Tumour necrosis factor induces phosphorylation primarily of the nitric-oxide-responsive form of glyoxalase I

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We have previously shown that TNF (tumour necrosis factor) induces phosphorylation of GLO1 (glyoxalase I), which is required for cell death in L929 cells. In the present paper, we show that the TNF-induced phosphorylation of GLO1 occurs primarily on the NO (nitric oxide)-responsive form of GLO1. In addition, analysis of several cysteine mutants of GLO1 indicated that Cys-138, in combination with either Cys-18 or Cys-19, is a crucial target residue for the NO-mediated modification of GLO1. Furthermore, the NO-donor GSNO (*S*-nitrosoglutathione) induces NO-mediated modification of GLO1 and enhances the TNF-induced phosphorylation of this NO-responsive form. GSNO also strongly promotes TNF-induced cell death. By the use

of pharmacological inhibition of iNOS (inducible NO synthase) and overexpression of mutants of GLO1 that are deficient for the NO-mediated modification, we have shown that the NO-mediated modification of GLO1 is not a requirement for TNF-induced phosphorylation or TNF-induced cell death respectively. In summary, these data suggest that the TNF-induced phosphorylation of GLO1 is the dominant factor for cell death.

Key words: cell death, glyoxalase I (GLO1), methylglyoxal, nitrosative stress, post-translational modification, tumour necrosis factor (TNF).

INTRODUCTION

The glyoxalase system, consisting of the enzymes glyoxalase I (GLO1) and II, is an integral component of cellular metabolism. A major function of the glyoxalase system is the detoxification of α -oxoaldehydes (α -ketoaldehydes), especially MG (methylglyoxal). Although the system was discovered in 1913, its full biological function has never been elucidated. The ubiquitous nature of the glyoxalase pathway suggests an important cellular function that is conserved through evolutionary selection. The work of the Nobel Laureate Szent-Györgyi suggests that GLO1 and its substrate MG are involved in the regulation of cellular growth, but a direct mechanistic link has yet to be identified (for a review, see [1]).

MG is a cytotoxic metabolite primarily produced by non-enzymatic phosphate elimination from the glycolytic intermediates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [2]. Increased production of MG leads to the formation of AGEs (advanced glycation end-products), which are involved in the development of a number of pathological conditions, including diabetes and aging-related diseases such as Alzheimer's disease [3]. Increased production of MG can also be induced by TNF (tumour necrosis factor) [4].

Overexpression of GLO1 has been linked to a number of diseases, including diabetes mellitus [5], Alzheimer's disease [6], high anxiety-like behaviour in mice [7] and, particularly, cellular proliferative disorders, including breast [8], prostate [9] and colon cancer [10]. Furthermore, overexpression of GLO1 is involved in the resistance of human leukaemia cells to anti-tumour-agent-induced apoptosis [11]. MG has long been regarded as a natural anticancer agent, and therefore GLO1 inhibitors have been developed as potential anticancer agents [12,13].

We have previously described the PKA (protein kinase A)-mediated phosphorylation of GLO1, which plays an essential role in TNF-induced necrosis in the fibrosarcoma cell line L929. Phosphorylated GLO1 is not involved in the detoxification pathway of MG, but instead mediates the cytotoxic effects of MG via a pathway that leads to MG-modification of specific target molecules (MG-derived AGE formation) [4]. However, the real biological function of phosphorylated GLO1 remains to be determined. The purpose of the present study was to analyse the different isoforms of GLO1 by high-resolution 2-DE (two-dimensional gel electrophoresis) in combination with Western blotting, to determine on which isoform the TNF-induced phosphorylation occurs and to determine its role in TNF-induced cell death.

MATERIALS AND METHODS

Cell lines and cultures

L929 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with GlutaMAXTM (Invitrogen) supplemented with heat-inactivated 10% (v/v) FCS (fetal calf serum) (Cambrex), 100 units/ml penicillin and 0.1 mg/ml streptomycin. Human carcinoma (HeLa) cells were grown in DMEM with GlutaMAXTM supplemented with 10% FCS. Both cell lines were cultured at 37°C in a humidified incubator under an 8% CO₂ atmosphere.

Reagents

Murine TNF was obtained from Roche Diagnostics. The PKA inhibitor H89, forskolin and NOS (nitric oxide synthase) inhibitor L-NMMA (*N*^G-monomethyl-L-arginine, monoacetate salt) were

Abbreviations used: AGE, advanced glycation end-product; 2-DE, two-dimensional gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GLO1, glyoxalase I; GSNO, *S*-nitrosoglutathione; IEF, isoelectric focusing; IFN α , interferon α ; MEF, mouse embryonic fibroblast; MG, methylglyoxal; L-NMMA, *N*^G-monomethyl-L-arginine, monoacetate salt; NF- κ B, nuclear factor κ B; NOS, nitric oxide synthase; iNOS, inducible NOS; PI, propidium iodide; PKA, protein kinase A; RBC, red blood cell; TNF, tumour necrosis factor; WT, wild-type.

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from Calbiochem. PI (propidium iodide) (Sigma) was used at a concentration of 30 μ M. The NO (nitric oxide) donor GSNO (*S*-nitrosoglutathione) and the selective iNOS (inducible NOS) inhibitor 1400W $\{N$ -[3-(aminomethyl)benzyl]acetamidine} were purchased from Invitrogen. The monoclonal anti-nitrotyrosine antibody was from Cell Signaling Technology.

