

Regulation of Fas antibody induced neutrophil apoptosis is both caspase and mitochondrial dependent

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Abstract Resolution of neutrophil mediated inflammation is achieved, in part, through induction of neutrophil apoptosis. This constitutively expressed programme can be delayed by inflammatory mediators and induced by ligation of the Fas receptor. However, functional activation of the neutrophil results in resistance to Fas signalled death. We evaluated the effects of Fas antibody engagement on caspase activation and mitochondrial permeability, and the impact of co-stimulation by lipopolysaccharide (LPS) or granulocyte macrophage-colony stimulating factor (GM-CSF) on these events. Fas engagement by an agonistic anti-Fas antibody resulted in enhanced caspase 3 and 8 activity and increased mitochondrial permeability. Studies with pharmacological inhibitors of caspase activity showed that activation of caspase 8 occurred before, and activation of caspase 3 occurred after mitochondrial disruption. The mitochondrial stabilising agent bongkreikic acid also inhibited caspase activation and apoptosis. LPS, GM-CSF and increased glutathione stabilised the mitochondria and inhibited caspase 3. Caspase 8 activity was also inhibited by co-stimulation through a mechanism independent of mitochondrial stabilisation. Glutathione directly inhibited caspase 3 and 8 activity. We conclude inhibition of Fas antibody induced apoptosis by inflammatory proteins is associated with augmented mitochondrial stability and reduced caspase 3 activity that may be glutathione mediated.

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Key words: Fas antibody; Neutrophil apoptosis; Caspase; Mitochondrion

1. Introduction

An inflammatory response is terminated, in part, through apoptosis of its cellular effectors [1]. Neutrophils die spontaneously by apoptosis *in vitro* and *in vivo*. This constitutively expressed programme can be regulated by a number of inflammatory mediators and processes [2–9].

Different apoptotic processes including Fas ligation mediate their effects through activation of a cascade of proteolytic enzymes known as caspases [10]. Mitochondrial permeabilisation has also been shown to activate caspases through the

release of cytochrome *c* (Apaf-2) [11]. Which pathway is central to Fas antibody induced neutrophil apoptosis is unknown. Inflammatory neutrophils are resistant to pro-apoptotic signals resulting from Fas ligation [7,12]. This resistance is associated with an increase in intracellular levels of reduced glutathione (GSH) [7] and altered caspase expression [12]. This delay in apoptosis is associated with the production of survival mechanisms [13–15]. How these mechanisms mediate their effects and if they involve caspase and mitochondrial components is unknown. We hypothesised that Fas antibody induced neutrophil apoptosis is caspase dependent and resistance of neutrophils to pro-apoptotic stimuli within an inflammatory milieu may result from altered caspase activity.

Here we demonstrate that anti-Fas antibody induced neutrophil apoptosis is associated with increased caspase 3 and 8 activity, and disruption of the mitochondrial membrane. Caspase inhibition studies suggest a role for caspase 8 in mitochondrial disruption and the subsequent activation of caspase 3. We show that mitochondrial disruption alone induces caspase 3 and apoptosis, independent of caspase 8 activity. We present evidence that lipopolysaccharide (LPS), granulocyte macrophage-colony stimulating factor (GM-CSF) and *N*-acetylcysteine (NAC) inhibit the effects of Fas ligation, by stabilising mitochondria and inhibiting caspase activity. Finally, we demonstrate that glutathione directly inhibits caspase activity in a cell free system.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin solution, L-glutamine, phosphate buffered saline (PBS) and foetal calf serum (FCS) were purchased from Gibco Life Technologies Ltd, Burlington, Ont., Canada. Z-DEVD-FMK and Z-IETD-FMK were purchased from Enzyme Systems Products, Dublin, CA, USA. Annexin V was from Genzyme, Cambridge, MA, USA. 3,3'-Diethyloxycarbocyanine iodide (DiOC₆) was purchased from Molecular Probes, Eugene, OR, USA. Dextran and Ficoll were from Pharmacia, Uppsala, Sweden. All other chemicals were supplied by Sigma-Aldrich, Dorset, UK, unless otherwise stated. Bongkreikic acid was kindly donated by Prof. J.A. Duine through Prof. Hiroshi Terada.

