CMLS, Cell. Mol. Life Sci. 59 (2002) 1388–1394
1420-682X/02/081388-07

CMLS Cellular and Molecular Life Sciences

Research Article

The role of H_2O_2 in the platelet-derived growth factor-induced transcription of the γ-glutamylcysteine synthetase heavy subunit

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Received 17 April 2002; received after revision 6 June 2002; accepted 17 June 2002

Abstract. This study demonstrates that platelet-derived growth factor (PDGF) increases transcription of the γ-glutamylcysteine synthetase (GCS) heavy subunit (GCS-HS) in NIH 3T3 fibroblasts via H_2O_2 and activation of protein kinase C (PKC). The data obtained using catalase, H_2O_2, phorbol-12-myristate 13-acetate (PMA) or a specific inhibitor of PKC demonstrate the possibility of a PDGF up-regulation pathway of GCS synthesis. Moreover, since PDGF mitogenic activity takes place through PKC activation and sphingosine-1-phosphate (S1P) production, the involvement of sphingosine kinase activity in the PDGF effect was also investigated. No clear direct relationship emerged between S1P production and any PDGF- or H_2O_2-induced increase in the GCS-HS mRNA level. However, for the first time, in SIP-stimulated NIH 3T3 cells, increased levels of GCS-HS mRNA were shown to be related to increases in the reduced glutathione synthesis rate similar to those obtained after PMA and PDGF stimulation.

Key words. PDGF; H_2O_2; protein kinase C; sphingosine-1-phosphate; γ-glutamylcysteine synthetase; NIH 3T3 fibroblasts.

γ-Glutamylcysteine synthetase (GCS) is the initial and rate-limiting enzyme of the GSH de novo synthesis pathway. GCS is composed of a light regulatory subunit and a heavy catalytic subunit which exhibits feedback inhibition by reduced glutathione (GSH) [1].

Expression of the GCS heavy subunit (GCS-HS) is induced under various conditions and by various agents including heavy metals, substances which deplete GSH, oxidants, antioxidants, and tumor necrosis factor [2, 3]. Increased GCS expression and GSH content have also been found in cells exposed to oxidative stress, in drug-resistant tumor cells, and during cell proliferation [3–5]. GSH is the most abundant intracellular reductant and detoxifying agent, but there is also evidence pointing to its involvement in the proliferation of both normal and cancer cells [5, 6]. In fact, GSH and oxidized glutathione (GSSG) may influence both the redox status and reactivity of specific thiol groups of redox-sensitive enzymes and transcriptional factors involved in cell cycle progression through thiol/disulfide exchange reactions [7, 8]. The possible role of GSH in the mitogenic signal cascade is supported by findings obtained by us and others regarding the kinase activity of growth factor receptors [4, 8–11]. We have demonstrated that in NIH 3T3 fibroblasts, a specific critical intracellular GSH level is necessary for maximal autophosphorylation and mitogenic activity of the platelet-derived growth factor (PDGF) receptor [9, 11].

Recently, we demonstrated that the GSH effect on the PDGF receptor is related to H_2O_2 production induced by PDGF through the activation of an NAD(P)H oxidase

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[11]. Moreover, other data show time- and PDGF dose-dependent increases in the intracellular GSH content related to the increase in both GCS enzymatic activity and the GCS-HS mRNA level [4]. Since the GSH increase induced by PDGF occurs 6 h after stimulation, GSH possibly also plays an important physiological role in the deactivation mechanism downstream of the PDGF signal. In fact, the progressive increase in the GSH/GSSG ratio may contribute to the restoration (i.e., reduction) of target proteins such as tyrosine phosphatases oxidized by reactive oxygen species (ROS). Taken together, these data demonstrate that PDGF stimulation of NIH 3T3 cells modulates GSH synthesis and that the cellular GSH content can be involved at different levels of the PDGF signal pathway.
Considering these previous results and that little is known about the up-regulation mechanisms of GCS synthesis related to cell proliferation, we investigated in NIH 3T3 cells the involvement of both H_2O_2 and specific transductors related to the PDGF-mediated signal pathway in the transcriptional regulation of GCS. In fact, H_2O_2 has been shown to be the messenger of various cytokines and growth factors, including PDGF [12]. Possible connections between these messengers and PDGF signaling were also investigated.