Plasmids

The cDNA encoding WT (wild-type) GLO1 was a gift from Dr K. Tew (Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, U.S.A.) and was subcloned into the pCAGGS vector. The inducible expression plasmid pSP64MxpA containing the murine Mx promoter that was used for the generation of stable L929 clones has been described previously [14]. Inserts encoding WT GLO1 were isolated as EcoRI fragments from the pCAGGS plasmid and cloned into the BamHI-opened pSP64MxpA expression vector. The different cysteine mutants of GLO1 were obtained using the QuikChange[®] site-directed mutagenesis kit (Stratagene) and the pSP64MxpA-wtGLO1 vector was used as template. The C18A mutation was introduced using the forward primer 5'-CGAGGCCGCCCTCAGTGCCTGCTC-CGACGCGGACCCC-3' and the reverse primer 5'-GGGG-TCCGCGTCCGAGCAGGCACTGAGGGCGGCCTCG-3'. The C19A mutation was introduced using the forward primer 5'-GGGGTCCGCGTCCGAGGCGCAACTGAGGGCGGCCTCG-3' and the reverse primer 5'-CGAGGCCGCCCTCAGTTGCGC-CTCCGACGCGGACCCC-3'. The double mutation C18A/C19A was introduced using the forward primer 5'-CGAGGCCGCC-CTCAGTGCCTCCGACGCGGACCCC-3' and the reverse primer 5'-GGGGTCCGCGTCCGAGGCGGCACTGAGGGCG-GCCTCG-3'. The C138A mutation was introduced using the forward primer 5'-CCTGATGTATACAGTGTCTGCTAAAAGGT-TTGAAGAACTGGG-3' and the reverse primer 5'-CCCAGTT-CTTCAAACCTTTTAGCAGCACTGTATACATCAGG-3'. The GLO1 mutant C138A was used as template to create the GLO1 mutants C18A/C138A and C19A/C138A with the respective primers described above. The GLO1 mutant C18A/C19A served as template to create the GLO1 mutant C18A/C19A/C138A with the primers used for mutating Cys-138. All mutations were confirmed by DNA sequencing. For transient transfections in the HeLa cell line, the different cysteine mutants were cloned under the control of a constitutive CMV (cytomegalovirus) promoter in the pCAGGS vector by a blunt-end ligation of the XbaI/Sall GLO1 fragments into the XhoI-opened mammalian expression vector. All constructs were verified by sequencing.

Purification of GLO1 from human RBCs (red blood cells) and production of polyclonal antibodies

All procedures, including centrifugation, were performed at 4°C. Human RBCs (250 ml) in a citrate buffer were received from a blood transfusion centre. RBCs were centrifuged at 2000 *g* for 7 min, and supernatant was removed. The RBC pellet was washed with PBS. The cells were lysed with 120 ml of cytosol extraction buffer [10 mM Tris/HCl, pH 7.4, 50 mM EDTA, 25 mM NaCl, 0.7 % Triton X-100, 100 mM PMSF and one Complete[™] protease inhibitor cocktail tablet (Roche) per 50 ml of cytosol extraction buffer]. The mixture was kept at 4°C for 15 min and centrifuged at 42000 rev./min for 2 h with an Ultracentrifuge (Beckman L7-55, rotor type 42.5). Supernatant was carefully removed and applied to a column of *S*-hexylglutathione (Sigma) immobilized on cross-linked 4 % beaded agarose. The column

was washed overnight with 1500 ml of PBS. GLO1 was eluted with 20 ml of 6 mM *S*-hexylglutathione and analysed by SDS/PAGE and silver staining. GLO1 was then concentrated using a Centricon filter with a 3000 Da cut-off at 7000 *g* in a Sorvall SA-600 rotor to a final volume of approx. 140 μ l. A small fraction (1/100) was then used for analysis by SDS/PAGE and silver staining. Because GLO1 was slightly contaminated with two other protein bands, GLO1 was purified further by gel-filtration using a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated in PBS and eluted at a flow rate of 1.2 ml/min. The fractions were analysed by SDS/PAGE and silver staining. The identity of the purified protein was confirmed by MS. The fractions containing the highest concentration of GLO1 were pooled and used as antigen for the production of rabbit polyclonal antibodies. The whole purification procedure was repeated four times. The specificity of the polyclonal anti-GLO1 antibody was tested against overexpressed human GLO1 in HeLa cells.

Purification of GLO1 from L929 cells

L929 cells were seeded 48 h before the experiment. After incubation with GSNO (250 μ M), the cells were rinsed three times with ice-cold PBS, and cell lysates were prepared in a CHAPS-containing cytosol extraction buffer (see above). The cell lysates were incubated with *S*-hexylglutathione-agarose beads (Sigma) for 2 h. The cell lysates were then removed and the beads were washed four times with PBS. GLO1 was eluted from the beads with 10 mM *S*-hexylglutathione in 10 mM Tris/HCl (pH 7.4), 50 mM EDTA and 25 mM NaCl.

2-Dimensional gel electrophoresis

IEF (isoelectric focusing) was carried out on 18 cm IPG (immobilized pH gradient) strips, pH 4–7 (GE Healthcare) according to the manufacturer's instructions. For the second dimension, proteins were separated by SDS/PAGE (12 % polyacrylamide).

Western blotting

Proteins were separated by SDS/PAGE (12 % polyacrylamide) and transferred on to a PVDF membrane (Hybond-P; GE Healthcare). The blots were incubated with an anti-(human GLO1) polyclonal antibody, followed by ECL[®] (enhanced chemiluminescence)-based detection (GE Healthcare).

Measurement of TNF-induced cell death by flow cytometry

L929 cells were seeded (2×10^5 /ml) 1 day before experiments in uncoated 24-well tissue culture plates (Sarstedt). Cell death was induced by the addition of TNF to the cell suspension. Cell death was measured by quantifying PI-positive cells using a FACSCalibur (Becton Dickinson) as described in [15]. The PI dye was excited with an argon-ion laser at 488 nm, and PI fluorescence was measured above 590 nm by using a long-pass filter. Approx. 3000 cells were routinely analysed. Cell death is expressed as the percentage of PI-positive cells in the total cell population.

