Evidence for glutathione involvement in platelet-derived growth-factor-mediated signal transduction

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Recent studies show that glutathione, while being involved in the well-known physiological processes of amino acid transport and detoxification, can also play a part in cell proliferation events. Cell treatment with L-buthionine sulfoximine, which causes glutathione depletion, is accompanied by a decrease in cell proliferation. At present no precise relationship between this thiol and any critical intermediate of the mitogenic cascade has been proved. In this study, conducted on NIH/3T3 murine

fibroblasts, we demonstrate a strict correlation between glutathione levels and platelet-derived growth-factor-receptor activation in response to stimulation and cell proliferation. The receptor autophosphorylation is severely impaired at low glutathione cellular levels. The interaction of glutathione with this growth-factor receptor *in vivo*, while being rather specific, is complex and may involve both cytosolic and extracellular receptor domains.

INTRODUCTION

Glutathione is the most abundant low-molecular-mass thiol found in mammalian cells. It plays a crucial role in many physiological functions, including amino acid transport and detoxification of electrophiles and oxidants [1]. The inter-organ metabolism and transport of GSH are involved in the maintenance of the intracellular thiol–disulphide redox status [2], which may influence the activity of many enzymic proteins [3] and transmembrane transport proteins [4]. Recently, it has been found that the reversible formation of GSH-mixed disulphides with proteins, a thiol–disulphide exchange reaction catalysed by thioltransferase activity, seems to be involved in the homeostatic maintenance of cellular thiols and in the regulation of some enzymic activity [5,6].

Previous studies have shown that GSH cellular levels can regulate DNA synthesis [7]. GSH, L-cysteine, 2-mercaptoethanol and other thiols may support the proliferation of rabbit lymphocytes stimulated by mitogens [8,9] and the growth of murine neoplastic cells [10], while inhibitors of GSH synthesis may abolish the proliferation of human lymphocytes [11]. Moreover, several cell lines show a correlation between their growth rate and GSH levels [12,13]; and cell treatment with L-buthionine sulfoximine (BSO), a specific inhibitor of γ -glutamylcysteine synthetase used to inhibit GSH synthesis [14], results in an antiproliferative effect that can be partially overcome by exogenous GSH [15,16].

In some cases cellular transformation is accompanied by an increase in GSH content and this may correlate with the resistance of tumour cells to radiation and therapeutic drugs [17,18]; pharmacological modifications of GSH metabolism have therefore been proposed as useful approaches to anti-cancer therapy [19,20].

The possible early involvement of GSH in the mitogenic signal cascade is supported by some pieces of evidence: findings obtained *in vitro* from isolated growth-factor receptors show that GSH can alter the kinase activity of both insulin and epidermal growth factor (EGF) receptors [21]. Kanner et al. propose that a thiol-group oxidation may inhibit receptor-mediated tyrosine

phosphorylation and activation of phospholipase C- γ in T-cells [22], whereas Terradez et al. show a reduction in protein kinase C activity and in cellular pH in Ehrlich ascite tumour cells that have been glutathione depleted [23].

Shaw and Chou show a peak in GSH content in NIH/3T3 murine fibroblasts during the early log phase of their proliferation [12], thus suggesting they are a model in which the relationship between glutathione levels and cell growth could be investigated.

Considering these results and the fact that intracellular GSH content can be altered under many physiological and pathological conditions [2,24–27], we studied the proliferation and the activation of platelet-derived growth-factor receptor (PDGF-r) in NIH/3T3 fibroblasts at different intracellular GSH contents. The purpose of this study was to identify a possible role of GSH in the modulation of the activity of PDGF-r and to clarify the relationship between GSH and the signals involved in cellular proliferation.

EXPERIMENTAL

Cell culture

NIH/3T3 murine fibroblasts were obtained from the Istituto Nazionale Tumori (Milan, Italy). They were routinely cultured at low density in a humidified atmosphere of 8% CO₂ at 37 °C in Dulbecco's modified Eagle's medium without sodium pyruvate, supplemented with 10% (v/v) fetal-calf serum (FCS). A431 human epidermoid carcinoma cells, overexpressing the epidermal growth-factor receptor (EGF-r), were grown in Dulbecco's modified Eagle's medium containing 1 g/litre glucose, supplemented with 10% FCS. During serum starvation, cells were cultured in medium containing 0.5% FCS.