Materials and methods

Cell culture and treatments

NIH 3T3 murine fibroblasts were obtained from the Istituto Nazionale Tumori (Milan, Italy). The cells were routinely cultured at low density in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS). During serum starvation, subconfluent 100-mm dishes of cells were cultured in a medium containing 0.5% FCS for 24 h. Starved cells were stimulated with PDGF-BB or mitogenic agents for different times. Starved cells were also treated with different H_2O_2 concentrations for 20 min, were subsequently washed, and fresh starvation medium was added. Catalase (6000 U/ml) was added to starvation medium for 24 h and 0.5 μg/ml actinomycin D for different times. In some experiments, starved cells were also treated for 1 h with 10 μM GF109203x (GF) or 20 mM DL-threodihydrosphingosine (DHS) or both. Phorbol-12-myristate 13-acetate (PMA) down-regulation was performed in 24 h-starved cells using 300 nM PMA added to starvation medium for the following 24 h.

Analysis of GCS-HS mRNA

Total mRNA was purified from the cells at different times from mitogenic stimulation and H_2O_2 treatment. Northern blot analysis of GCS-HS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as previously reported [4]. Densitometric analysis of GCS-HS mRNA levels was normalized using GAPDH mRNA values.

Determination of GSH synthesis rate and total GSH levels

Cells starved for 24 h were stimulated or not with PMA or sphingosine-1-phosphate (S1P) for different times, and then treated with 0.2 mM diethylmaleate (DEM) for 20 min to deplete the cytosolic GSH content and to minimize feedback inhibition of GSH on GCS. The cells ($\sim 10 \times 10^6$) were washed, collected by scraper and centrifuged at $125 \times g$ for 10 min. The precipitate was resuspended in 0.5 ml of 150 mM Tris buffer containing 5 mM $MgCl_2$, 2 mM 1,4-dithiothreitol at pH 7.5. The cells were lysed by sonication at 4 °C. The homogenate was centrifuged at $12{,}550 \times g$ for 30 min to obtain the supernatant (cell-free extract). The dynamic rate of GSH synthesis was determined by coupling the two enzymatic steps for GSH synthesis, as reported by others [13]. Synthesized GSH was determined as the 2,4-dinitrophenyl derivative by HPLC [4]. The reaction mixture contained 5 mM cysteine, 5 mM glutamic acid, 5 mM glycine, 10 mM ATP, and 100 mM Tris/HCl buffer pH 8, containing 50 mM KCl, 20 mM $MgCl_2$ and 2 mM EDTA. The reaction was started by addition of cell-free extract preincubated or not with 1 mM buthionine sulphoximine (BSO) at 37 °C for 30 min to specifically inhibit GCS activity [13]. The reaction was blocked after 0, 10, 20 and 30 min of incubation at 37 °C by the addition of 5% $HClO_4$. The linearity of the reaction was verified over 30 min. The GSH synthetic rate was expressed as nmol/min/per milligram protein.
Intracellular GSH levels were also determined by the HPLC method [4] in starved cells stimulated or not with PMA or S1P for different times.

Protein determination

The protein concentration was determined by the Bradford method [14]. Bovine serum albumin (Sigma) was used as standard.

Statistical analysis

Statistical analysis was undertaken using Student's t-test. $p \leq 0.05$ was considered significant.

Results and discussion

The role of H_2O_2 in the PDGF-induced increase of GCS-HS mRNA levels

To assess the role of H_2O_2 as the ROS responsible for the PDGF-induced increase in GCS-HS mRNA content [4], we examined the effect of catalase. Catalase, a highly reactive enzyme, has been widely used to block the biological activities of H_2O_2 induced by specific ligands, either by entering the cells or by acting on the external surface of the membrane [15]. As shown in figure 1, pretreatment

