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H7, a protein kinase C inhibitor, increases the glutathione content of neuroblastoma cells

Francisco J. Romero[a], Juan Llopis[b], Vicente Felipo[c], María-Dolores Miñana[c], Joaquín Romá[a] and Santiago Grisolía[c]

[a]*Departament de Fisiologia, Universitat de València, E-46010 Valencia, Spain,* [b]*Department of Toxicology, Karolinska Institutet, S-104 01 Stockholm, Sweden* and [c]*Instituo de Investigaciones Citológicas de Valencia, E-46010 Valencia, Spain*

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It is shown that the intracellular glutathione (GSH) concentration of neuroblastoma-2a cells in culture increases with a maximum at 24 h after starting treatment with 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), an inhibitor of protein kinase C (PKC). Other inhibitors of this and other protein kinases, e.g. sphingosine, staurosporine, and HA 1004, at the concentrations tested, had a less marked or negligible effect on intracellular GSH concentration. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was also tested and showed no significant effect 24 h after addition.

Glutathione; Neuroblastoma cell; Protein kinase C; Cellular differentiation; H7; 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine

1. INTRODUCTION

It has been recently reported that 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine, called H7, a known inhibitor of protein kinase C (PKC), promoted differentiation in neuroblastoma-2a cells as indicated by the induction of neuritogenesis, increase in acetylcholinesterase activity [1] and decrease in DNA synthesis [2]. This effect was reversed by the PKC activator 12-*O*-tetradecanoyl-13-phorbol-acetate (TPA), which confirms the involvement of PKC [3]. It has been claimed that PKC is sensitive to thiol–disulfide exchange; hence, its activity may be sensitive to the redox state of the cellular environment [4]. In the latter report, it was discussed that the oxidation state of PKC might modulate its activity. In this regard, H_2O_2 promoted inactivation of partially purified brain PKC [5]; conversely quinone-induced oxygen radical generation lead to activation of the soluble hepatocyte protein to its high V_{max} form [4].

The main objective of this study was to study the changes induced by PKC inhibition on the intracellular concentration in neuro-2a cells of GSH, the most abundant non-protein thiol in mammalian cells.

2. MATERIALS AND METHODS

The clonal line neuro-2a, C1300 mouse neuroblastoma, was obtained from the American Type Culture Collection, Rockville, MD, and grown at 37°C in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, 100 IU penicillin/ml, and 100 μg of streptomycin/ml as previously described [2,3]. H7, TPA, staurosporine and sphingosine were from Sigma Chemical Co. (St. Louis, MO). HA-1004 was from Seikagaku America Inc. (Rockville, MD). All reagents were of the highest purity available. PKC inhibitors and other substances were dissolved in either DMSO or 70% ethanol. These solvents had no effect on cell viability, neuritogenesis, or GSH concentration. Cells were seeded at 200,000/ml and after 24 h subculture, fresh culture medium containing the inhibitors and/or other substances was added. Cell viability was assessed by Trypan blue exclusion after detachment, and total GSH concentration according to [6] and is expressed as GSH equivalents ([GSH] + 2[GSSG]).

Correspondence address: F.J. Romero, Departament de Fisiologia, Unidad de Toxicología Experimental y Neurotoxicología, Facultat de Medicina i Odontologia, Universitat de València, Av. Blasco Ibañez 17, E-46010 Valencia, Spain. Fax: (34) (6) 386-4173.

3. RESULTS AND DISCUSSION

The effect of H7 on GSH concentration in neuroblastoma-2a cells is shown in Fig. 1; 24 h incubation of neuro-2a cells with 0.5 mM H7 induced an approximately 70% increase in GSH concentration. However, unlike neurite outgrowth, which was apparent 4 h after starting H7 treatment, no change in GSH concentration was observed at this time. With the highest concentration of H7 used, cell viability was not significantly different from the control (Fig. 2). The fact that GSH concentration increases during differentiation fits well with previous reports showing changes in GSH during the cell cycle in different cell lines [7,8]. Moreover, activation of cells with TPA in different cellular and in vivo models leads to the opposite effect, i.e. decrease of GSG concentration [9,10], and also to the release of cell–cell contact inhibition [11]. In order to establish the contribution of PKC to the changes of GSH concentration observed in Fig. 1, we tested different inhibitors and activators of PKC, as well as HA-1004, which inhibits