2.2. Neutrophil isolation

Neutrophils were isolated from six different healthy volunteers by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient [5] and these cells were used in all experiments. Isolated neutrophils were resuspended at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin solution. Cells were incubated in polypropylene tubes to prevent adherence. Neutrophil purity, assessed by size and granularity on flow cytometry, was consistently greater than 95% (each *n* represents results from one individual volunteer).

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Abbreviations: NAC, *N*-acetylcysteine; GSH, glutathione; GM-CSF, granulocyte macrophage-colony stimulating factor; DiOC₆, 3,3'-diethyloxycarbocyanine iodide; FADD, Fas-associating protein with death domain

2.3. Quantification of apoptosis

Neutrophil apoptosis was quantitated as the percent of cells with hypodiploid DNA [5] and annexin V binding. Cells were centrifuged at $200 \times g$ for 10 min, then gently resuspended in 500 μ l of hypotonic fluorochrome solution (50 μ g/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100) and stored in the dark at 4°C before analysis using a Coulter ELITE cytofluorometer (Coulter Electronics, Bedfordshire, UK). For annexin V binding experiments, neutrophils (1×10^6) were pelleted and incubated with 200 μ l of annexin V antibody buffer (10 μ M) for 20 min at 4°C and then washed with cold PBS before analysis by flow cytometry. All measurements were performed under the same instrument settings.

2.4. Caspase activity assay

Cell lysates were prepared from 10×10^6 neutrophil membrane fractions after different treatments. Aliquots of the lysates (250 μ l) were diluted in assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS) containing 20 μ M CBZ-Asp-Glu-Val-Asp-AFC (caspase 3 substrate) or CBZ-Ile-Glu-Thr-Asp-AFC (caspase 8 substrate) (Enzyme Systems, Livermore, CA, USA), and incubated for 45 min at room temperature. The release of AFC was detected by continuous measurement in a luminescence spectrometer (Perkin Elmer LS30, London, UK) with an excitation of 400 nm and an emission slit at 505 nm. Specific activity is measured as pmol/s mg protein.

2.5. Mitochondrial permeability assay

Neutrophil mitochondrial permeability was measured using DiOC₆ (final concentration 40 nM, excitation wavelength 488 nm, emission 529 nm) [16]. Briefly, neutrophils (1×10^6 /ml) were incubated with DiOC₆ for 20 min at 37°C and then washed with cold PBS. Samples were stored on ice until analysis by flow cytometry. All cells were included for analyses by side and forward scatter.

2.6. Western blot analysis

Whole cell protein extracts were isolated from 2×10^6 human neutrophils, and run on a 12% SDS polyacrylamide gradient gel, then electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA, USA). Blots were incubated with caspase 3 primary antibody (1:1000, Transduction Laboratories, Lexington, KY, USA) in 1% BSA TBS and 0.1% Tween 20 for 1 h at room temperature and then incubated with horseradish peroxidase conjugated anti-mouse IgG at 1:5000 dilution for 1 h. Blots were developed using an enhanced chemiluminescence system.

2.7. Statistics

Statistical analysis was carried out using ANOVA with Scheffé correction. Significance was assumed for values of $P < 0.05$.