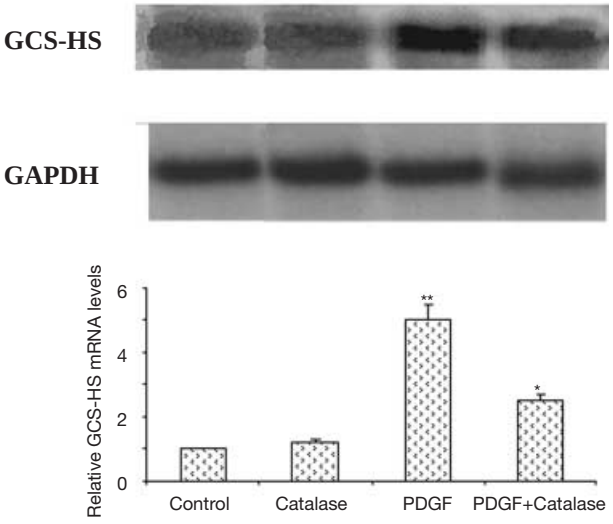

Figure 1. Effect of catalase on GCS-HS mRNA levels in PDGF-stimulated NIH 3T3 cells. Starved cells, pretreated or not with 6000 U/ml catalase, were stimulated for 3 h with 20 ng/ml PDGF. Starved cells were the control. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were compared with GAPDH mRNA levels used as quantitative control and GCS-HS mRNA values were expressed relative to control ± SE of three different preparations. * $p \leq 0.05$ compared to values measured in PDGF-stimulated cells without catalase. ** $p \leq 0.005$ compared to control.

of NIH 3T3 cells with catalase for 24 h decreased the PDGF-induced GCS-HS mRNA level 3 h after stimulation by about 50% compared to the values measured in PDGF-stimulated cells without catalase. The catalase effect was determined after 3 h because previous studies indicate this as the time required to obtain the maximum increase in GCS-HS mRNA level after PDGF stimulation [4]. These results indicate that intracellular H_2O_2 is involved in the PDGF effect on GCS-HS mRNA levels.

To confirm the involvement of H_2O_2, NIH 3T3 cells were exposed to 0.5 mM H_2O_2 for 20 min, since a transient production of 0.1 – 1 mM H_2O_2 after PDGF stimulation has already been demonstrated [12]. Figure 2 shows an increase of about five-fold compared to control in GCS-HS mRNA content 6 h after H_2O_2 treatment. The same results were obtained using 1 mM H_2O_2 (data not shown).

Effect on GCS-HS mRNA content of other PDGF signalling-related transductors

Subsequently, the effect of various mitogenic signal transductors on the GCS-HS mRNA content was tested and compared with the PDGF effect. These experiments were performed to identify other intracellular messengers through which PDGF and H_2O_2 could modulate GCS synthesis. Figure 3 shows a significant increase in GCS-HS mRNA content after 3 h stimulation with PDGF, PMA (which mimics diacylglycerol), S1P, sphingosine (SPH), and C2-ceramide (C2), these last two compounds being

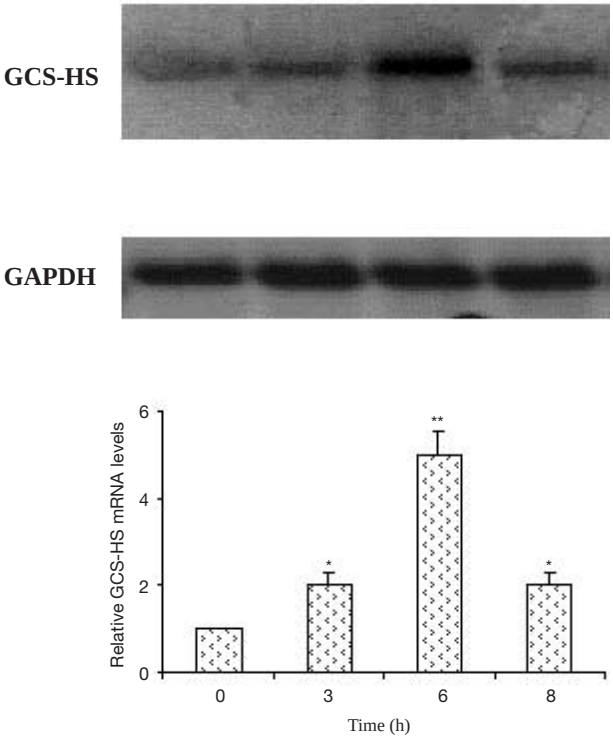

Figure 2. GCS-HS mRNA levels in starved NIH 3T3 cells at different times following H_2O_2 treatment. Starved cells were treated with 0.5 mM H_2O_2 for 20 min. Total mRNA was extracted at various times after H_2O_2 treatment. 0 time was the control. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. * $p \leq 0.05$, ** $p \leq 0.005$ compared to control.