Evaluation of mitogenic response

Cells were serum starved for 24 h, then cultured in fresh medium supplemented with 10% FCS or 20 ng/ml platelet-derived growth factor (PDGF)-BB (Peprotech) for an additional 24 h. To modify the cellular GSH content during stimulation, we alternatively added to the medium either 15–50 μ M BSO or

Abbreviations used: BSO, L-buthionine sulfoximine; EGF, epidermal growth factor; EGF-r, epidermal growth-factor receptor; PDGF, platelet-derived growth factor; PDGF-r, platelet-derived growth-factor receptor; FCS, fetal-calf serum; γ -GT, γ -glutamyltransferase; DTT, dithiothreitol.

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1 mM GSH, or 50 μ M BSO plus 1 mM GSH. The fibroblasts' ability to proliferate was evaluated by pulse-labelling them with 1 μ Ci/ml of [3 H]thymidine during the last 4 h of starvation (basal proliferation level) or during the last 4 h of stimulation periods, as previously described [28]. Each experimental point was assessed in quadruplicate. Additional dishes, treated in exactly the same way (except for thymidine labelling), were prepared and destined for cellular GSH evaluation.

Determination of PDGF-r activation

Subconfluent 35 mm dishes of NIH/3T3 fibroblasts were starved for 24 h in medium containing 0.5% FCS. BSO or GSH was added to the starvation medium when we varied the GSH content before mitogen stimulation.

Fibroblasts were then stimulated for 5 min at 37 °C by adding 20 ng/ml PDGF-BB to the medium. In some experiments fresh medium alternatively containing 1 mM GSH or 1 mM cysteine or 0.1 mM dithiothreitol (DTT) was added to the cells 5 min before growth-factor stimulation.

At the end of treatment, dishes were placed on ice and cells were washed twice with ice-cold PBS at pH 7.4. The lysis buffer [66 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol and Bromphenol Blue] was directly added to the dishes. The cell lysates were boiled for 10 min and centrifuged at 12400 *g* for 10 min, then the total protein content was determined by the bicinchoninic-acid-solution (Pierce) protein reagent assay. Equal protein amounts from whole-cell extracts were subjected to SDS/PAGE on an 8% (w/v) gel and electrotransferred to a nitrocellulose membrane (Sartorius). Tyrosine-phosphorylated proteins were visualized by incubating the membrane with RC20 anti-phosphotyrosine antibody conjugated to horseradish peroxidase (Transduction Laboratories), using the enhanced chemiluminescence (Amersham) detection method, as previously described [29]. A densitometric analysis of bands was performed.

The correspondence of the 180 kDa band to the PDGF-r was confirmed by a specific recognition with polyclonal anti-(PDGF-r) antibodies (Upstate Biotechnology Incorporated).

The amount of PDGF-r in the experiments was assessed by using the same antibody in the immunodetection of electrophoresed and blotted cell-membrane fractions. The membrane fractions were purified by lysing cells in hypotonic buffer [10 mM Hepes (pH 7.5)/10 mM NaCl/1 mM KH_2PO_4 /5 mM NaHCO_3 /1 mM CaCl_2 /0.5 mM MgCl_2 /5 mM EDTA/leupeptin (10 μ g/ml)/aprotinin (20 μ g/ml)/1 mM PMSF]. Lysates were then sonicated, clarified at 1000 *g* for 5 min and centrifuged at 100000 *g* for 30 min at 4 °C. The pellet was dissolved in lysis buffer supplemented with 1% (v/v) Triton X-100 and again centrifuged at 100000 *g* for 30 min. The supernatant was collected as membrane fraction.

The cellular GSH level was evaluated under every different set of experimental conditions.

