ELSEVIER

Biochemical Pharmacology 62 (2001) 319–328

Biochemical Pharmacology

Control of survival of proliferating L1210 cells by soluble guanylate cyclase and p44/42 mitogen-activated protein kinase modulators

Flavio Flamigni*, Annalisa Facchini, Ivana Stanic, Benedetta Tantini, Francesca Bonavita, Claudio Stefanelli

Dipartimento di Biochimica G. Moruzzi, Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

Received 11 November 2000; accepted 16 January 2001

Abstract

Intracellular signaling pathways involved in the survival of proliferating L1210 leukemia cells were investigated by using specific modulators. Among the various inhibitors tested, only 1*H*-[1,2,4]oxadiazole [4,3-*a*]quinoxalin-1-one (ODQ), a soluble guanylate cyclase (sGC) inhibitor, was found to induce a marked increase in caspase activity, which was associated with a loss of cell viability and a reduction in cGMP content. ODQ also provoked the processing of caspases-3 and -9, release of cytochrome *c* and, as early events, reduction of Bcl-2 content and dephosphorylation of Bad at Ser 112. Furthermore, YC-1, an sGC activator, and 8-Br-cGMP, a cell-permeant analogue of cGMP, exerted some protection against various apoptotic stimuli, such as serum deprivation or spermine accumulation. Although PD98059 (2′-amino-3′-methoxyflavone), an inhibitor of the p44/42 mitogen-activated protein kinase (MAPK) pathway, did not increase basal caspase activity, and ODQ did not affect p44/42 MAPK phosphorylation significantly, phorbol myristate acetate stimulated p44/42 MAPK and reduced caspase activation induced by ODQ, serum deprivation, and spermine in a p44/42-dependent manner. SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1*H*-imidazole), a p38 MAPK inhibitor, also partially protected against ODQ-induced apoptosis by increasing p44/42 MAPK phosphorylation. In conclusion, these results suggest that sGC may be relevant both for survival of L1210 cells under basal growing conditions and for protection against various apoptotic stimuli. p44/42 MAPK activation may also confer some protection from apoptosis, but apparently through a pathway largely independent of cGMP.

Keywords: ODQ; Mitogen-activated protein kinase; Caspase; Apoptosis; Signal transduction; Leukemia

1. Introduction

Inappropriate regulation of apoptosis is associated with a variety of diseases [1–3]. In particular, the failure of dividing cells to initiate apoptosis in response to DNA damage has been implicated in the development and progression of cancer and leukemia. On the other hand, since apoptosis represents an active, gene-directed mechanism, it should eventually be possible to control this process for therapeutic purposes. Indeed, the efficacy of chemotherapeutic drugs may rely on their ability to provoke apoptosis preferentially in neoplastic cells.

The activation of a family of aspartate-specific proteases termed caspases [1] is recognized as a critical event in the execution phase of apoptosis, although, quite recently, even caspase-independent pathways have been described in some models [4]. Initiator caspases, such as caspases-8 and -9, activated by death receptor- and mitochondria-related events respectively, can induce the cleavage/activation of effector caspases, such as caspases-3 and -7, eventually leading to the disassembly of cellular structures characteristic of apoptotic cell death.

Rigorous signaling requirements and a complex network of regulatory molecules appear to maintain a tight control of the activation status of caspases. The role played by differ-

* Corresponding author. Tel.: +39 051 209 1216; fax: +39 051 209 1224.

E-mail address: fflamign@biocfarm.unibo.it (F. Flamigni).

Abbreviations: L-NMMA, N^G-monomethyl-L-arginine; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazole [4,3-*a*]quinoxalin-1-one; PI3K, phosphoinositide 3-kinase; PKA, cyclic AMP-dependent protein kinase; PMA, phorbol myristate acetate; sGC, soluble guanylate cyclase; and SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00646-3

ent signal transduction molecules in the control of cell survival is the subject of intense discussion. In this regard, key proteins may be PI3K, its downstream effector Akt, and p44/42 MAPK, which are activated by growth stimuli and generally considered as prosurvival mediators [5–7]. In contrast, p38 MAPK, another member of the MAPK family preferentially activated by cytokines and stress stimuli, appears to favor apoptosis in many cell models [7–9]. NOS is another important signaling enzyme that has been reported to have an impact on cell survival [3,9,10]. Although in many experimental systems NO may favor cell death and even apoptosis, in others NO production may exert a protective effect, either through sGC-dependent or -independent mechanisms [3,11]. Cyclic AMP-dependent pathways may also regulate cell death and survival [9,12]. However, these signaling proteins should not be viewed as located in completely separated pathways, and they may actually interact in a complex and cell-specific manner.

Survival-inducing and death-promoting pathways may be integrated at the level of Bcl-2 family proteins [13]. The balance between prosurvival and proapoptotic members of this family can regulate critical events in apoptosis, such as cytochrome *c* release from mitochondria and the activation of caspase-9 and downstream caspases.

The intracellular pathways that control apoptosis of leukemia cells are only partially known. In the present study, we used specific inhibitors of key signaling proteins to define the pathways involved in the survival of proliferating L1210 leukemia cells. We found that ODQ, an sGC inhibitor [14], can cause a marked increase in caspase activity and cytotoxicity, whereas pharmacological stimulation of sGC or p44/42 MAPK can exert a protective effect versus various apoptotic stimuli.