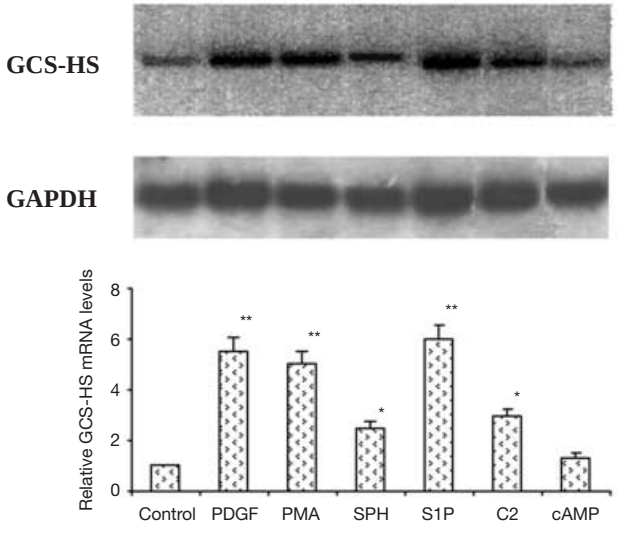

Figure 3. GCS-HS mRNA levels in starved NIH 3T3 cells after stimulation by various mitogenic agents. Starved cells were stimulated or not (control) for 3 h with 20 ng/ml PDGF, 100 nM PMA, 30 μM sphingosine (SPH), 10 μM S1P, 10 μM C2-ceramide (C2), or 100 μM cAMP. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. * $p \leq 0.05$, ** $p \leq 0.005$ compared to control.

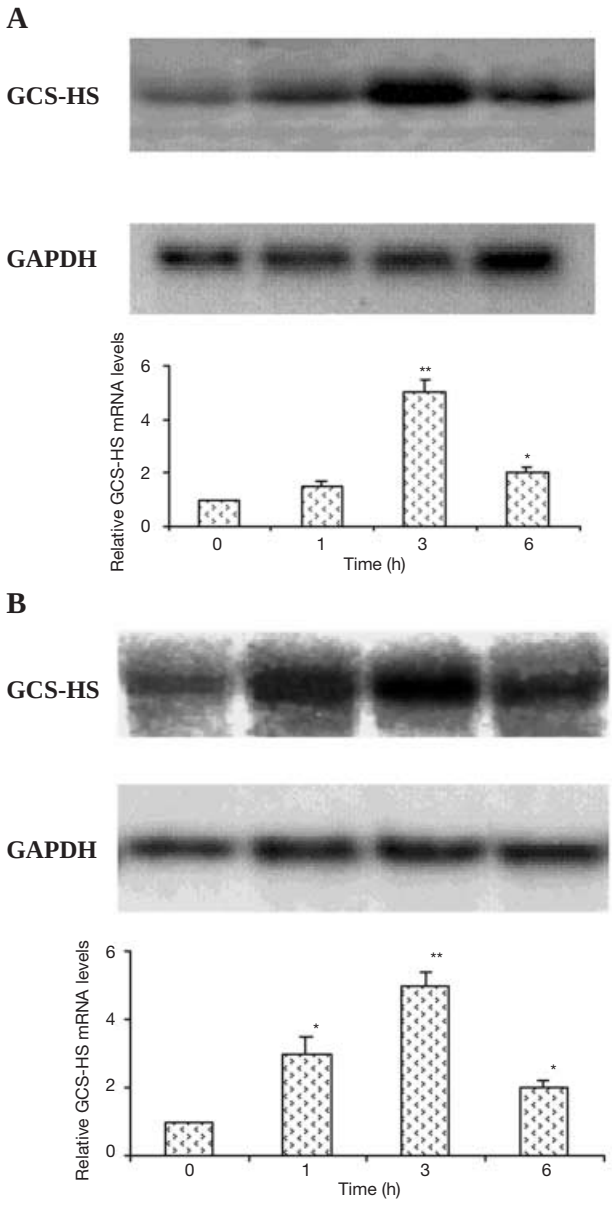

Figure 4. GCS-HS mRNA levels in starved NIH 3T3 cells at various time points after PMA or S1P stimulation. Starved cells were stimulated for different times with 100 nM PMA (*A*) or 10 µM S1P (*B*). 0 time was the control. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. * $p \leq 0.05$, ** $p \leq 0.005$ compared to control.