3. Results and discussion

3.1. Anti-Fas antibody induced neutrophil apoptosis is associated with increased caspase 3 and 8 activity and enhanced mitochondrial permeability

Engagement of the Fas receptor leads to the initiation of the caspase cascade and induction of apoptosis [10]. Previous studies have demonstrated that an agonistic antibody to Fas (clone CH-11) induces neutrophil apoptosis [7,17]. The ability of anti-Fas antibody to induce neutrophil apoptosis was confirmed in this study by staining of DNA with propidium iodide, and by assessment of annexin V binding (the latter recognises phosphatidylserine expressed on the exterior of apoptotic cells). Propidium iodide DNA staining resulted in a significant ($P < 0.05$) increase in apoptosis from $26 \pm 5\%$ apoptosis in control neutrophils to $61 \pm 10\%$ apoptosis in Fas antibody treated neutrophils at 24 h. The percent neutrophils expressing FITC labelled annexin V at 6 h was significantly increased with anti-Fas treatment ($48 \pm 12\%$ apoptosis) compared to control neutrophils ($25 \pm 7\%$ apoptosis). Anti-Fas also induced a significant increase in caspase 3 and 8 activity at 4 and 6 h (Fig. 1a). Cleavage of pro-caspase 3

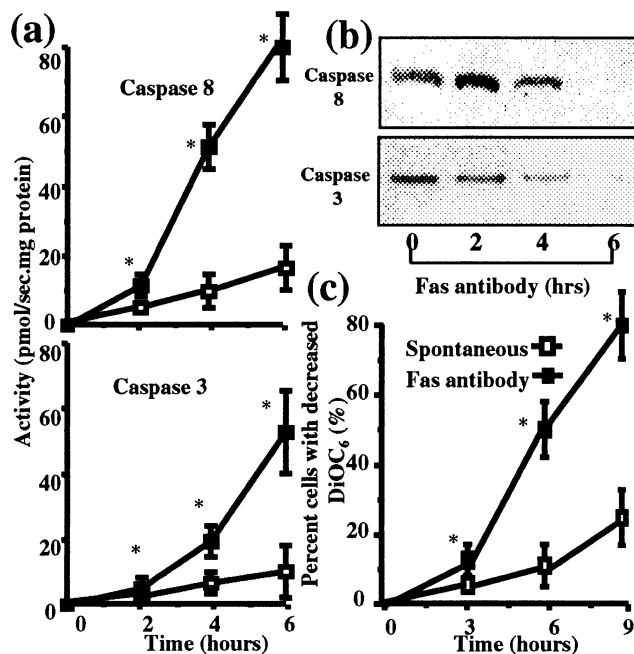


Fig. 1. Effects of anti-Fas antibody on (a) caspase 3 and 8 activity, (b) protein expression and (c) mitochondrial permeability. Normal neutrophils were incubated with anti-Fas antibody (CH-11, 100 ng/ml) and assessed for (a) caspase 3 and 8 activity over time (0–6 h) using AFC labelled substrates, CBZ-Asp-Glu-Val-Asp-AFC (caspase 3) or CBZ-Ile-Glu-Thr-Asp-AFC (caspase 8). b: Total protein was also isolated at 0, 2, 4 and 6 h and SDS-PAGE Western blotting carried out to determine the expression of pro-caspase 3 and 8. Blot represents one of three experiments. c: Mitochondrial permeability was assessed at 0, 3, 6 and 9 h by assessing DiOC₆ fluorescence intensity using flow cytometry. * $P < 0.05$ vs spontaneous neutrophils, $n = 6$.

and 8 was confirmed by Western blotting, which demonstrated that Fas ligation decreases pro-caspase 3 and 8 protein expression (Fig. 1b). The caspase antibodies used in this study do not recognise active forms of the corresponding caspase as has been shown by Sanghavi et al. [20]. Many studies have demonstrated that ligation of the Fas receptor causes recruitment of FADD/MORT1 and activation of caspase 8 [10]. In addition recent studies have demonstrated a role for caspases in tumour necrosis factor- α (TNF- α) induced neutrophil apoptosis [18].