Determination of cellular glutathione level

Cells [(3–8) $\times 10^6$] were washed twice with PBS and, after trypsin treatment, centrifuged at 125 *g* for 10 min. The precipitate was washed again and then dissolved in 100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. This suspension was used for both protein and GSH determinations. Protein content was measured by Bradford's method [30] after cellular lysis by 0.5 M NaOH. Total glutathione, reduced (GSH) and oxidized (GSSG), was measured by the method of Baker et al. [31]. Briefly, cellular suspension was supplemented with sulphosalicylic acid to a final concentration of 0.5% (w/v), when it was sonicated and centri-

fuged at 10600 *g* for 15 min. The supernatant was used for GSH determination by cyclic reduction of GSSG by glutathione reductase and NADPH followed by reduction of the colorimetric reagent 5,5'-dithiobis-(2-nitrobenzoic acid) by GSH. Absorbance was recorded at 405 nm. All samples were assayed in duplicate and results are expressed as nmol of GSH/mg of protein. GSH and GSSG were also measured by HPLC, as described by Fariss and Reid [32], to confirm the results obtained. Moreover, by this method it is also possible to measure the intracellular content of glutamic acid (amino acid constituent of GSH) and to verify the degradation of extracellular GSH, which is operated by γ -glutamyltransferase (γ -GT) activity, the only known membrane enzyme able to break down the γ -glutamyl linkage of GSH.

Enzyme assay

Cells (20 $\times 10^6$) were washed twice with PBS, collected by scraper and centrifuged at 125 *g* for 10 min. The precipitate was resuspended using 2 mM Tris buffer containing 50 mM mannitol, pH 7. Cells were lysed by sonication at 4 °C and the homogenate was centrifuged at 125 *g* for 10 min in a microcentrifuge; the supernatant was again centrifuged at 29000 *g* for 20 min. The precipitate was collected, washed and centrifuged, and then the pellet, containing partially purified plasma membranes, was dissolved in the same buffer and used for γ -GT activity determination with a commercially available assay kit (Boehringer Mannheim), using γ -glutamyl-*p*-nitroanilide and glycylglycine as substrates. After 60 min at 37 °C, the reaction mixture was centrifuged at 10600 *g* for 5 min and *p*-nitroaniline formation in the supernatant was determined at 405 nm [33].

γ -GT activity was also measured using whole cells by the method described above, and also reported by others [13].

Thioltransferase activity was measured in cell homogenate by the method of Axelsson et al. [34].

Statistical analyses

Statistical analyses were evaluated by computer program using Student's *t*-test on a Philips PR07BM743. *P* < 0.05 was considered to be significant.

RESULTS

Effect of alterations to GSH intracellular levels on the proliferation of NIH/3T3 cells

NIH/3T3 cells were serum starved for 24 h and then stimulated with either FCS or PDGF-BB added to the basal medium (controls) in the presence of 15 or 50 μ M BSO, or 1 mM GSH, or 50 μ M BSO plus 1 mM GSH, for the subsequent 24 h.

The use of BSO allowed us to specifically decrease the GSH cellular content by the inhibition of γ -glutamylcysteine synthetase, the regulatory enzyme of GSH synthesis. In the concentration range of BSO used we did not observe any toxicity for cells, as is also reported in the literature [19,35]. Both GSH intracellular levels and cell proliferation were then evaluated.

Table 1 shows a significant decrease in GSH levels in 15 μ M BSO-treated cells after stimulation with FCS (–60%) and PDGF (–80%) compared with control cells. A higher decrease in GSH content was measured in 50 μ M BSO-treated cells: –90% and –99% after FCS and PDGF stimulation respectively. These results indicate a remarkable decrease in GSH content, mainly in 50 μ M BSO-treated cells, during PDGF stimulation.

A significant increase in GSH content was obtained, with respect to 50 μ M BSO-treated cells, when 1 mM GSH plus

Table 1 Levels of GSH in serum and PDGF-stimulated NIH/3T3 cells after BSO and GSH treatments

After 24 h serum starvation, NIH/3T3 cells were serum- or PDGF-stimulated in the absence (control) and presence respectively of BSO, BSO plus GSH and GSH for the following 24 h. GSH was measured by Baker's method [31] and is expressed as nmol/mg of protein. Data are means $\pm$ S.E.M. of three experiments, each performed on duplicate samples. GSH content in starved cells: 22 ± 3 nmol/mg of protein. Data are significantly different: from respective control cells, * $P < 0.005$, ** $P < 0.05$; from 50 μ M BSO-treated cells, † $P < 0.05$; from starved cells, ‡ $P < 0.05$.