Stable L929 cell lines overexpressing WT GLO1 and mutants

Because there is a large clonal variability in sensitivity to TNF-induced cell death in L929 cells, we used an inducible expression system so that TNF sensitivity can be compared in the same clone upon induced expression of GLO1, circumventing the problem of clonal variability. Murine fibrosarcoma L929 cells

were transfected with pSP64MxpA GLO1 and several Cys → Ala mutants of GLO1 respectively. In this expression vector, GLO1 is under the control of the murine Mx promoter, which is inducible with IFN α (interferon α). After transfection, G418-resistant clones were screened and retained when they showed low-level leak expression in the non-induced condition and strong expression in the induced condition. Three clones for WT GLO1 and mutants and three mock clones were selected for use in further experiments. For induction, cells were incubated with 500 units/ml IFN α for 16 h before TNF treatment.

Statistics

Data are presented as means $\pm$ S.D. Significance between groups was determined using an unpaired two-tailed Student's *t* test. Significance was established at *P* < 0.05.

RESULTS

TNF induces PKA-mediated phosphorylation of the NO-responsive form of GLO1

We have previously described the TNF-induced phosphorylation of GLO1 mediated by PKA in L929 cells by the use of *in vivo* [32 P]orthophosphate labelling and Western blotting in combination with low-resolution 2-DE [4]. To characterize further the TNF-induced phosphorylation of GLO1 in L929 cells, we performed a detailed study of the isoforms of GLO1 by high-resolution 2-DE on a pH 4–7 gradient in combination with Western blotting. L929 cells were treated with TNF (1000 units/ml) for 1.5 h. Cell lysates were analysed by 2-DE, and representative Western blots from control and TNF-treated L929 cells are shown in Figure 1(A). As this Figure clearly shows, multiple isoforms of GLO1 exist. A similar isoform pattern of GLO1 was also observed in all other cell types tested so far, including mouse β -cells MIN6, mouse pro-B-cells Ba/F3, human HeLa cells and primary MEFs (mouse embryonic fibroblasts) (see Figure 1B). To study the modifications of GLO1 on Western blots of 2-DE gels, we used the position of the most intensive isoform of GLO1 (pI of 5.0) as a reference and have designated this isoform α ; α refers to the non-NO-responsive form of GLO1 (see below). The two most acidic isoforms of GLO1 did not change upon treating the cells with TNF, and so they are not discussed further. As shown in Figure 1(A), a new GLO1 isoform appeared in the TNF condition (indicated by an arrowhead in the upper-right-hand panel) and this was concomitant with the disappearance of the most basic isoform of GLO1 (this isoform is indicated by an arrow in the upper-left-hand panel). We have shown previously that the TNF-induced phosphorylation of GLO1 could be completely inhibited by the PKA inhibitor H89 [4]. We therefore used H89 to examine whether the induction of the new GLO1 isoform in the TNF condition was due to phosphorylation. As shown in the lower panels of Figure 1(A), in the presence of H89, the TNF-induced induction of the new GLO1 isoform was inhibited completely, while the most basic isoform of GLO1 remained. These results indicate that the new GLO1 spot induced by TNF is derived from phosphorylation of the most basic isoform of GLO1, which consequently shifts to a more acidic position on the 2-DE gel.

It has been reported that GLO1 is a NO-responsive protein and that treatment of endothelial cells, as well as purified human GLO1, with several NO donors, including GSNO, results in the appearance of a more basic isoform of GLO1 on 2-DE gels [16,17]. Therefore, using the NO donor GSNO, we investigated whether the basic isoform corresponds to the NO-responsive form of GLO1. As shown in Figure 1(C), treatment of L929 cells with

Figure 1 TNF-induced phosphorylation of GLO1 mediated by PKA occurs on the NO-responsive form

(A) TNF induces phosphorylation on the most basic isoform of GLO1. Western blots of 2-DE gels (pH 4–7) developed with an anti-(human GLO1) polyclonal antibody are shown. TNF treatment was performed for 1.5 h (1000 units/ml). Pre-incubation with the PKA inhibitor H89 (5 μ M) was performed for 1.5 h. C, control cells; TNF, TNF-treated cells; H89, control PKA inhibitor; TNF + H89, TNF-treated cells that were pre-incubated with the PKA inhibitor. The most intensive spot denoted α was used as a reference and corresponds to the non-NO-responsive form of GLO1. Note the induction of a new GLO1 isoform (arrowhead) in TNF-treated cells that is completely prevented by the PKA inhibitor H89. (B) An similar 2-DE pattern (pH4–7) of GLO1 isoforms was found in several other mouse and human cells. (C) The most basic isoform of GLO1 corresponds to the NO-responsive form. L929 cells were incubated with the NO donor GSNO (250 μ M) for 1 h. Western blots of the 2-DE gels (pH 4–7) were developed with the anti-(human GLO1) polyclonal antibody. C, control cells; GSNO, GSNO-treated cells. Note the strong induction of the most basic isoform of GLO1 in the presence of GSNO (spot indicated by an arrow). Ac, acidic; Bs, basic.

250 μ M GSNO for 1 h resulted in a prominent increase of the most basic isoforms of GLO1. Similar results were also obtained with 100 μ M GSNO. These data indicate that the most basic isoform of GLO1 corresponds to the NO-responsive form of GLO1 and that the TNF-induced phosphorylation of GLO1 primarily occurs on the NO-responsive form.