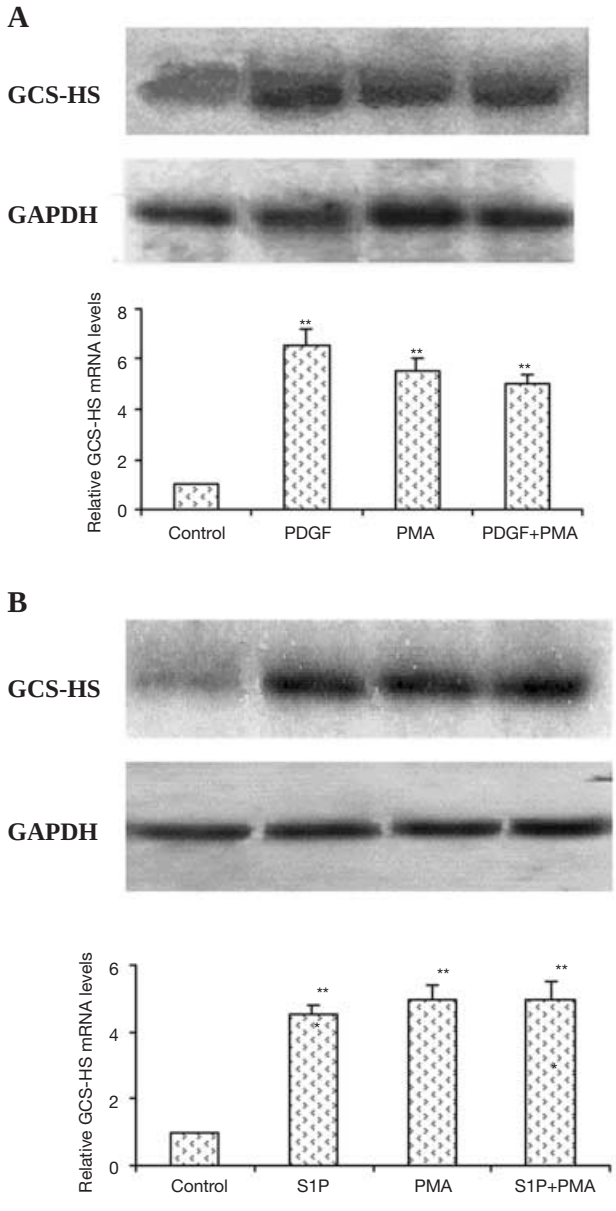

Figure 5. GCS-HS mRNA levels in starved NIH 3T3 cells after stimulation by PDGF, PMA or S1P. Starved cells were stimulated or not (control) for 3 h with 20 ng/ml PDGF or 100 nM PMA or both (*A*) or 10 µM S1P or 100 nM PMA or both (*B*). Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. ** $p \leq 0.005$ compared to control.

precursors of S1P. The highest levels of mRNA were detected after PMA or S1P stimulation and were similar to those measured by PDGF stimulation, an increase of about five-fold compared to the control (fig. 3). Therefore, the effect of PMA and S1P on GCS-HS mRNA content in relation to PDGF stimulation or H_2O_2 treatment was studied, since PDGF mitogenic action in Swiss 3T3 fibroblasts occurs with both S1P and phosphatidylinositol-3,4,5 phosphate [16]. In fact, PDGF increases S1P content by an enzymatic activation of sphingomyelinase, ceramidase, and sphingosine kinase (SK) in Swiss 3T3 cells [17]. S1P is essential for the proliferation of these cells and for the activation of mitogenic-activated protein kinase. Figure 4 A, B shows that both PMA and S1P induce the maximum increase in GCS-HS mRNA levels 3 h after stimulation. The time-dependent behavior of the mRNA content of GCS-HS shown in Figure 4 corresponds to that previously determined in PDGF-stimulated cells [4]. Moreover, the increase in GCS-HS mRNA content measured after 3 h with simultaneous stimulation by

PDGF and PMA or PMA and S1P was not cumulative (fig. 5A, B). Similar results were obtained after PDGF-plus S1P stimulation (not shown). These findings suggest that these messengers use the same pathways to increase GCS-HS content.

Effect of actinomycin D on the transcriptional activation of GCS-HS

To determine whether the increased content of GCS-HS mRNA was due to increased transcription, the half-life of GCS-HS mRNA was assessed in actinomycin D-treated cells both in the presence and absence of the inducing agents (fig. 6). The ratio GCS-HS mRNA to GAPDH mRNA was used to calculate degradation rates and GCS mRNA half-life. As shown in figure 6, in RNA degredation actinomycin D-treated cells was similar (about 7 h) whether or not they had been stimulated with PDGF, and the half-life value was about 7–8 h. Moreover, no increase was observed in GCS-HS mRNA content 3 h after PDGF stimulation in actinomycin D-treated cells. These results indicate that posttranscriptional modification of GCS-HS mRNA cannot account for the increase in GCS-HS mRNA in NIH 3T3 cells after PDGF stimulation. Similar results were also obtained after H_2O_2, PMA and S1P stimulation (data not shown). Furthermore, they are in accordance with previous data showing no GSH synthesis activation in cells pretreated with actinomycin D or cycloheximide after PDGF stimulation [4].