	GSH content (nmol/mg of protein)				
	Control cells	15 μ M BSO	50 μ M BSO	50 μ M BSO + 1 mM GSH	1 mM GSH
FCS stimulation	18.0 ± 2.0	$7.0 \pm 0.6^{**}$	$1.5 \pm 0.4^*$	$10.0 \pm 1.6^\dagger$	$32.0 \pm 5.0^{**}$
PDGF stimulation	$32.0 \pm 3.0^\ddagger$	$6.0 \pm 1.0^{**}$	$0.44 \pm 0.05^*$	$6.0 \pm 1.0^\dagger$	27.0 ± 2.0

Table 2 [3 H]Thymidine incorporation into NIH/3T3 cells after 24 h serum or PDGF stimulation in the presence or absence of BSO, BSO plus GSH or GSH

Cells were serum starved for 24 h, and then in these cells the basal levels of [3 H]thymidine incorporation were measured by pulse (4 h) labelling. Starved cells were then stimulated with 10% FCS or 20 ng/ml PDGF-BB for 24 h in the presence or absence (control) of BSO, BSO plus GSH or GSH, and [3 H]thymidine incorporation was again measured. Values of incorporation are reported as the increase above the basal level. Data are means $\pm$ S.E.M., $n = 4$. Data are significantly different: from control, * $P < 0.005$, ** $P < 0.05$; from 50 μ M BSO-treated cells, † $P < 0.005$.

	[3 H]Thymidine incorporation (increase above the basal level)				
	Control cells	15 μ M BSO	50 μ M BSO	50 μ M BSO + 1 mM GSH	1 mM GSH
FCS stimulation	22.0 ± 2.0	$17.0 \pm 1.0^{**}$	$10.0 \pm 1.0^*$	$21.4 \pm 2.0^\dagger$	18.0 ± 2.0
PDGF stimulation	25.0 ± 2.0	$15.0 \pm 0.7^*$	$5.0 \pm 0.5^*$	$14.0 \pm 1.7^\dagger$	20.5 ± 1.0

50 μ M BSO were added together to the basal medium during stimulation. It is important to note that GSH increase in treated cells was higher in PDGF- (12-fold) than in FCS-stimulated (6-fold) cells, but final levels were higher in FCS- than in PDGF-stimulated cells. Moreover, under both sets of conditions GSH values did not again reach those observed in control stimulated cells.

Finally, when 1 mM GSH was added to the basal medium, a 77% increase in GSH was measured in FCS-stimulated cells compared with the control. This GSH increase seems to be related to its low level in control FCS-stimulated cells; in fact, the same GSH addition did not affect its content in PDGF-stimulated cells. These GSH changes were not due to differences in protein concentration since it remained similar under all the experimental conditions.

Cellular proliferation was evaluated as [3 H]thymidine incorporation. Table 2 shows the increase in thymidine incorporation in stimulated cells with respect to the basal level measured after 24 h of serum starvation. A significant decrease in thymidine incorporation was measured in both 15 μ M and 50 μ M BSO-treated cells compared with untreated control cells. However, this effect was more striking in 50 μ M BSO-treated cells during PDGF stimulation (4-fold decrease) than during FCS stimulation (1-fold decrease). This effect was completely reversed when 1 mM GSH was added to the basal medium, together with 50 μ M BSO, in FCS stimulation experiments; during PDGF stimulation [3 H]thymidine incorporation also increased (2-fold with respect to 50 μ M BSO-treated cells), but it did not reach the

Figure 1 Tyrosine phosphorylation pattern after PDGF stimulation of cells previously treated with increasing BSO concentrations

Cells were serum starved for 24 h in the presence of increasing BSO concentrations, and then stimulated for 5 min with 20 ng/ml PDGF-BB (lanes 2, 4, 6, 8). Whole-cell lysates were analysed by SDS/PAGE followed by immunoblot with anti-phosphotyrosine antibodies conjugated to peroxidase and by enhanced chemiluminescence detection. Control cells were not treated with BSO. BSO concentrations were: lanes 1 and 2, 0; lanes 3 and 4, 15 μ M; lanes 5 and 6, 50 μ M; lanes 7 and 8, 250 μ M. Arrowhead: PDGF-r. Densitometric analysis of PDGF-r band (percentage variations with respect to lane 2): lane 4, -15%; lane 6, -38.8%; lane 8, -43.1%.

level of BSO-untreated cells (control). It is important to note that the picture of changes in thymidine incorporation during treatments is similar to that of changes in GSH content. Moreover, Table 2 shows that the addition of 1 mM GSH in the absence of growth factors did not significantly affect thymidine incorporation.