To substantiate further the role of PKA in the TNF-induced phosphorylation of GLO1, we used forskolin, an allosteric activator of adenylate cyclase that catalyses the formation of cAMP, which in turn activates PKA. We tested the effect of forskolin (Figure 2) on the TNF-induced phosphorylation of GLO1 and the pathways in which phosphorylated GLO1 is involved: namely, TNF-induced cell death and the TNF-induced formation of a specific MG-derived AGE [4]. The TNF-induced phosphorylation of the NO-responsive form of GLO1 is shown in Figure 2(A) (TNF, phosphorylated forms of GLO1 are indicated by arrowheads). As is clear from this Figure, in the presence of forskolin (TNF + F), the amount of TNF-induced phosphorylation of the NO-responsive form of GLO1 was not significantly

Figure 2 Synergistic effect of forskolin on the TNF-induced phosphorylation of GLO1, TNF-induced cell death and the TNF-induced formation of a specific MG-derived AGE

(A) Effect of forskolin (F) on the TNF-induced phosphorylation of GLO1. L929 cells were pre-incubated with 5 μM forskolin for 0.5 h and then incubated further with 1000 units/ml TNF for 1 h. Note that, in the presence of forskolin, TNF also induced phosphorylation of the non-NO-responsive form of GLO1. Forskolin treatment alone induced phosphorylation of GLO1. Ac, acidic; Bs, basic; C, control. (B) Effect of forskolin on TNF-induced cell death in L929 cells. White bars, control cells; grey bars, 200 units/ml TNF; black bars, 2 μM forskolin-treated cells; hatched bars, TNF + 2 μM forskolin. Note, in the presence of forskolin, the 3-fold increase in cell death at early time points of TNF treatment (2.5 h). Results are means ± S.D. ** $P < 0.001$. (C) Effect of forskolin on the TNF-induced formation of a specific-MG-derived AGE (indicated by an arrow). Cells were pre-incubated for 0.5 h with forskolin. Equal amounts of protein were loaded.

higher than that induced by TNF alone. However, a significant phosphorylation of the non-NO-responsive form of GLO1 (shift of the α form to the acidic side of the gel) was induced by this combined treatment. This also indicates that the PKA-mediated phosphorylation of GLO1 is not exclusive to the NO-responsive form. Forskolin alone (F) also induced phosphorylation of GLO1 in L929 cells (arrowhead in the lower-left-hand panel of Figure 2A). In conclusion, these data indicate that the total amount of phosphorylated GLO1 in TNF-treated cells increases significantly in the presence of forskolin and that activation of PKA by forskolin alone in L929 cells leads to phosphorylation of GLO1. Still, whether GLO1 is a direct substrate for PKA remains to be determined.

TNF-induced cell death in L929 cells increases dramatically in the presence of forskolin (Figure 2B). This synergistic effect (we observed a 3-fold increase in cell death in the presence of forskolin compared with cell death induced by TNF alone) was most pronounced at lower doses of TNF and at early time points of TNF treatment (2.5 h). At this time point, no cell death was observed with forskolin alone. It was only at later time points that forskolin induced a low percentage of cell death (Figure 2B).

We have shown previously that phosphorylated GLO1 is not involved in the detoxification of MG, but instead is on the pathway leading to the formation of a specific MG-derived AGE. Furthermore, we showed that formation of the latter is prevented by the specific GLO1 inhibitor, *S*-*p*-bromobenzyglutathione cyclo-

pentyl diester, and the PKA inhibitor H89, which are both inhibitors of the TNF-induced phosphorylation of GLO1 [4]. We therefore also tested the effect of forskolin on the TNF-induced formation of this specific MG-derived AGE by Western blotting using the monoclonal antibody mAbB6 that recognizes MG-derived AGEs [4,18]. The formation of the specific MG-derived AGE in L929 cells was weakly induced at 1.5 h of TNF treatment alone and was more pronounced at 2 h of TNF treatment (Figure 2C). However, in the presence of forskolin, the TNF-induced formation of the specific MG-derived AGE increased dramatically (indicated by an arrow in Figure 2C). Note that forskolin by itself did not induce the formation of the specific MG-derived AGE. This is also in line with our previous work, which indicates that the TNF-induced MG-derived AGE is formed only under conditions in which cell death occurs [4].

In summary, all these data substantiate further the role of PKA in the TNF-induced phosphorylation of GLO1, in TNF-induced cell death and the TNF-induced formation of the specific MG-derived AGE.

Cysteine residues are involved in the NO-mediated modification of GLO1

Post-translational modifications of proteins by NO occur mainly as nitration of tyrosine residues and nitrosylation of cysteine residues [19]. Human GLO1 contains six tyrosine residues and four cysteine residues. First, using an anti-nitrotyrosine monoclonal antibody, we examined whether nitration of a tyrosine residue was involved in the NO-mediated modification of GLO1. Purified GLO1 (see the Materials and methods section) from control and GSNO-treated L929 cells was analysed by 2-DE. The Western blots were detected consecutively with the anti-nitrotyrosine antibody and the anti-GLO1 antibody. Although we could clearly detect the NO-responsive form of GLO1 in the GSNO-treated sample with the anti-GLO1 antibody (indicated by an arrow in Figure 3A), the anti-nitrotyrosine antibody failed to detect any isoform of GLO1. This result suggests that the NO-mediated modification of GLO1 is not caused by nitration of a tyrosine residue.

