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Biochemical Pharmacology 66 (2003) 1521–1526
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

A novel role of protein kinase C-δ in cell signaling triggered by glutathione depletion

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Received 31 January 2003; accepted 11 April 2003

Abstract

Current evidence demonstrates that protein kinase C (PKC) belongs to a group of cell-signaling molecules that are sensitive targets for redox modifications and functional alterations that mediate oxidant-induced cellular responses. Our studies have demonstrated that diminished intracellular GSH was associated to inactivation of classic isoforms and increased activity of novel PKCs, and triggered molecular signals important for cell survival. Loss of GSH and oxidative damage are probably an early signaling event in apoptotic death, which is characterized by the activation of PKC-δ. Apoptotic process consequent to GSH depletion was inhibited by rottlerin, a PKC-δ-specific inhibitor, which exerted a negative effect on oxyradical production. Therefore, it may be concluded that PKC-δ activity is related to reactive oxygen species production and is involved in the pathway leading to apoptosis and growth arrest.

Keywords: Glutathione; L-Buthionine-*S*,*R*-sulphoximine; Reactive oxygen species; Protein kinase C; Rottlerin; Apoptosis

1. Introduction

Alterations in the cellular redox state regulate several signal transduction pathways that lead to functional changes in cells and tissues. The balance between oxidants and antioxidants in biological systems can indirectly influence cell phenotype through an effect on redox active molecules involved in many cell responses, such as proliferation, growth arrest, and death [1,2]. Cellular redox potential is largely determined by GSH, a tripeptide of glutamate, cysteine, and glycine, that is synthesized from its constituent amino acids in the cytosol of virtually all mammalian cells.

When a tissue is exposed to oxidant compounds, such as hydrogen peroxide and/or hydroxylradicals, GSH may be oxidized to GSSG through the GSH peroxidase reaction; the ratio GSH/GSSG cannot be maintained at its physiological value and GSSG accumulates in spite of glutathione reductase activity [3].

Thus, a direct oxidative injury and/or a decline of cytosolic GSH are able to influence protein oxidation state [4,5] and many oxidatively modified target proteins have been demonstrated to initiate intracellular signaling events. The only signaling protein in which direct activation by oxidative modifications of its structure has been reported is PKC [6–8]. PKCs are a family of homologous enzymatic isoforms that are variously distributed, structurally correlated, and differentially involved in the transduction of signals for cell proliferation, differentiation, and apoptosis [9].

PKC isoforms are divided into three categories according to the cofactors required for optimal phospholipid-dependent catalytic activity: conventional or classic (α, βI,

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Abbreviations: AP-1, activator protein 1; PKC, protein kinase C; DAG, diacylglycerol; ROS, reactive oxygen species; BSO, buthionine-*S*,*R*-sulphoximine; DEM, diethylmaleate.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00507-0

βII, γ) PKCs, which are calcium-dependent and stimulated by second-messenger diacylglycerol (DAG); novel (δ, ε, η, θ) PKCs, which are calcium-independent but stimulated by DAG; and atypical (ι, λ, ζ) PKCs, which require neither calcium nor DAG for optimal activity [10].

PKC isoforms contain unique structural features that are susceptible to oxidative modification: the N-terminal regulatory domain contains cysteine-rich motifs which are readily oxidized by oxidants and thereby stimulate PKC activity [11], while the C-terminal catalytic domain contains several reactive cysteines that are modified by various antioxidants (selenocompounds and Vitamin E analogues) [12] and thereby decrease PKC activity.

Our previous *in vivo* and *in vitro* studies [13–16] demonstrated that pro-oxidant doses of carbon tetrachloride and ethanol inhibit hepatic PKC activity and induce subsequent proteolytic degradation. On the other hand, low levels of these oxidants stimulated liver kinase activity. We have recently shown that pathophysiological concentrations of 4-hydroxynonenal specifically activate the PKC-β isoform, inducing marked stimulation of cathepsin D transport towards the endosomal-lysosomal compartment and exocytosis of the mature form from rat hepatocytes [17]. 4-Hydroxynonenal-mediated activation of PKC-β isoenzymes increases the release of the chemokine monocyte chemotactic protein-1 [18] from macrophage-like cell line and exerts a regulatory effect on the production of amyloid-β protein by human neurons [19].

The aim of the present study was to identify the possible molecular mechanisms by which oxidative stress might be translated into cellular responses. First, we investigated the role of PKC isoforms as cellular sensors able to intercept intracellular redox changes and promote an adaptation to injury. Subsequently, we tried to demonstrate the involvement of reactive oxygen species (ROS) production in cellular reaction mediated by alterations of PKC-dependent signaling.