These results indicate that the antiproliferative effect of BSO can be ascribed to the related reduction in intracellular GSH level. The inhibitory effect of GSH depletion upon cell proliferation seemed to be particularly striking under PDGF-BB stimulation; this fact led us to investigate a possible alteration in the pattern of tyrosine phosphorylation that derives from the growth-factor-receptor activation.

Relation between PDGF-BB receptor phosphorylation and intracellular GSH level

Cells were serum starved in the presence or absence (control) of increasing BSO concentrations, and then stimulated with PDGF-BB for 5 min. The subsequent analysis of the cell lysates, through SDS/PAGE and immunoblotting with anti-phosphotyrosine antibodies, showed a specific reduction in the tyrosine phosphorylation of a band of approx. 180 kDa which, by a specific immunodetection, was confirmed to be the PDGF-r; this decrease became more evident as BSO rose, and it reached a plateau between 50 μ M and 250 μ M BSO (Figure 1). This lowering of receptor phosphorylation correlated with the measured cellular GSH levels, which attained a minimum and remained constant in the same BSO concentration range (50–250 μ M) (Table 3). The evaluation of PDGF-r content in cell-membrane fractions confirmed that no variations were caused by glutathione depletion during BSO treatment (results not shown).

When NIH/3T3 fibroblasts were serum starved in the presence of 50 μ M BSO and 1 mM GSH, receptor phosphorylation was completely recovered (Figure 2). Indeed in BSO-plus-GSH-treated cells an approx. 3.3-fold increase in GSH content with respect to BSO-treated cells was measured (Table 3). Only a slight increase in PDGF-r phosphorylation was observed following the addition of 1 mM GSH during starvation of cells in the absence of BSO, even if under these conditions GSH cellular content doubled with respect to untreated cells (Table 3). This

Table 3 Levels of GSH in starved NIH/3T3 cells after BSO and GSH treatment

Cells were serum starved for 24 h in the absence (control) and presence of BSO, BSO plus GSH and GSH. GSH was measured by Baker's method [31] and is expressed as nmol/mg of protein. Data are means $\pm$ S.E.M. of five experiments, each performed on duplicate samples. Data are significantly different from control: * $P < 0.005$; from 50 μ M BSO-treated cells, † $P < 0.001$.

GSH content (nmol/mg of protein)					
Starved cells (control)	15 μ M BSO	50 μ M BSO	250 μ M BSO	50 μ M BSO + 1 mM GSH	1 mM GSH
22 $\pm$ 3.0	6.5 $\pm$ 1.1*	1.6 $\pm$ 0.2*	1.4 $\pm$ 0.5	7.0 $\pm$ 0.9†	44.0 $\pm$ 5.0*

Figure 2 Tyrosine phosphorylation pattern after PDGF stimulation of cells previously treated with BSO and/or GSH

Cells were serum starved for 24 h in the presence of the following treatments: lanes 1 and 2, no treatment; lanes 3 and 4, 50 μ M BSO; lanes 5 and 6, 1 mM GSH; lanes 7 and 8, 50 μ M BSO and 1 mM GSH. Cells were then stimulated with 20 ng/ml PDGF-BB for 5 min (lanes 2, 4, 6, 8). Whole-cell lysates were run on a 8% polyacrylamide gel, then subjected to immunoblot with anti-phosphotyrosine antibodies as described in the text. Arrowhead: PDGF-r. Densitometric analysis of PDGF-r band (percentage variations with respect to lane 2): lane 4, -27.9%; lane 6, +8%; lane 8, -1.3%.

result correlates with the lack of enhancement of the mitotic response (Table 2). So we can exclude a direct effect of BSO itself on PDGF-r phosphorylation and we can conclude that a basal level of GSH is necessary for maximum activation of PDGF-r. It is worth noting that glutathione itself has no stimulatory effect on PDGF-r, since no receptor phosphorylation is evident in the absence of the specific growth factor and FCS.

If we compare the results obtained in these experiments with those of thymidine incorporation, it can be seen that the level of growth-factor-receptor activation correlates with the extent of cell proliferation.

Effect of extracellular GSH on PDGF-r phosphorylation

One fact must still be clarified: is a certain level of GSH necessary inside or outside the cell for PDGF-r activation? When we incubated the fibroblasts with 1 mM GSH or 1 mM GSH plus 50 μ M BSO for 24 h, we increased both its cellular and its extracellular concentration. Therefore we devised an experiment in which cells would be exposed to a high concentration of GSH in their medium for a period short enough not to significantly increase its cellular concentration.