Human GLO1 contains four cysteine residues, of which Cys-138 is conserved from bacteria to humans. Cys-18 and Cys-19 are only present in mouse and human GLO1. Cys-60 is only present in human GLO1, and this residue has been reported to be involved in substrate binding [20]. To identify the critical cysteine residues that could be involved in the NO-mediated modification of GLO1, we made use of the fact that transient overexpression of WT human GLO1 in HeLa cells results in the spontaneous induction of the NO-responsive form of overexpressed GLO1 (spot indicated by an arrow in Figure 3B). This allowed us to screen for cysteine mutants of GLO1 that were deficient for NO-mediated modification at physiological concentrations of NO. Because Cys-60 is only present in human GLO1, and since we observed the NO-responsive form of GLO1 in several mouse cell lines, including L929 cells, it is unlikely that this residue is involved in the NO-mediated modification of GLO1. Indeed, the level of the NO-responsive form of the overexpressed human GLO1 C60A mutant protein was comparable with that of WT GLO1 (Figure 3B). Overexpression of the other single mutants C18A, C19A and C138A of human GLO1 is shown in Figure 3(B). The NO-mediated modification was still induced on the C18A and C19A mutant proteins. In contrast, the NO-mediated modification of the GLO1 C138A mutant was considerably impaired. In several independent experiments, and in contrast with the other mutants, overexpression of the C138A

Figure 3 Involvement of cysteine residues in the NO-mediated modification of GLO1

(A) Western blot of a 2-DE from purified GLO1 derived from control and GSNO-treated L929 cells (250 μ M). The Western blot was detected in consecutive order with the anti-nitrotyrosine and the anti-GLO1 antibodies (Ab). (B) HeLa cells were transiently transfected with cDNAs encoding WT and different cysteine mutants of GLO1. Mock-transfected cells were used as a control for overexpression. Cells were lysed 24 h after transfection. Western blots of the 2-DE gels (pH 4–7) were developed with an anti-GLO1 polyclonal antibody. Note the spontaneous induction of the NO-responsive form of overexpressed WT GLO1. The induction of the NO-responsive form is drastically impaired in the C138A and C18A/C19A mutants, and completely impaired in the C18A/C138A, C19A/C138A and C18A/C19A/C138A mutants. The difference in pI value of the NO-responsive forms of some of the GLO1 mutant proteins is presumably due to phosphorylation. Ac, acidic; Bs, basic.

mutant never resulted in the induction of a focused spot of the NO-responsive form. These data indicate that Cys-138 plays an important role in the NO-mediated modification of GLO1.

In a next step, we overexpressed the double mutants of GLO1, C18A/C19A, C18A/C138A and C19A/C138A, and the triple mutant C18A/C19A/C138A. The 2-DE pattern of these mutants is also shown in Figure 3(B). The NO-mediated modification of the C18A/C19A mutant was greatly impaired. Note that the NO-mediated modification of this mutant did not result in a focused spot, although Cys-138 was still present. This result suggested that the NO-mediated modification of GLO1 involved Cys-138 in combination with either Cys-18 or Cys-19. Accordingly, mutation of either Cys-18 or Cys-19, in combination with Cys-138, resulted in a complete absence of the NO-responsive form at physiological concentrations of NO. This was also the case for the triple-cysteine mutant. In addition, by administration of GSNO (250 μ M) to the cells, the NO-responsive form of the triple-cysteine mutant of GLO1 was not induced (Figure 3B). This result further confirmed the exclusive role of cysteine residues in the NO-mediated modification of GLO1.

In summary, these data show that cysteine residues are involved in the NO-mediated modification of GLO1 and that the most conserved residue, Cys-138, in combination with either Cys-18 or Cys-19, plays a crucial role in this modification.

Figure 4 Effect of GSNO on TNF-induced cell death and the phosphorylation of GLO1

(A) L929 cells were incubated in the presence of 30 units/ml TNF and/or different concentrations of GSNO (1 h pre-incubation) for the indicated periods. Cell death was measured by quantifying PI-positive cells by FACS as described in the Materials and methods section. White bars, control GSNO (250 μ M); grey bars, TNF; black bars, TNF + 100 μ M GSNO; hatched bars, TNF + 250 μ M GSNO. Note the strong synergistic effect of GSNO on TNF-induced cell death. Results are means $\pm$ S.D. for triplicate cultures. (B) Western blots showing the TNF-induced phosphorylation of GLO1 in the presence of GSNO. L929 cells were pre-incubated with 250 μ M GSNO for 1 h and then treated with 1000 units/ml TNF for 1.5 h. Western blots of the 2-DE gels (pH 4–7) were developed with an anti-GLO1 polyclonal antibody. Note the multiple spots to the right of α that correspond to multiple phosphorylations on the NO-responsive form of GLO1. Ac, acidic; Bs, basic.

The NO donor GSNO enhances the TNF-induced phosphorylation of the NO-responsive form of GLO1 and strongly promotes TNF-induced cell death

We investigated further whether the NO-mediated modification of GLO1 also contributed to TNF-induced cell death. TNF-induced cell death in L929 cells was measured as a function of time in the presence or absence of the NO donor GSNO. As shown in Figure 4(A), GSNO by itself did not induce cell death. Pre-incubating L929 cells for 1 h with 250 μ M GSNO strongly promoted TNF-induced cell death ($\pm$ 100 % increase compared with TNF alone). This synergistic effect was particularly pronounced at lower doses of TNF (100 units/ml or less) and also at earlier time points of TNF treatment. Pre-incubation of L929 cells for 1 h with 100 μ M GSNO also promoted TNF-induced cell death, but to a lesser extent.