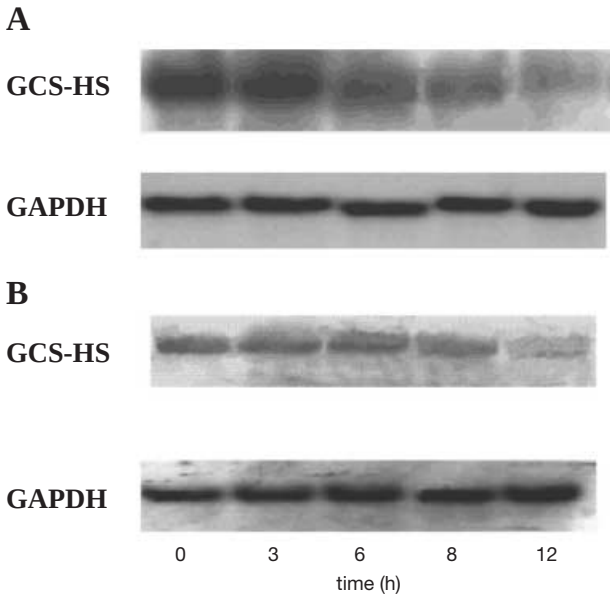

Figure 6. Effect of actinomycin D on GCS-HS mRNA levels in PDGF-stimulated NIH 3T3 cells. Northern hybridization analysis of GCS-HS and GAPDH mRNA in starved cells treated for different times (0–12 h) with 0.5 µg/ml actinomycin D (*A*) and Actinomycin D plus 20 ng/ml PDGF (*B*). The half-life of GCS-HS mRNA in actinomycin D-treated cells stimulated or not with PDGF was about 7 h. The half-life was determined by densitometric analysis and calculated from the ratio GCS-HS mRNA to GAPDH mRNA. Data represent one of three experiments.

Involvement of protein kinase C and SK activation in PDGF or H_2O_2 induction of GCS-HS expression

Since protein kinase C (PKC) and SK activity are related to PDGF signalling, the involvement of these enzymes in the PDGF and H_2O_2 induction of GCS-HS expression was investigated. NIH 3T3 cells were first treated with specific inhibitors of PKC and SK, GF [18] and DHS [16], respectively, and then stimulated by PDGF, PMA, or H_2O_2. As shown in figure 7, GF caused a reduction in the PDGF-induced increase of GCS-HS mRNA by about 50% compared to the control. This percentage of inhibition is the maximum value obtained even when higher concentrations of GF were used (not shown). No significant change in the GCS-HS mRNA content of PDGF-stimulated cells was determined in the presence of DHS alone; likewise, DHS did not increase the GF effect (fig. 7). (Note that DHS was used at the concentration that inhibits SK activity in these cells [16]). Higher concentrations of DHS were also used, but similarly, no effect was observed; the same results were obtained after PMA stimulation or H_2O_2 treatment (data not shown) using these inhibitors. These results indicate that PDGF activates GCS-HS mRNA synthesis by H_2O_2 and this signal is prevalently mediated through PKC activity. The involvement of PKC in H_2O_2 induction of GCS mRNA synthesis was also confirmed by down-regulation experiments. NIH 3T3 cells contain the