NIH/3T3 fibroblasts were serum starved for 24 h either in the presence or absence of 50 μ M BSO, or 50 μ M BSO plus 1 mM GSH, and then treated with or without (control) 1 mM GSH for 10 min. During the last 5 min, cells were stimulated with PDGF

Figure 3 Effect of a brief exposure to GSH on PDGF-r phosphorylation

During a 24 h serum starvation, cells were treated as follows: lanes 1–3, no treatment; lanes 4 and 5, 50 μ M BSO; lanes 6 and 7, 50 μ M BSO and 1 mM GSH. GSH (1 mM) was then given to cells for 10 min (lanes 3, 5, 7); all cells were additionally stimulated with 20 ng/ml PDGF-BB for 5 min. GSH-treated cells were supplemented with PDGF during the last 5 min of GSH treatment. Whole cell lysates were obtained and subjected to SDS/PAGE and immunoblot with anti-phosphotyrosine antibodies. Lane 1, cells not stimulated with PDGF. Arrowhead: PDGF-r. Densitometric analysis of PDGF-r band (percentage variations with respect to lane 2): lane 3, +39.6%; lane 4, -39.1%; lane 5, -66.4%; lane 6, +2.8%; lane 7, +36.8%.

(20 ng/ml) and then their tyrosine phosphorylation pattern was analysed through SDS/PAGE and immunoblotting (Figure 3). An increase in PDGF-r phosphorylation with respect to control was observed upon 10 min GSH treatment in all cells except those treated only with 50 μ M BSO during the serum starvation that preceded growth-factor stimulation. When fibroblasts were starved in a medium containing only 50 μ M BSO, they reached a very low cellular GSH concentration (Table 3) and again showed reduced PDGF-r phosphorylation: in this case no recovery in receptor activation was evident after 10 min treatment with exogenous GSH. In no case was any significant increase in cellular glutathione concentration observed after a 10-min GSH treatment. GSH levels under these experimental conditions were similar to those reported in Table 3.

These results indicate that PDGF-dependent receptor phosphorylation can be enhanced by increasing the GSH concentration outside the cell, but only if a critical internal GSH level is preserved. It is also evident that, at least as far as the extracellular effect of GSH is concerned, the observed variation in tyrosine phosphorylation in response to PDGF cannot be ascribed to a different PDGF-r turnover in GSH-enriched and in GSH-depleted cells respectively, as the effect of the treatment becomes evident in a very short time.

A brief treatment with two different reducing agents (1 mM cysteine or 0.1 mM DTT) before PDGF stimulation caused a similar increase in growth-factor-receptor phosphorylation (Figure 4), thus indicating that at least the effect of extracellular GSH could be linked to its reducing activity. Moreover, to verify if the effects obtained in all the reported experiments were partly due to the degradation products of GSH, we measured the activity of γ -GT, but it was not detectable whether we used entire cells or partially purified membranes. Moreover, the HPLC analysis of glutamic acid showed no increase in this amino acid when GSH was added to the culture medium, indicating no GSH degradation.

Specificity of GSH effect on PDGF-r phosphorylation

Other authors have demonstrated that the phosphorylation of different growth-factor receptors (insulin receptor, EGF-r) is

Figure 4 Effect of a brief exposure to various thiols on PDGF-r phosphorylation

Cells were serum starved for 24 h and then subjected to the following treatments for 10 min: lanes 1 and 5, no treatment; lane 2, 1 mM GSH; lane 3, 1 mM cysteine; lane 4, 0.1 mM DTT. During the last 5 min of the preceding treatment, 20 ng/ml PDGF-BB was added to the culture medium. Whole cell lysates were analysed by SDS/PAGE and immunoblotting with anti-phosphotyrosine antibodies. Lane 1, cells not stimulated with PDGF. Arrowhead: PDGF-r. Densitometric analysis of PDGF-r band (percentage variations with respect to lane 5): lane 2, +16.8%; lane 3, +18.8%; lane 4, +11.3%.

influenced *in vitro* by GSH [21]. To test the specificity of GSH effects on growth-factor receptors *in vivo* we performed experiments with A431 cells (overexpressing EGF-r). As was previously done for NIH/3T3 fibroblasts, these cells were serum starved and treated with BSO concentrations of up to 150 μ M before stimulation with EGF, but no evident effect of GSH decrease on EGF-r phosphorylation was observed. (GSH levels in serum-starved A431 cells: 120 ± 25 nmol/mg of protein; in 150 μ M BSO-treated cells, 10 ± 1 nmol/mg of protein.)