Mitochondrial permeability (Fig. 1c) was also significantly increased at 3, 6 and 9 h. This disruption has also been associated with the early stages of apoptosis in a number of cell types [16,19]. Cytochrome *c* (Apaf-2), a mitochondrial protein, activates caspase 3 through caspase 9 (Apaf 3) cleavage [10]. Indeed it is uncertain whether caspases or mitochondria serve as the central effectors of apoptosis: caspases open megachannels in mitochondria resulting in apoptosis, while mitochondrial protein release is required for caspase activation [20,21].

3.2. Effects of caspase 3 and 8 inhibitors on anti-Fas antibody induced caspase activity and mitochondrial permeability

To determine the sequence of events by which engagement of Fas induces apoptosis, neutrophils were treated with anti-Fas antibody 1 h after incubation with the caspase 3 inhibitor Z-DEVD-FMK, 100 μ M, or the caspase 8 inhibitor, Z-IETD-FMK, 100 μ M. Anti-Fas antibody induced apoptosis was

significantly inhibited at 18 h by both inhibitors (Fig. 2a). Apoptosis was also assessed by annexin V binding at 6 h. Similar effects of caspase inhibition were seen when apoptosis was evaluated by annexin V binding (DEVD-FMK $31 \pm 8\%$ apoptosis, Z-IETD-FMK $29 \pm 9\%$ apoptosis, $P < 0.05$). Inhibition of apoptosis by either inhibitor was associated with a corresponding decrease in caspase 3 activity (Fig. 2a). Caspase 8 inhibition both prevented caspase 8 activity and increased mitochondrial permeability (Fig. 2a), suggesting that activation of caspase 8 precedes mitochondrial disruption, whereas caspase 3 follows mitochondrial disruption.

3.3. Mitochondrial stability inhibits Fas antibody induced apoptosis

Bongkreikic acid (BA) stabilises the mitochondrial membrane, preventing its disruption. Pre-incubation of neutrophils

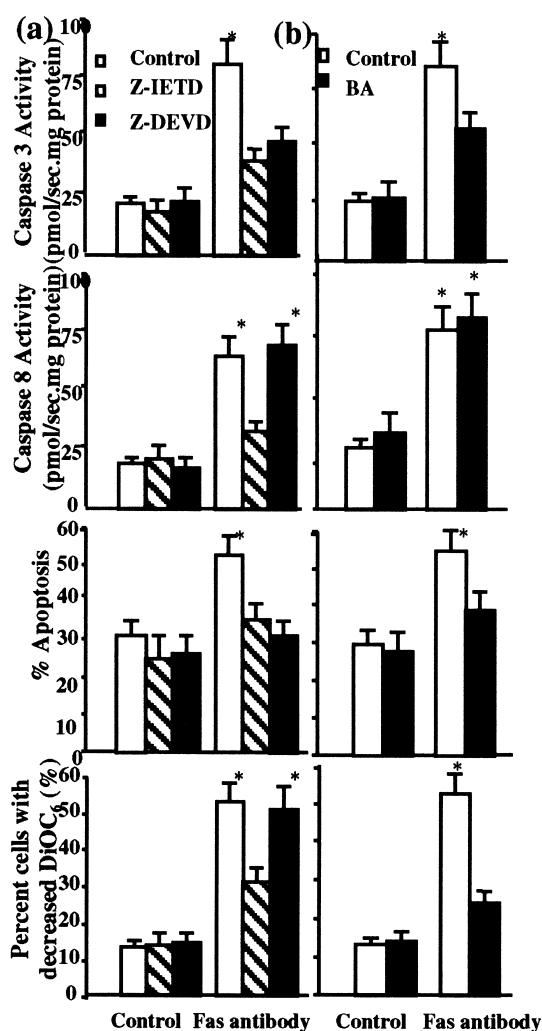


Fig. 2. Effects of caspase inhibitors (a) and BA (b) on anti-Fas antibody mediated apoptosis. Neutrophils (1×10^6 /ml) were preincubated with (a) Z-IETD-FMK (100 μ M) and Z-DEVD-FMK (100 μ M) or (b) BA (62 μ M) for 1 h and then treated with or without anti-Fas antibody (CH-11, 100 ng/ml). Apoptosis was assessed by propidium iodide DNA staining after 18 h culture in vitro. Aliquots of cells (10×10^5) were collected at 6 h and assessed for mitochondrial permeability using DiOC₆ staining detected by flow cytometry. Caspase 3 and 8 activity was also assessed using AFC labelled substrates and the rate of AFC fluorescence release was calculated and expressed as pmol/s mg protein. * $P < 0.05$ vs control, $n = 6$.