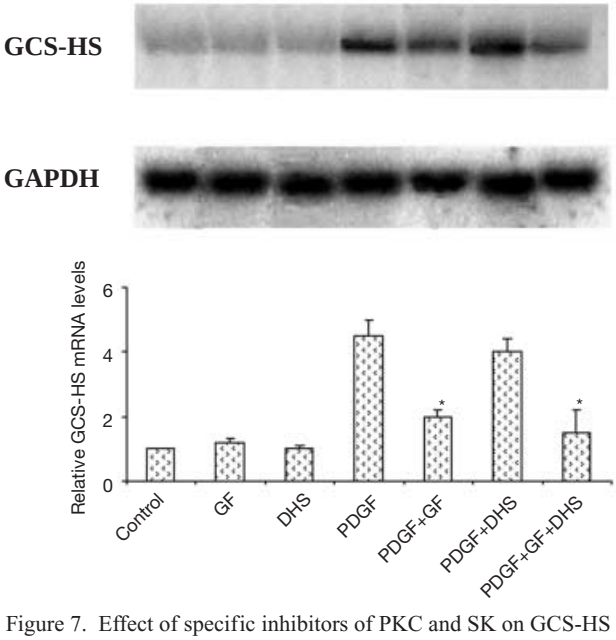

Figure 7. Effect of specific inhibitors of PKC and SK on GCS-HS mRNA levels in PDGF-stimulated NIH 3T3 cells. Starved cells pretreated or not with 10 µM GF or 20 µM DHS or both for 1 h were stimulated for 3 h with 20 ng/ml PDGF. Not stimulated and not pretreated starved cells were the controls. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. * $p \leq 0.05$ com pared to values measured both in PDGF- and PDGF+DHS-stimulated cells.

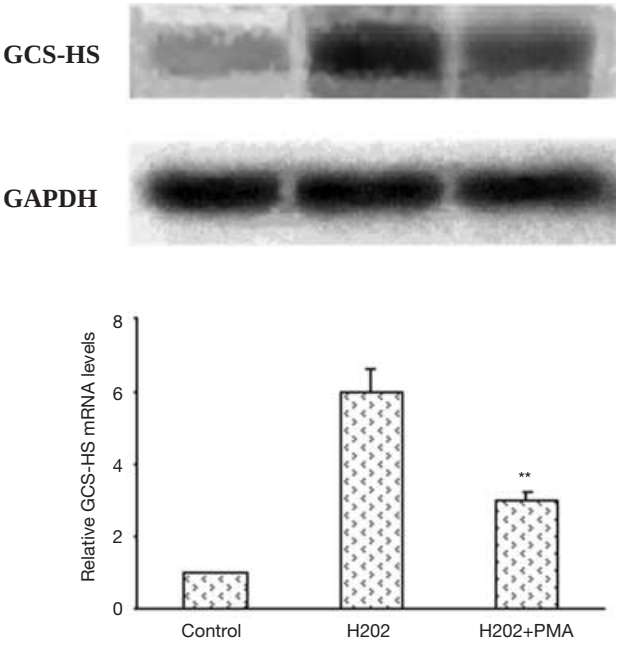

Figure 8. Effect of PMA down-regulation on GCS-HS mRNA levels in NIH 3T3 cells after H_2O_2 exposure. Starved cells pretreated or not for 24 h with 300 nM PMA were exposed to 0.5 mM H_2O_2 for 20 min. PMA-pretreated cells not exposed to H_2O_2 were the control. Total mRNA was extracted 6 h after H_2O_2 treatment. Top: Northern hybridization analysis of GCS-HS and GAPDH mRNA. Bottom: densitometric analysis. GCS-HS mRNA levels were measured and expressed as reported in figure 1. ** $p \leq 0.005$ compared to values measured in H_2O_2-exposed cells in the absence of PMA.

α, δ, ε, and ζ PKC isoenzymes, of which the α, ε and δ isoenzymes are down-regulated by at least 80% upon treatment of cells with 300 nM PMA for 24 h [19]. Figure 8 shows that in NIH 3T3 cells pretreated with 300 nM PMA for 24 h, the GCS-HS mRNA level increase induced by H_2O_2 fell by about 50% compared to values measured in H_2O_2-exposed cells in the absence of PMA treatment. These data, taken together, indicate that both PDGF and exogenous H_2O_2 can directly activate GCS-HS expression through PKC activity. However PDGF may possibly up-regulate GCS-HS mRNA content by induced PKC activation generating endogenous H_2O_2. In fact, cytokines and growth factors activate a membrane enzyme, NADPH oxidase, which generates H_2O_2 through a complex activation mechanism including PKC activity [20]. Therefore, these findings demonstrate a link between H_2O_2 and PKC in the PDGF transcriptional activation of GCS-HS; by contrast, no direct relationship between PDGF or H_2O_2 effect and S1P production was observed. However, other factors besides PKC could be involved in the PDGF and H_2O_2 activation of GCS-HS expression; indeed, only a partial reduction of activation was obtained in cells pretreated with GF or GF plus DHS. In the presence of DHS, PDGF stimulation may increase the content of the S1P precursors, SPH and C2, and these could enhance the level of GCS-HS mRNA level, as shown in figure 3.