DISCUSSION

This work demonstrates for the first time that *in vivo* the activation of PDGF-r in NIH/3T3 cells is modulated by changes in the basal level of GSH. As regards cellular proliferation, we observed a direct correlation between reduction in GSH content and thymidine incorporation decrease.

Data reported in the literature in different cells suggest a role for GSH in cell proliferation [12,13,36,37]; however, a direct cause-effect relationship between changes in cellular GSH levels and regulation of cell growth has not been demonstrated. A correlation between GSH cellular levels and mitogenic stimulation of quiescent NIH/3T3 fibroblasts has been found [12]. Kang and Enger [36] also showed in A549 human lung carcinoma cells that BSO treatment decreases cellular GSH and inhibits cell growth, but did not demonstrate a direct relation between these two observations. Subsequently these authors measured increases in GSH levels after serum stimulation of quiescent normal rat kidney fibroblasts (NRK49F) [37] and came to the conclusion that GSH increase is associated with cysteine and glutamic acid repletion and not with growth factor stimulation.

We do not share the same opinion, considering that after 24 h of serum starvation, GSH levels in NIH/3T3 cells increased after PDGF stimulation (Table 1). Considering that no increase in GSH level is obtained by serum stimulation and new medium addition, a specific induction of GSH synthesis by high levels of PDGF could be hypothesized.

Moreover Kang and Enger [36] found that thymidine incorporation decrease depends on BSO treatment itself and not on GSH depletion. These results are different from ours; we found that on adding 1 mM GSH to BSO-treated cells (Table 2) the antiproliferative effect of BSO could be overcome. This

excludes a direct effect of BSO and confirms the strict relationship with GSH content.

Recently Kang et al. [13,16] explained the antiproliferative effect of BSO on A549 cells through its inhibition of the uptake of glutamine or cysteine, essential factors for cellular growth, and not through the decrease in GSH content itself, even though other authors report that a depletion of GSH in endothelial cells and fibroblasts stimulates glutamate and cysteine uptake through their specific transport systems [38]. In Kang's opinion the attenuation exerted by exogenous GSH on the BSO antiproliferative effect was not due to the re-establishment of a critical cellular GSH level, but to the recovery of BSO-inhibited cysteine or glutamine accumulation through the activity of γ -GT. We could exclude similar events in NIH/3T3 cells, since in these fibroblasts γ -GT activity is undetectable. Moreover, we did not measure any increase in glutamic acid by HPLC analysis when 1 mM GSH was added to starved or stimulated cells. Rather, we found an increase in cellular GSH, even when its synthesis was inhibited by BSO. So we conclude that NIH/3T3 cells can take up exogenous GSH, probably by a transmembrane transporter and not by extracellular GSH degradation caused by γ -GT activity, subsequent uptake of degradation products and intracellular resynthesis. GSH seems to enter the cell by a mediated transport system, which might be regulated, considering that in the presence of high GSH cellular levels no increase is measured when GSH is added to the culture medium (1 mM GSH-treated cells after PDGF stimulation, Table 1). Many specific transport systems for GSH in different cells have been described [39] and recently Yi et al. [40] demonstrated that cDNA of the rat canalicular transporter hybridizes with single transcripts isolated from rat brain, kidney, lung and small intestine.

From our data it is evident that a critical level of intracellular GSH (50 μ M BSO plus 1 mM GSH treatment, corresponding to a GSH concentration of 10 nmol/mg of protein, Table 2) is necessary for a maximal mitogenic stimulation and that PDGF-stimulated cells are more sensitive to BSO treatment, suggesting that FCS may provide cells with some other unidentified elements that render them capable of maintaining more elevated GSH levels and proliferative ability.

Strong evidence then indicates that GSH can interact with or modulate the signal cascade that proceeds from the cited growth-factor receptor. One way to investigate the site of interaction is to examine the activation profile that cells display after stimulation, in terms of protein tyrosine phosphorylation. The analysis of the tyrosine phosphorylation pattern in response to PDGF stimulation shows that GSH depletion is associated with a specific reduction in PDGF-r autophosphorylation. No effect of GSH depletion was evident on the amount of surface PDGF-r.