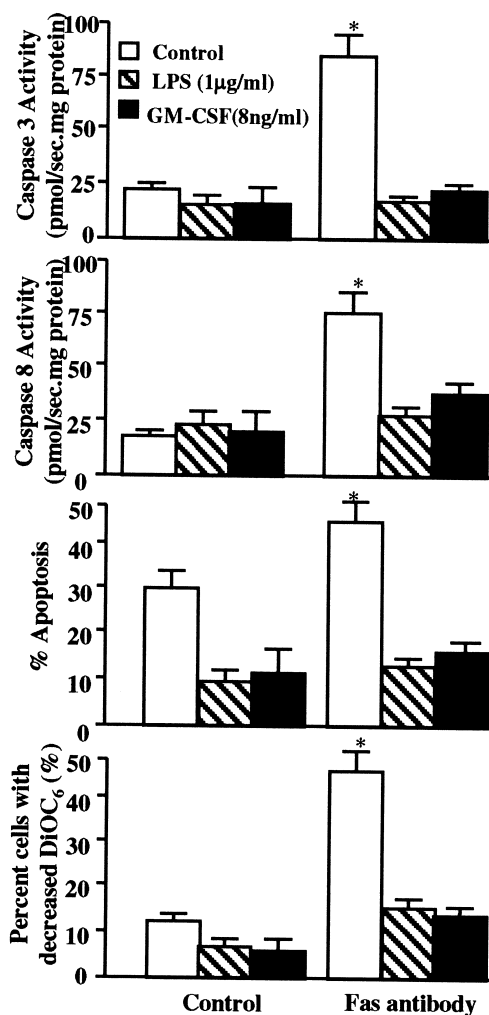


Fig. 3. Effects of LPS and GM-CSF on anti-Fas antibody induced apoptosis, mitochondrial and caspase changes. Neutrophils (1×10^6 /ml) were preincubated with LPS (1 μ g/ml) and GM-CSF (8 ng/ml) for 1 h and then treated with anti-Fas antibody (CH-11, 100 ng/ml). a: Neutrophil apoptosis was assessed by propidium iodide DNA staining (18 h) and mitochondrial permeability (6 h) using DiOC₆ staining detected by flow cytometry. b: Caspase 3 and 8 activity was also assessed after 6 h, using AFC labelled substrates. The rate of AFC fluorescence release was calculated and expressed as pmol/s mg protein. * $P < 0.05$ vs control neutrophils, $n = 5$.

for 1 h with 62 μ M BA significantly inhibited anti-Fas antibody induced mitochondrial disruption and apoptosis (Fig. 2b), indicating that mitochondrial disruption is necessary for the induction of apoptosis by Fas engagement. BA was also able to significantly decrease corresponding increases in caspase 3 activity but had no effect on caspase 8 activity (Fig. 2b). There may be some direct activation of caspase 3 by caspase 8, which has been suggested in other studies, as BA was unable to completely block the effects of anti-Fas antibody even though it did completely stabilise the mitochondrial membrane.

Caspase 3 and 8 inhibitors and the mitochondrial stabiliser BA were unable to prevent spontaneous apoptosis indicating that activity of these caspases may not be directly involved in spontaneous neutrophil apoptosis. We would postulate that there are other caspases involved in this spontaneous process that mediate their effects independent of caspase 3 and 8.

