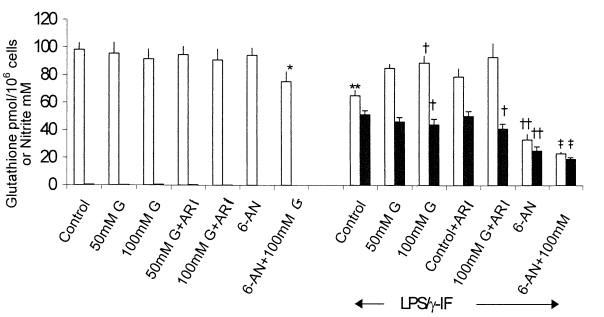

Fig. 4. Effect of medium glucose (50 and 100 mM) on J774 cell glutathione and nitrite production following cytokine activation. Each value is the mean ± S.E.M. of six observations. Open bars: glutathione; closed bars: nitrite. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs non-activated control, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs LPS/λ-IF activated control and $^{\ddagger}P < 0.05$ vs LPS/λ-IF activated 6-AN treated.

activated cells, where competition for NADPH is far more acute, marked glutathione depletion occurred before 24 h. A similar decrease in NO production has also been reported in cerebellar slices using an alternative nicotinamide analogue, 3-acetylpyridine. However, in this instance the change was attributed to in vivo structural damage to Purkinje cells resulting from deafferentation of climbing fibres, rather than to metabolic perturbations in the redox state [23].

Other factors must be considered, the most important being the synthesis of BH_4, which is also inhibited by 6-AN [24]. NO synthase becomes an uncoupled NADPH oxidase when either BH_4 or arginine concentrations fall below a critical level. Under these conditions, NOS produces less NO, but more superoxide. Hence, when NADPH demand cannot be met, BH_4 depletion could compound the down-regulation of NO production resulting from limited availability of NADPH.

An association has been reported between cell vulnerability to the cytotoxic effects of NO and glucose availability [25]. Our data support this association, as the decrease in glutathione due to cellular activation observed at physiological glucose levels was abolished when the glucose concentration was raised. This could be related to the phenomenon of glucose sparing by glutamine discussed above, and suggests that at physiological levels glucose becomes a limiting factor in NADPH production in activated macrophages.

In our experiments a high concentration of glucose failed to change the level of glutathione in resting cells, but did produce a small and significant decrease in NO production during activation. This decrease caused by high glucose was additive to that caused by 6-AN, providing evidence that competition for NADPH was compounded. However, this glucose effect was not associated with polyol pathway activity, in agreement with recent studies in J774 and mesangial cells [26,27]. Aldose reductase was also not involved in the reversal of glutathione depletion during cytokine activation. These data point to an action of glucose on NO production through some route other than NADPH competition, which may be depletion of cellular arginine [27].

In conclusion, our data demonstrate that NADPH derived from the PP is essential to maintain normal redox homeostasis in macrophages actively synthesising NO.

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