As already observed in cellular proliferation experiments, there is a direct correlation between the extent of receptor phosphorylation and cellular GSH levels: receptor phosphorylation decreases and stabilizes to a constant low level of between 50 μ M and 250 μ M BSO treatment, when cellular GSH content has reached its minimum value. Besides, we can observe that the same GSH critical level (approx. 10 nmol/mg of protein), is necessary to obtain levels of both proliferation and PDGF-r phosphorylation similar to control. Owing to the reversal of the BSO effect by adding exogenous GSH, we can exclude the possibility that BSO has an effect on PDGF-r that is different from the one mediated by the lowering of glutathione content, at least in the concentration range used.

The increase in PDGF-r activation, observed when cells with a normal GSH content are incubated for a short period with GSH, cysteine or DTT before growth-factor stimulation, indi-

cates that a certain GSH cellular level is necessary for extracellular GSH to perform its positive action upon the receptor phosphorylation. This effect is probably due to its reducing property because it can be also mediated by other thiols.

Clark and Konstantopoulos [21] studied the effect of thiol reagents on insulin and EGF receptors. GSH stimulates *in vitro* basal receptor phosphorylation, but is without effect on EGF-r and inhibits the insulin receptor in the presence of their ligands. Furthermore, exogenous GSH did not affect insulin receptor activation and structure at up to 50 mM *in vivo*. In contrast, DTT appeared to activate phosphorylation of insulin receptor in association with reduction of its extracellular disulphides, but was without effect on EGF-r. They concluded that, since the cell lines used (CHOT and A431) did not seem to be permeable to GSH, and these effects were observed principally on isolated receptors, the complex thiol interaction should occur in the cytoplasmic receptor domains, and the differences observed, depending on the presence of the ligand, were probably due to changes in receptor conformation upon activation. Moreover, DTT is a dithiol with an oxidation potential greater than that of GSH. Consequently DTT leads to complete reduction of disulphides, whereas GSH, being a monothiol, may also form mixed disulphides.

As regards PDGF-r, several disulphide bonds are present in the immunoglobulin-like domains on the extracellular side of the receptor [41]. Therefore the effect of external GSH may actually be due to its reducing action, but so far nothing has been determined with respect to an effect of this reduction on PDGF-r activation in response to the growth factor.

As for the GSH effect on the cytoplasmic side of the PDGF-r, we cannot exclude the possibility of a direct interaction of GSH with PDGF-r and a possible alteration of its conformation, as in the case of the cited study on EGF and insulin receptors [21]. This may also be confirmed by the necessary presence of a certain internal level of GSH to obtain PDGF-r stimulation and receptor activation enhancement by external GSH in the presence of PDGF.

We cannot exclude a possible effect of variations in the amount of GSSG and in the GSH/GSSG ratio on the regulation of PDGF-r activity. However, the lowering of glutathione caused by BSO treatment makes the determination of GSSG extremely difficult, because it remains very near to the lower detection limit of the HPLC method.

It seems to be possible that the oxidation of protein thiol groups resulting from the lowering of GSH content could be the reason for the reduced PDGF-r autophosphorylation. As recently demonstrated for some enzymes [5,6], protein activity may be regulated through thiol-disulphide exchange reactions catalysed by thioltransferases that use GSH as the specific substrate. In the case of PDGF-r too, specific thiol groups may become reactive towards GSH, depending on the cellular redox state. Actually these enzymes are present in NIH/3T3 cells, and we measured a specific activity of 0.013 ± 0.001 $\mu\text{mol}/\text{min}$ per mg of protein that does not change after cell treatment with BSO or GSH.

Our results in A431 cells, GSH-depleted by BSO treatment, show that a significant GSH decrease does not affect the phosphorylation of the EGF-r. These data point to a certain specificity of the effect of GSH variations on the activation of PDGF-r *in vivo*.

Summarizing, these results, taken together, show that the interaction of thiol-reactive agents with receptors is a complex process. However, it seems to be confirmed that PDGF-r activation, and consequently the cellular proliferation of NIH/

3T3, may be modulated by cellular changes in GSH levels that may occur under many physiological and pathological conditions.

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