3.4. Effects of LPS and GM-CSF on anti-Fas antibody induced caspase activity and mitochondrial permeability

Cellular resistance to Fas/Fasligand and TNF/TNFR1 induced apoptosis has been demonstrated in a number of cell systems. NF- κ B activation renders lymphocytes, fibroblasts and macrophages refractory to the pro-apoptotic effects of TNF [22,23]. Interleukin-1 β stimulates resistance in Jurkat cells to Fas induced apoptosis [24]. Co-stimulation with LPS and GM-CSF induces neutrophil resistance to Fas antibody induced apoptosis [7]. Similarly, inflammatory neutrophils isolated from the lungs of LPS treated rats are resistant to anti-Fas antibody and TNF induced apoptosis [12]. As shown in Fig. 3, neutrophils preincubated with LPS (1 μ g/ml) and GM-CSF (8 ng/ml) for 1 h before exposure to anti-Fas antibody showed a significant reduction in rates of apoptosis. Similar results were seen with annexin V binding; anti-Fas antibody induced neutrophil apoptosis was reduced from $48 \pm 12\%$ to $10 \pm 5\%$ with LPS and $15 \pm 4\%$ with GM-CSF. Resistance to apoptosis was associated with inhibition of mitochondrial per-

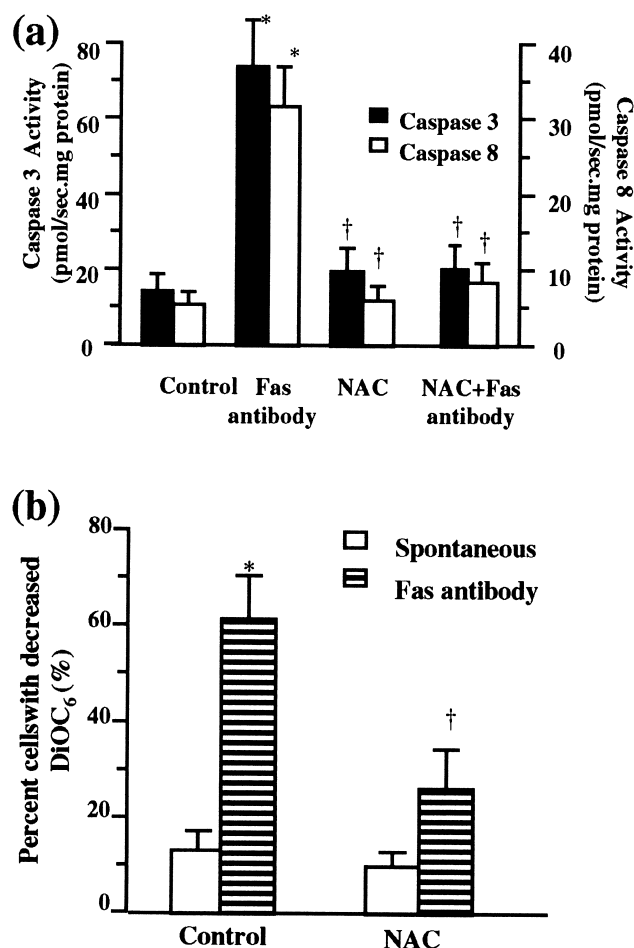


Fig. 4. Effects of NAC on anti-Fas antibody and mCICCP induced caspase and mitochondrial changes. a: Neutrophils (1×10^6 /ml) were preincubated with NAC (25 mM) for 1 h and then treated with anti-Fas antibody (CH-11, 100 ng/ml). Caspase 3 and 8 activity was assessed after anti-Fas antibody incubation for 6 h, using AFC labelled substrates. The rate of AFC fluorescence release was calculated and expressed as pmol/s mg protein. b: Mitochondrial disruption was also assessed at the same time, using DiOC₆ staining detected by flow cytometry. * $P < 0.05$ vs control neutrophils, † $P < 0.05$ vs Fas antibody treated neutrophils, $n = 6$.