Analysis of the TNF-induced phosphorylation of GLO1 in L929 cells in the presence of GSNO revealed that the level of phosphorylated GLO1 (spots indicated by arrowheads) on the NO-responsive form was higher compared with a TNF-treated sample in the absence of GSNO (Figure 4B). The multiple spots to the right of α (arrowheads) correspond to multiple phosphorylations (shifts to the acidic side) of the NO-responsive form of GLO1. It is

Figure 5 Effect of overexpression of Cys → Ala mutants of GLO1 on the synergistic action of GSNO on TNF-induced cell death in L929 cells

(A) Expression levels of ectopically expressed GLO1 in WT clones and clones overexpressing the C18A/C19A/C138A mutant (Mu) and endogenously expressed GLO1 in mock clones (Mo) under induced (i) and non-induced (ni) conditions. The numbers after Mo, WT and Mu refer to the clone number. Note that all GLO1-overexpressing clones have leak expression in the non-induced condition, as compared with the expression level of endogenous GLO1 in the mock clones. Equal amounts of protein were loaded for all clones. (B) Percentage of TNF-induced cell death expressed as the percentage of PI-positive cells after 5 h of 30 units/ml TNF treatment of the different clones under non-induced (ni) and induced (i) conditions and in the absence and presence of 50 μ M GSNO. (C) Synergistic effect of GSNO on TNF-induced cell death of the different clones under non-induced (ni) and induced (i) conditions for the same experiment as shown in (B). The synergistic effect is expressed as the fold increase in percentage of TNF-induced cell death in the presence of GSNO over the percentage of TNF-induced cell death without GSNO for the same conditions.

worth mentioning that treatment of L929 cells with GSNO alone not only strongly induced the NO-responsive form of GLO1, but also was often accompanied by a limited phosphorylation of this form. These results suggest that there is an intimate link between the NO-mediated modification and phosphorylation of GLO1.

To investigate whether the synergistic effect of GSNO on TNF-induced cell death was mediated by the NO-mediated modification of GLO1, we overexpressed cysteine mutants of GLO1 deficient for the NO-mediated modification. Stable L929 clones that inducibly overexpressed the C18A/C19A/C138A, C18A/C138A and C19A/C138A mutants and WT GLO1 were selected. Because the double mutants behaved similarly to the triple mutant, only the triple mutant (Mu) is shown in Figure 5, together with L929 clones overexpressing WT GLO1 (WT) and mock-transfected clones (Mo). The expression level of endogenous GLO1 (Mo clones) and of the ectopically expressed proteins in the non-induced (ni) and induced (i) conditions is shown in Figure 5(A). As is evident from this Western blot, the clones overexpressing GLO1 had considerable leak expression in

the non-induced conditions, a phenomenon that we had observed previously with this expression vector [14]. TNF-induced cell death in the various clones under the induced and non-induced conditions and in the presence and absence of GSNO was measured for three independent experiments, and a representative experiment is shown in Figure 5(B). Figure 5(C) shows the synergistic effect of GSNO in the various clones under the non-induced and induced conditions of the same experiment as shown in Figure 5(B). TNF-induced cell death in clones overexpressing GLO1 (WT and mutants) was lower compared with the mock clones, even under the non-induced condition (white bars). This effect is most likely to be caused by overexpression of GLO1 (= leak expression as compared with the levels of endogenous GLO1 in the mock clones), which contributes to resistance towards TNF-induced cell death. However, upon induced expression of ectopic GLO1 (WT and Mu), TNF-induced cell death (grey bars) was higher compared with cell death in the mock clones. This apparent discrepancy may be due to the fact that two opposing effects of GLO1 contribute to TNF-induced cell death.

Overexpression of GLO1 may, on the one hand, counteract the TNF-induced increased levels of MG [4] which may inhibit cell death, and on the other hand, lead to more phosphorylated GLO1 which promotes TNF-induced cell death. The synergistic effect of GSNO on TNF-induced cell death in clones that overexpressed GLO1 was much higher compared with that in the mock clones, indicating that overexpression of GLO1 sensitizes for the synergistic effect of GSNO on TNF-induced cell death. However, the synergistic effect in clones overexpressing the mutants of GLO1 was not significantly different from that of clones overexpressing WT GLO1. This result suggests either that the cysteine mutants of GLO1 cannot act as dominant-negative mutants or that phosphorylation of GLO1 is the dominant factor for TNF-induced cell death. The latter is also in line with the fact that the PKA inhibitor H89, which strongly inhibits TNF-induced phosphorylation of GLO1 on the NO-responsive form, also strongly inhibits TNF-induced cell death [4]. It is also very plausible that several other target proteins of NO mediate the synergistic effect of GSNO on TNF-induced cell death. For example, several proteins involved in the TNF-induced activation of NF- κ B (nuclear factor κ B) can be inhibited by NO [21], and it is well known that inhibition of NF- κ B activation promotes TNF-induced cell death [22,23].

NO-mediated modification of GLO1 is not required for the TNF-induced phosphorylation of GLO1

To investigate further whether the NO-mediated modification of GLO1 was required for TNF-induced phosphorylation of GLO1, we used pharmacological inhibitors of NO synthase. L-NMMA is a general inhibitor of the constitutive [eNOS (endothelial NOS) and nNOS (neuronal NOS)] and iNOS. This inhibitor (1 mM for 2 h) only slightly reduced the level of the NO-responsive form of GLO1 and had no effect on TNF-induced cell death in L929 cells (results not shown). TNF is known to be a strong inducer of iNOS [24] and can also induce the NO-mediated modification of GLO1 (D. Rondas and K. Vancompernelle, unpublished work). We therefore used 1400W, which is a slow, tight-binding and highly selective inhibitor of iNOS [25]. L929 cells were pre-incubated with 40 μ M 1400W for 2 h and then incubated further with 1000 units/ml TNF for 2 h. Cell lysates were analysed by 2-DE and the Western blots of GLO1 are shown in Figure 6(A). 1400W by itself had no significant effect on the basal level of the NO-responsive form of GLO1. However, in the presence of 1400W, TNF not only induced phosphorylation of the NO-responsive form of GLO1 (arrowheads to the right of α), but also induced limited phosphorylation of the non-NO-responsive form of GLO1 (arrowheads to the left of α). This may explain why 1400W only modestly inhibited TNF-induced cell death (a maximum inhibition of 30 % was obtained at 40 μ M, Figure 6B). It is noteworthy that, in the presence of 1400W alone, we often observed a limited phosphorylation of GLO1. This result suggests further that there is an intimate link between NO signalling and the phosphorylation of GLO1. In summary, these data suggest further that the TNF-induced phosphorylation of GLO1 is not exclusive to the NO-responsive form, but that phosphorylation can also be induced on the non-NO-responsive form of GLO1.