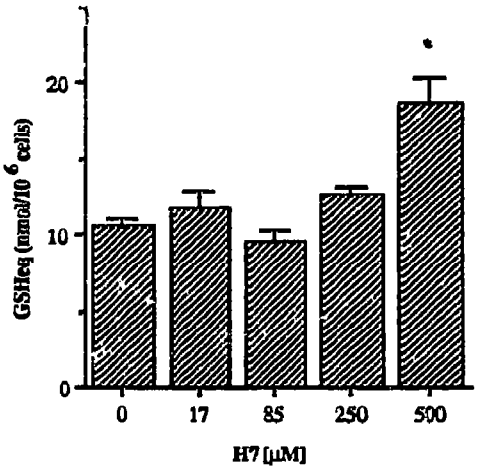

Fig. 1. Effect of H7 on GSH concentration in neuro-2a cells after 24 h treatment. Results are means ± S.E.M. of at least 4 different determinations. *$P \leq 0.005$.

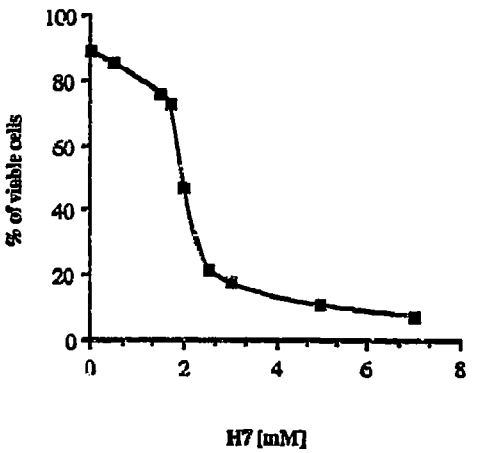

Fig. 2. Toxicity of H7 on neuro-2a cells after 24 h treatment.

the protein kinases dependent on cAMP and cGMP with a lower K_i than for PKC [12]. Their effect is shown in Table I. Sphingosine and staurosporine, at the concentrations tested, did not affect GSH levels. These inhibitors cannot be tested at higher concentrations because they are toxic for the cells. HA-1004 did not affect GSH levels, indicating that the effect of H7 is not due to inhibition of protein kinases dependent on cAMP or cGMP. TPA did not affect GSH levels in neuro-2a cells.

The results presented show that the H7-induced differentiation of neuroblastoma cells not only inhibits their growth as tumor cells, but results in a high concentration of GSH. It should be noted that 500 μM H7 is needed to induce marked changes in GSH levels. However, differentiation of neuro-2a, with stimulation of neurite growth, can be observed at much lower doses (i.e. 85 μM) [1–3]. This indicates that the two phenomena could not be correlated. It has been shown that there is a good correlation between induction of neuritogenesis by H7 and inhibition of protein kinase C [2]. The requirement of larger amounts of H7 to induce the accumulation of GSH could be attributed to the necessity of extensive inhibition of PKC or could indicate that the effect is not due to inhibition of PKC. It is known that the isoforms of protein kinase C have different responses to activators and inhibitors [13,14] and that down-regulation is also different for each isoform [15]. Also, the PKC isotypes have differential substrate specificity [16] and are involved in different functions [17]. It has been reported that only the type II of PKC is associated with the membrane skeleton from neuronal growth cones [18]. Therefore, it is also possible that different isoforms of PKC, with very different susceptibility to H7 inhibition, could be involved in the induction of neuritogenesis and in the control of intracellular GSH content in this cell type.

The rise in GSH induced by H7 could be one more feature of the morphological (neurite outgrowth) and functional (acetylcholinesterase activity increase) differentiation of this tumoral cell type observed upon inhibition of PKC. The involvement of PKC in the regulation of intracellular GSH levels in neural cells has not been reported and could be important for the basic understanding of the role of PKC and also could have therapeutical implications.

Table I

Effect of different inhibitors and activators of protein kinase C on the GSH concentration of neuroblastoma-2a cells

Addition	GSHeq concentration (nmol/10^6 cells)
None	10.51 ± 1.74 (10)
Sphingosine 50 μM	12.95 ± 0.54 (4)
Staurosporine 80 nM	11.84 ± 1.03 (6)
HA1004 250 μM	9.67 ± 2.06 (4)
TPA 25 nM	10.47 ± 2.46 (8)

Results are expressed as the mean ± S.D. with the number of observations in parenthesis. Additions were made 24 h after subculture, and collection of samples 24 h after additions.

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