2. Results

2.1. Modulating effect of cellular GSH levels on the activity of specific isoforms

Given the central role of GSH in cellular redox homeostasis, it is important to determine its influences on PKC redox modifications. High concentrations of GSH (>3 mM) have been demonstrated to inhibit PKC isoenzymes *in vitro* through a non-redox mechanism, since the depletion of thiol antioxidant *per se* may create a permissive environment for PKC activity [20,21].

To investigate the effects of GSH depletion on PKC, we used two different compounds: buthionine-*S*,*R*-sulphoximine (BSO), a selective and irreversible transition state inhibitor of γ-glutamylcysteine synthetase, the enzyme catalyzing the first step in glutathione biosynthesis, and diethylmaleate (DEM), which is able to reduce cellular stores of GSH through direct interaction.

Isolated rat hepatocytes were incubated for 90 min in the presence of different concentrations of L-BSO (0.25–1 mM), which caused a progressive loss of reduced GSH up to 48% at 1 mM BSO. Exposure to DEM concentrations (50, 100 μM) lowered GSH by 30–60% in a dose-dependent way [22] (Fig. 1). Neither treatment significantly modified GSSG level.

In this context, the enzymatic activity of classic PKCs was depressed by 55–75% at 0.5–1 mM BSO and 50–100 μM DEM while the amount of the protein remained

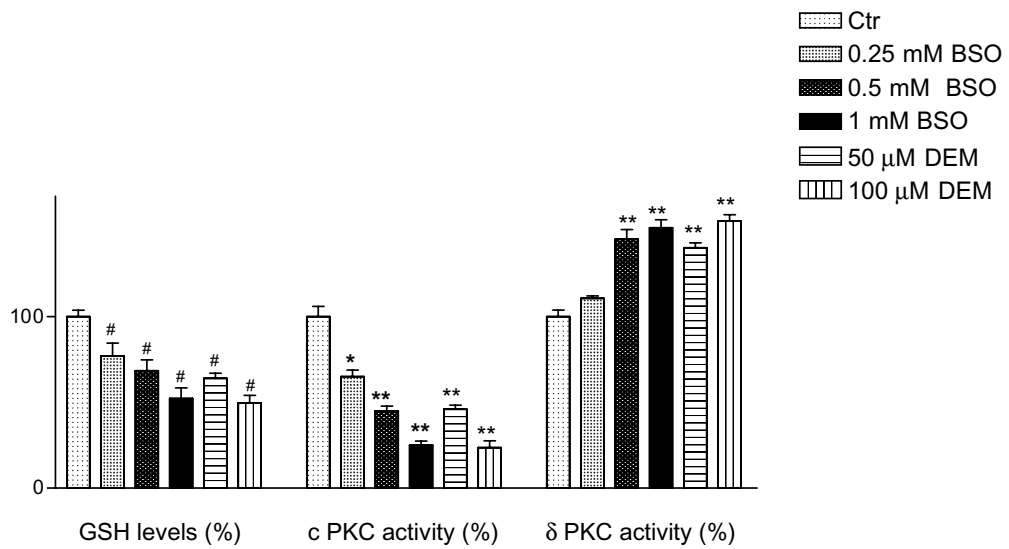

Fig. 1. Glutathione levels (expressed as % vs. control = 15.8 ± 1.0 nmol/10^6 cells) and PKC isoform activities of control, BSO- and DEM-treated isolated hepatocytes. All PKC isoforms were immunoprecipitated with specific antibodies and their enzymatic activities were assayed with H1 histone as substrate in the presence (cPKCs) or absence (PKC-δ) of calcium. Proteins were separated by 12.5% SDS–polyacrylamide gel and the relative intensity of phosphorylated H1 histone was measured by densitometric analysis [22]. $^{*}P < 0.05$ vs. control; $^{**}P < 0.001$ vs. control; $^{\#}P < 0.01$ vs. control.