DISCUSSION

We characterized the different isoforms of GLO1 in more detail by 2-DE on a narrow pH gradient in combination with Western blotting and studied their role in TNF-induced cell death. Multiple isoforms of GLO1 exist, and these isoforms are present in all cell lines tested so far. We determined that the TNF-induced

Figure 6 TNF-induced phosphorylation of GLO1 is not exclusive to the NO-responsive form

(A) L929 cells were pre-incubated with the iNOS inhibitor 1400W (20 μ M) for 2 h. TNF treatment was performed for 1.5 h (1000 units/ml). Western blots of the 2-DE gels (pH 4–7) were developed with an anti-GLO1 polyclonal antibody. 1400W, cells pre-incubated with the iNOS inhibitor; TNF + 1400W, TNF treated cells in the presence of 1400W. Note the TNF-induced phosphorylation of GLO1 on the NO-responsive form (arrowheads to the right of α) as well as on the non-NO-responsive form of GLO1 (arrowheads to the left of α). Ac, acidic; Bs, basic. (B) Effect of 1400W on TNF-induced cell death. L929 cells were pre-incubated for 2 h with different concentrations of 1400W and then treated with TNF (100 units/ml) for 5 h. Cell death was measured by quantifying PI-positive cells by FACS as described in the Materials and methods section. Results are means $\pm$ S.D. for triplicate cultures. * P < 0.05.

phosphorylation of GLO1, which is mediated by PKA, occurs primarily on the NO-responsive form of GLO1. In the presence of the NO donor GSNO, the TNF-induced phosphorylation of the NO-responsive form of GLO1 was promoted and GSNO was also strongly synergistic with TNF-induced cell death. By the use of pharmacological inhibition of iNOS and overexpression of mutants of GLO1 that are deficient for the NO-mediated modification, we could show that the NO-mediated modification of GLO1 is not a requirement for TNF-induced phosphorylation or TNF-induced cell death respectively. In addition, in the presence of forskolin (an activator of adenylate cyclase that catalyses the formation of cAMP, which in turn activates PKA), TNF also induced phosphorylation of the non-NO-responsive form of GLO1. Furthermore, some of our data indicate that there is an intimate link between the NO-mediated modification of GLO1 and the phosphorylation of GLO1.

By overexpression of GLO1 mutants, we could show that the NO-mediated modification of GLO1 involves cysteine residues. Human GLO1 contains four cysteine residues of which Cys-138 is conserved from bacteria to humans [10]. Cys-18 and Cys-19 are present only in human and mouse GLO1, but not in plant, yeast or bacterial GLO1 [20]. Our results indicate that, Cys-138 in combination with either Cys-18 or Cys-19, are crucial target residues for the NO-mediated modification of GLO1. In contrast, Cys-60, which is present only in human GLO1, is not involved in the NO-mediated modification of GLO1. This residue is located in the hydrophobic cavity of the active site and is involved in substrate binding [16,17]. It is therefore unlikely that the reversible inactivation of the MG-detoxification capacity of GLO1 in the presence of GSNO (although at high concentrations, 1 mM) is due to modification of this residue, as has been suggested previously [16].

The critical cysteine residues that are involved in the NO-mediated modification of GLO1 are all located on the outside of the molecule in the three-dimensional structure [20]. Furthermore, treatment of L929 cells with increasing concentrations of GSNO (10–250 μ M) results in the increased presence of a faster migrating form of GLO1 on non-denaturing gels (V. de Hemptinne, D. Rondas and K. Vancompernelle, unpublished work), suggesting a conformational change. Therefore the impact of the NO-mediated modification on the enzymatic activity of GLO1 is more likely to be due to structural changes that are induced by this modification. The exact nature of the NO-mediated modification remains to be determined. The increase in net charge of the NO-responsive form of GLO1 indicates the formation of positive charges on the protein. The formation of an S-nitrosothiol or disulfide bond formation with glutathione in GLO1 would cause no alteration or a decrease in net charge of the protein respectively. Therefore these types of modification in GLO1 are unlikely. It may also be plausible that the covalent modification of the NO-responsive form of GLO1 is not directly derived from an NO species, but may also be another type of covalent modification that is induced indirectly by NO.

The biological function of phosphorylated GLO1 is not yet known. We have shown previously that phosphorylated GLO1 is no longer involved in the MG-detoxification pathway, but instead is involved, either directly or indirectly, in a pathway that leads to MG-modification of proteins [4]. Because the GLO1 activity is not inhibited in TNF-treated cells [4], it is unlikely that the NO-mediated modification of GLO1 may contribute to TNF-induced cell death by simply inhibiting the activity of GLO1. Rather, the NO-mediated modification may facilitate or promote the phosphorylation of GLO1 by, for example, inducing a change in the three-dimensional structure.