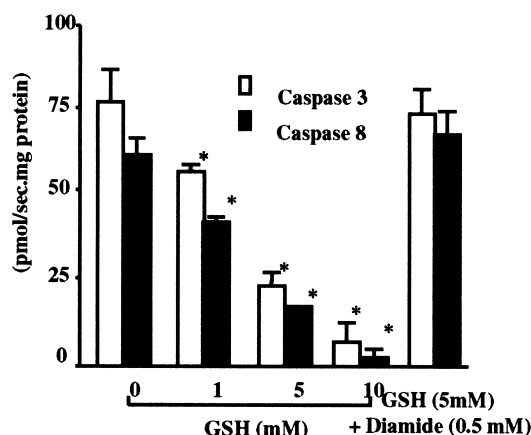


Fig. 5. Glutathione regulation of caspase 3 and 8 activity. Cell lysates were collected from 10×10^6 neutrophils incubated with anti-Fas antibody (CH-11, 100 ng/ml) for 6 h. Lysates were then incubated with 20 μ M of fluorogenic substrates for caspase 3 (CBZ-Asp-Glu-Val-Asp-AFC) and caspase 8 (CBZ-Ile-Glu-Thr-Asp-AFC) with increasing concentrations of glutathione (1–10 mM). Diamide (0.5 mM, which chemically breaks down glutathione) was incubated with the glutathione (5 mM) solution for 1 h before addition as above. Caspase activity was calculated as the rate of AFC fluorescence release and expressed as pmol/s mg protein. * $P < 0.05$ vs 0 mM GSH, $n = 3$.

meability (Fig. 3), and reduced caspase 3 and 8 activity (Fig. 3), suggesting that LPS and GM-CSF inhibit the caspase cascade at a step prior to caspase 8 activation.

3.5. Effects of NAC on anti-Fas induced caspase activity and mitochondrial permeability

Augmentation of intracellular glutathione with NAC results in resistance to anti-Fas induced apoptosis [7]. The protective effect of NAC was associated with inhibition of caspase 3 and 8 activity induced by anti-Fas antibody (Fig. 4a) and a significant reduction in the percentage of cells with mitochondrial injury (Fig. 4b). These results indicate that glutathione not only inhibits caspase 8 activity, but also stabilises the mitochondrial membrane. Glutathione has been shown to block the ability of granzyme B to cleave poly(ADP-ribose) polymerase, a caspase 3 dependent process [25]. In addition, dithiocarbamate disulphide, a thiol regulating agent, inhibits proteolytic processing of the caspase 3 pro-enzyme in Jurkat T lymphocytes treated with anti-CD95 antibodies [26].

3.6. Direct effect of glutathione on caspase activity

Glutathione was also shown to have a direct effect on caspase activity. Cell lysates, from neutrophils incubated for 6 h with anti-Fas, were incubated with fluorogenic substrates for caspase 3 and 8 with differing, physiologic concentrations of glutathione (1–10 mM) and assessed for caspase activity (Fig. 5). Glutathione resulted in significant dose dependent inhibition of caspase 3 and 8 activity. These effects were specific for glutathione as diamide (0.5 mM, which chemically degrades glutathione) significantly inhibited the effects of glutathione on caspase activity.

This study demonstrates that the inflammatory mediators that delay and inhibit Fas antibody induced apoptosis mediate their effect through stabilisation of the mitochondrial membrane and caspase inhibition. These processes could also be induced by increased glutathione, which was shown

to directly inhibit caspase activity. Increased glutathione levels during neutrophil activation may represent an important mechanism to protect the cell against oxidative stress encountered during bacterial ingestion. In addition, thiols may protect the cell against caspase activation, allowing the cell to survive longer and respond to an infectious challenge. Manipulation of intracellular thiols may provide a mechanism for regulating apoptosis in activated neutrophils, and thus prevent the persistence of inflammation and neutrophil mediated tissue injury.

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