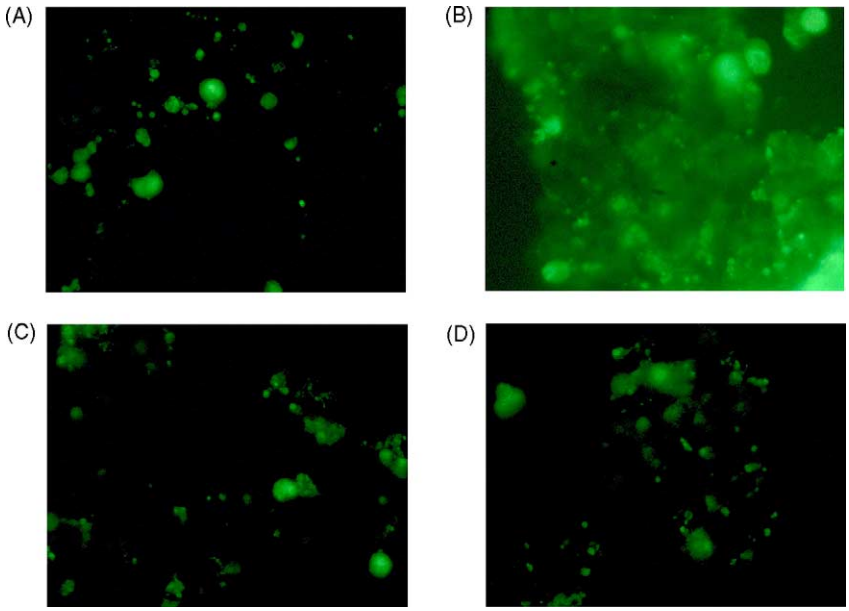

Fig. 2. ROS detection in SK-N-BE-2-C cells. (A) Control; (B) 1 mM BSO; (C) 3 μM rottlerin; (D) 3 μM rottlerin + 1 mM BSO. Cells were incubated for 20 min at 37° with 20 μM 2′,7′-dichlorofluorescein diacetate, a cell-permeable, non-precursor of dichlorofluorescein that can be used as an intracellular probe for oxidative stress. Accumulation of dichlorofluorescein in cells was measured by an increase in fluorescence at 530 nm when the sample was excited at 485 nm.

unchanged. Interestingly, the functional activity of the δ isoform increased 1.5-fold at 0.5–1 mM BSO and 50–100 μM DEM (Fig. 1), without changes in protein levels.

To further investigate molecular mechanisms of cell response to glutathione loss, SK-N-BE-2-C cells, a human neuroblastoma cell line, were exposed to a single dose of BSO (1 mM), which has been demonstrated to be efficacious in the clinical treatment of neuroblastoma [23–26].

A parallel decrease (10%) in GSH and GSSG levels, without ROS production, was detected after 1-hr treatment and was associated to a transient stimulation (1.0- to 1.5-fold) of the functional activities of all PKC isoforms. Longer times (24 hr) of BSO treatment were characterized by marked (44%) ROS generation, and by a shift in the GSH/GSSG balance; these oxidative events were accompanied by selective stimulation (5-fold) of PKC-δ activity and inactivation (60%) of PKC-α.

2.2. PKC-δ: possible links between GSH loss and the apoptotic process

Treatment of isolated rat hepatocytes with BSO or DEM, at concentrations able to activate PKC-δ, induces a 15–20% increase in the number of apoptotic cells [22]. To investigate the role of this isoenzyme in BSO-induced apoptosis, we applied rottlerin, a selective PKC-δ inhibitor, and found that it was able to counteract the apoptotic process and activator protein 1 (AP-1) activation. AP-1 is a redox-sensitive transcription factor and plays an important role in cellular response to oxidative stress; many reports have connected the increase in AP-1 activation with apoptosis in various conditions of cellular stress: ethanol intoxication, dexamethasone treatment, and derangement of the microtubular network [27–30].

Similarly, BSO-induced GSH loss in the neuroblastoma cell line, which was associated with ROS production and PKC-δ activation, induced morphological changes characteristic of early apoptosis (20% annexin-V-positive cells). Rottlerin pretreatment was able to prevent ROS generation and apoptosis of neuroblastoma cells (Fig. 2).

3. Discussion

GSH depletion and direct oxidative damage are two different but related processes influencing the oxidation state of many proteins involved in the signal transduction pathways that regulate cell growth [31]; among these signaling proteins, PKCs are a family of isoenzymes sensitive to oxidative stress, and therefore able to act as molecular intermediates that intercept intracellular redox changes and contribute to promote cell response.

Moreover, many studies [32–35] have shown that GSH loss can be an initial injury able to trigger a cascade of events (ROS generation, and probably mitochondrial dysfunction) which could lead to cell injury and finally to apoptosis [36–39]. Recently, ROS production, following glutathione depletion, has been demonstrated to represent a crucial event in the commitment to apoptosis of a human B lymphoma cell line [40]. ROS are also associated with cellular proliferation, differentiation, and may play a dual role in apoptosis, either as activators of permeability transition or as a consequence of this transition, depending on the death stimulus [41,42].

Our findings provide evidence that experimental GSH loss determines a modulation of selective PKC isoform activities both in isolated rat hepatocytes and neuroblastoma cells, thus generating a similar cellular response at different times.

In a continuous cell line, such as neuroblastoma cells, we observed an early parallel decrease in GSH and GSSG levels after 1-hr BSO exposure, associated to a transient stimulation of the functional activities of all PKC isoforms studied. This phenomenon may be an intermediate step in an adaptive cell response contributing to cell survival.

When BSO exposure was prolonged (24 hr), marked ROS production and a shift in the GSH/GSSG balance were found. This cell redox state was associated to an increase in PKC-δ activity and to inactivation of PKC-α leading to apoptosis commitment.