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REFERENCES

- Kalapos, M. P. (1999) On the promine/retine theory of cell division: now and then. *Biochim. Biophys. Acta* **1426**, 1–16
- Thornalley, P. J. (1996) Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification – a role in pathogenesis and antiproliferative chemotherapy. *Gen. Pharmacol.* **27**, 565–573
- Dukic-Stefanovic, S., Schinzel, R., Riederer, P. and Munch, G. (2001) AGEs in brain ageing: AGE-inhibitors as neuroprotective and anti-dementia drugs? *Biogerontology* **2**, 19–34
- Van Herreweghe, F., Mao, J., Chaplen, F. W., Grooten, J., Gevaert, K., Vandekerckhove, J. and Vancompernelle, K. (2002) Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methylglyoxal-derived AGE. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 949–954
- Thornalley, P. J. (1990) The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem. J.* **269**, 1–11
- Chen, F., Wollmer, M. A., Hoerndli, F., Munch, G., Kuhla, B., Rogae, E. I., Tsolaki, M., Papassotiropoulos, A. and Gotz, J. (2004) Role for glyoxalase I in Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 7687–7692
- Hovatta, I., Tennant, R. S., Helton, R., Marr, R. A., Singer, O., Redwine, J. M., Ellison, J. A., Schadt, E. E., Verma, I. M., Lockhart, D. J. et al. (2005) Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. *Nature* **438**, 662–666
- Rulli, A., Carli, L., Romani, R., Baroni, T., Giovannini, E., Rosi, G. and Talesa, V. (2001) Expression of glyoxalase I and II in normal and breast cancer tissues. *Breast Cancer Res. Treat.* **66**, 67–72
- Davidson, S. D., Cherry, J. P., Choudhury, M. S., Tazaki, H., Mallouh, C. and Konno, S. (1999) Glyoxalase I activity in human prostate cancer: a potential marker and importance in chemotherapy. *J. Urol.* **161**, 690–691
- Ranganathan, S., Walsh, E. S., Godwin, A. K. and Tew, K. D. (1993) Cloning and characterization of human colon glyoxalase-I. *J. Biol. Chem.* **268**, 5661–5667
- Sakamoto, H., Mashima, T., Kizaki, A., Dan, S., Hashimoto, Y., Naito, M. and Tsuruo, T. (2000) Glyoxalase I is involved in resistance of human leukemia cells to antitumor agent-induced apoptosis. *Blood* **95**, 3214–3218
- Thornalley, P. J., Edwards, L. G., Kang, Y., Wyatt, C., Davies, N., Landan, M. J. and Double, J. (1996) Antitumor activity of S-p-bromobenzylglutathione cyclopentyl diester *in vitro* and *in vivo*: inhibition of glyoxalase I and induction of apoptosis. *Biochem. Pharmacol.* **51**, 1365–1372
- Kavarana, M. J., Kovaleva, E. G., Creighton, D. J., Wollman, M. B. and Eiseman, J. L. (1999) Mechanism-based competitive inhibitors of glyoxalase I: intracellular delivery, *in vitro* antitumor activities, and stabilities in human serum and mouse serum. *J. Med. Chem.* **42**, 221–228
- Vancompernelle, K., Boonfaes, T., Mann, M., Fiers, W. and Grooten, J. (2000) Tumor necrosis factor-induced microtubule stabilization mediated by hyperphosphorylated oncoprotein 18 promotes cell death. *J. Biol. Chem.* **275**, 33876–33882
- Grooten, J., Goossens, V., Vanhaesebroeck, B. and Fiers, W. (1993) Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. *Cytokine* **5**, 546–555
- Mitsumoto, A., Kim, K. R., Oshima, G., Kunimoto, M., Okawa, K., Iwamatsu, A. and Nakagawa, Y. (2000) Nitric oxide inactivates glyoxalase I in cooperation with glutathione. *J. Biochem. (Tokyo)* **128**, 647–654
- Mitsumoto, A., Kim, K. R., Oshima, G., Kunimoto, M., Okawa, K., Iwamatsu, A. and Nakagawa, Y. (1999) Glyoxalase I is a novel nitric-oxide-responsive protein. *Biochem. J.* **344**, 837–844
- Oya, T., Hattori, N., Mizuno, Y., Miyata, S., Maeda, S., Osawa, T. and Uchida, K. (1999) Methylglyoxal modification of protein: chemical and immunochemical characterization of methylglyoxal-arginine adducts. *J. Biol. Chem.* **274**, 18492–18502
- Gow, A. J., Farkouh, C. R., Munson, D. A., Posencheg, M. A. and Ischiropoulos, H. (2004) Biological significance of nitric oxide-mediated protein modifications. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L262–L268
- Cameron, A. D., Olin, B., Ridderstrom, M., Mannervik, B. and Jones, T. A. (1997) Crystal structure of human glyoxalase I: evidence for gene duplication and 3D domain swapping. *EMBO J.* **16**, 3386–3395
- Marshall, H. E., Hess, D. T. and Stamler, J. S. (2004) S-nitrosylation: physiological regulation of NF- κ B. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 8841–8842
- Marshall, H. E. and Stamler, J. S. (2002) Nitrosative stress-induced apoptosis through inhibition of NF- κ B. *J. Biol. Chem.* **277**, 34223–34228
- Shishodia, S. and Aggarwal, B. B. (2002) Nuclear factor- κ B activation: a question of life or death. *J. Biochem. Mol. Biol.* **35**, 28–40
- Cauwels, A., Van Molle, W., Janssen, B., Everaerd, B., Huang, P., Fiers, W. and Brouckaert, P. (2000) Protection against TNF-induced lethal shock by soluble guanylate cyclase inhibition requires functional inducible nitric oxide synthase. *Immunity* **13**, 223–231
- Garvey, E. P., Oplinger, J. A., Furfine, E. S., Kiff, R. J., Laszlo, F., Whittle, B. and Knowles, R. G. (1997) 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.* **272**, 4959–4963

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