Moreover, no change in S1P-induced GCS-HS mRNA activation was measured after cell treatment with GF (data not shown); thus, there is apparently no PKC involvement in the S1P effect. These data suggest that the effect of S1P and its precursors is mediated by other factors, and partial involvement of intracellular S1P production in PDGF activation of GCS-HS mRNA synthesis cannot be excluded. However, this aspect needs further investigation, as does the effect of extracellular S1P on GCS-HS mRNA transcription. Indeed, S1P has recently been shown to be the ligand for specific G protein-coupled receptors of the EDG class (endothelial differentiation gene) [21], which have also been identified in NIH 3T3 cells [22]. Therefore, even if S1P has been demonstrated to act as an intracellular messenger of the PDGF signal, the effects of extracellular S1P may differ in part from those mediated by PDGF [21, 23]. Moreover, considering that GCS-HS mRNA level increases measured by simultaneous stimulation with PMA and S1P are not cumulative, these messengers conceivably induce the activation of the same factors downstream of signalling. The promoter region of the gene for the GCS heavy chain possesses binding sites for specific transcriptional factors, such as AP-1 and NF-κB, and their activation may induce GCS expression [3]. H_2O_2, PMA, and S1P may activate both these transcriptional factors [3, 24, 25], which could constitute the final common targets in the GCS transcriptional activation mediated by PDGF in NIH 3T3 cells.

Relationship between GCS activity and the PMA- or S1P-induced increase in GCS-HS mRNA

Previously, increases in GCS activity and GSH levels were related to PDGF-induced increases in GCS-HS mRNA content [4]. Therefore, to verify that PMA- or S1P-induced GCS-HS transcriptional activation was actually related to increased GCS activity and GSH synthetic rate, cell-free experiments were performed in both stimulated and non-stimulated cells. Six to 8 h after stimulation by PMA or S1P, the cytosolic synthetic rate of GSH increased by about 50% compared to the control (fig. 9). These increases are related to similar increases in GSH content after the same times of stimulation by PMA or S1P (data not shown). The GSH synthesis rate was effectively linked to GCS activity, since no GSH production was detected by HPLC when BSO, a specific inhibitor of GCS, was added to cell extracts. Previously, similar increases (of about 50%) of GCS activity and GSH levels were also measured in NIH 3T3 cells 6 h after PDGF stimulation [4].

In conclusion, this study demonstrates the existence of a possible up-regulation pathway in NIH 3T3 cells for GCS expression induced by PDGF through H_2O_2 and PKC activity. In addition, no direct relationship was observed between S1P production and the effect of PDGF or H_2O_2 on the increase in GCS-HS transcription. However, for the first time, S1P stimulation has been demonstrated to in-

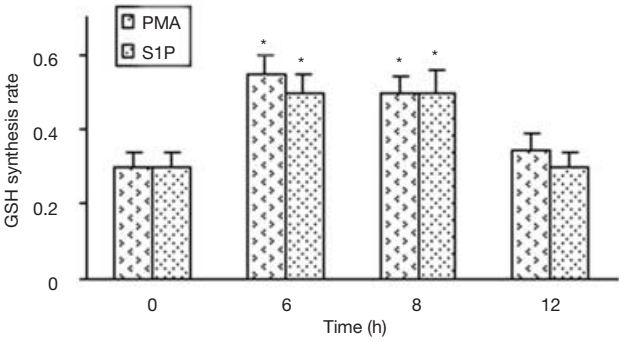

Figure 9. GSH synthesis rate in cell-free extracts of NIH 3T3 cells at various times after PMA or S1P stimulation. Starved cells stimulated with 100 nM PMA or 10 μM S1P for different times were treated with 0.2 mM DEM for 20 min to eliminate intracellular GSH and to minimize feedback inhibition by GSH. The GSH synthesis rate was determined by HPLC and expressed as nmol/min per milligram protein. Data are the mean ± SE of three experiments. * $p \leq 0.05$, compared to values measured at 0 time (control).

crease GCS-HS mRNA content in NIH 3T3 cells, and this effect is related to the increase in GCS activity. Further study is needed to clarify whether any connection exists between PDGF and S1P in the upstream regulation mechanisms of GCS synthesis. This could prove useful, because the knowledge of factors related both to transduction signal pathways and to the regulation of GSH synthesis might also have clinical value. Indeed, these factors could be new targets for therapies to treat diseases involving GSH, the intracellular redox state, and cell proliferation.

Acknowledgements. This work was supported by grants from MURST, and by a donation from the Cassa di Risparmio di Firenze.

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