Analogous changes in PKC isoform activities and the appearance of apoptotic features were observed in isolated rat hepatocytes treated with 1 mM BSO for 90 min, which is an optimal exposure time for maintaining the viability of control cells. This rapid response is probably due to the short lifespan of hepatocyte suspensions, which determines a greater sensitivity to GSH depletion.

The different time-related modulation of PKC activities observed in these two different experimental systems may depend on cellular context. Some cells (neuroblastoma) may adapt, for a restricted period, to a condition of GSH loss by setting up molecular pathways that concur to maintain an equilibrated redox environment and are able to prime the apoptotic process when the consequences of redox imbalance generate irreversible cell damage. In other cells (hepatocytes) redox changes prime more rapid apoptosis, thereby quickening the molecular steps involved in this pathway.

Taken together, these results show that the functional inactivation of classic PKCs and the increase in the activity of PKC-δ were accompanied by the appearance of morphological apoptotic features.

Finally, by applying the same stimulus (GSH depleting agents) to two different experimental systems, we have shown that cells react by triggering apoptosis through the same PKC-dependent signaling pathway. This hypothesis is sustained by the fact that the use of rottlerin revealed PKC-δ activation to be a limiting step in the promotion of apoptosis (Fig. 3). Rottlerin has been widely used in many different cellular systems, and only recently it has been reported [43] that it might act as a mitochondrial uncoupling agent, decreasing the ATP level and indirectly limiting cellular reactions.

The preventive action of rottlerin is not only directly related to inactivation of the δ isoform, as demonstrated in hepatocytes, but also to an inhibitory effect on oxyradical production, as shown in neuroblastoma cells.

Our findings on the behavior of classical PKCs are in agreement with other papers; it has been reported that the apoptosis inducer ceramide induces inactivation of PKC-α [44] and the overexpression of exogenous PKC-α results in its increased mitochondrial localization, increased Bcl-2 phosphorylation, and suppression of apoptosis [45].

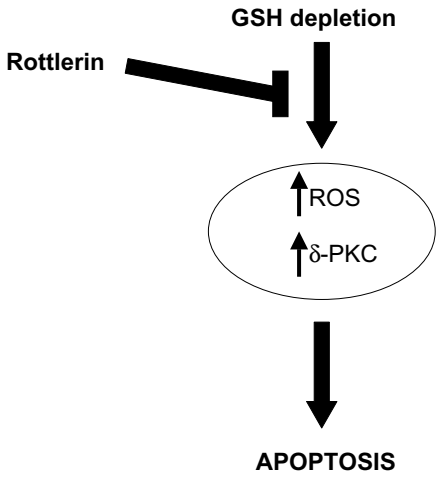

Fig. 3. Proposed model for rottlerin-mediated preventive effects. This compound, able to counteract ROS levels and PKC-δ activation, can inhibit apoptotic process, demonstrating the importance of these events in morphological changes of GSH-depleted cells.

Moreover, the identification of pro- and anti-apoptotic isoforms suggests that PKC may function as a molecular sensor, promoting cell survival under favorable conditions and killing off abnormal or damaged cells [9].

PKC-δ has been demonstrated to be involved in both apoptotic and anti-apoptotic phenomena, and may well represent a switch between these two pathways; the cellular commitment is probably related to PKC-δ localization, its phosphorylation state, and interaction with other signaling proteins [46–48].

In this study, we have shown the correlation between PKC-δ activation and apoptosis in both cell models by using rottlerin, and in neuroblastoma cells we have demonstrated that δ isoform activity is related to ROS production. However, the relationship between these events is not completely clear. Our unpublished data indicate that PKC-δ localizes at the mitochondria and there works as a cellular sensor able to intercept redox changes contributing to apoptotic machinery.

Indeed, it has been demonstrated that PKC-δ plays multiple roles in apoptosis upstream and downstream of mitochondria [9,49,50] and it is unknown whether it exerts a direct effect at the level of the mitochondria or an indirect effect *via* regulation of another component of the apoptotic pathway.

Several lines of evidence suggest that oxidative stress and glutathione depletion play a key role in the pathophysiology of many age-related degenerative diseases, characterized by perturbations of normal cell homeostasis. Given the role of PKC-δ in the induction of early apoptotic changes and considering that it is specifically activated by

a condition of oxidative stress and antioxidant decline, the modulation of PKC-δ activity might be useful in the clinical approach to many human diseases and in the identification of new therapeutic strategies.

Acknowledgments

This work was supported by MURST ''COFIN 2000: Molecular signaling by GSH depletion'', MIUR ''COFIN 2001: Biomarkers of aging'', and FIRB 2001 Poli G. (Operative Unit Marinari U.M.).

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