2. Materials and methods

2.1. Materials

Anti-phospho-specific and total Bad, p44/42 MAPK, and p38 MAPK antibodies were purchased from New England Biolabs, Inc. Anti-caspase-9 p10 (H-83), caspase-3 (H-277), cytochrome *c*, Bcl-2, Bax, e(endothelial), i(inducible), and n(neuronal)NOS antibodies were obtained from Santa Cruz. Other antibodies were from Pharmingen. ODQ, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002),2′-amino-3′-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2- (4-methylsulfonylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), 8-Br-cGMP, 2′,5′-dideoxyadenosine, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A), SNAP, PMA, chelerythrine, anisomycin, and *N*-hexanoyl-D-sphingosine (ceramide) were obtained from Alexis Biochemicals. L-NMMA, spermine, etoposide, and staurosporine were obtained from Sigma.

2.2. Cell culture and treatments

L1210 (lymphocytic leukemia) cells were routinely grown in RPMI-1640 medium supplemented with fetal bovine serum as previously reported [15,16]. For experiments, quiescent cells were stimulated to growth by dilution at $2–3 \times 10^5$/mL in fresh medium containing serum. After 24 hr, cells were treated with the indicated agents. Unless stated otherwise, compounds were added at the following final concentrations: 100 μM ODQ, 20 μM LY294002, 50 μM PD98059, 10 μM YC-1, 100 μM L-NMMA, 100 μM SNAP, 10 μM SB203580, 1 mM 8-Br-cGMP (treatment repeated after 10 hr of incubation), 100 ng/mL of PMA, 0.5 mM 2′,5′-dideoxyadenosine, 10 μM MDL-12,330A, 5 μM chelerythrine, 1 μM staurosporine, 1 μg/mL of anisomycin, 20 μM etoposide, 20 μM ceramide, and 5 mM spermine. At the time indicated after treatment, cells were harvested by centrifugation and washed with PBS. Cell viability was determined by trypan blue exclusion by counting cells with a hemocytometer and was calculated as the ratio of unstained living cells to the total amount of cells.

2.3. Determination of caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) during a 15-min incubation at 37°, as detailed elsewhere [17]. Since the DEVD sequence represents a substrate for caspase-3 and other members of the caspase family [18], this activity will be referred to as caspase activity. Caspase activity is expressed as units/mg protein, where 1 unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute.

2.4. cGMP determination

Cell pellets were deproteinized with 0.6 M $HClO_4$, frozen, and thawed twice, and then centrifuged at 15,000 *g* for 10 min. The pellets were used for protein determination, while the supernatants were neutralized with 5 M K_2CO_3 and then assayed for cGMP content by a radioimmunoassay kit (Amersham). cGMP content is expressed as pmol/mg protein.

2.5. Western blot analyses

Western blot of total and phosphorylated p44/42 MAPK and p38 MAPK was carried out as described previously [15,19]. In the case of caspase-3, caspase-9, Bcl-2, eNOS, iNOS, and nNOS, total cell extracts were obtained as described for p44/42 MAPK [15], and aliquots corresponding

to 50 μg protein were analyzed by SDS–PAGE (12% gel) according to the manufacturer's instructions. Briefly, after blotting, the nitrocellulose membrane was blocked with 5% non-fat milk for 1 hr, washed with Tris-buffered saline (1 mM Tris, pH 8, 15 mM NaCl), and probed overnight at 4° (caspase-3 and caspase-9) or at room temperature for 90 min (Bcl-2 and NOS isoforms) with specific primary antibody. After a further wash, the membrane was incubated with the secondary antibody for 30 min. For Western blot analyses of phospho-Bad, aliquots corresponding to 500 μg protein were incubated with anti-Bad antibody overnight at 4° and then with protein A-Sepharose for a further 1 hr at room temperature. The immunoprecipitate was resuspended with loading buffer (0.25 M Tris–HCl, pH 6.8, 2% SDS, 5% glycerol, 0.002% bromphenol blue, 4% β-mercaptoethanol) and then boiled for 3 min. The samples were analyzed using SDS-PAGE (15% gel). Separated proteins were transferred onto a nitrocellulose membrane for 1 hr. The membrane was saturated with 5% non-fat milk for 1 hr and then incubated with phospho-specific Bad (Ser 136) and Bad (Ser 112) antibodies overnight at 4°. Bands were revealed by the Amersham ECL detection system. The release of cytochrome *c* from mitochondria was examined by Western blot in cytosolic extracts obtained by subcellular fractionation [16,20]. The content of Bax, Bim, Bid, and Bik was determined in cytosolic and mitochondrial fractions [16,20] by Western blotting carried out as described above for Bcl-2. Western blot experiments shown in the figures were repeated at least twice with similar results.

3. Results

3.1. Caspase activation and cytotoxicity by ODQ, an sGC inhibitor

In order to identify some factors that govern the survival of leukemia cells, a number of agents known to interfere with specific signaling pathways were tested for their ability to induce caspase activity in proliferating L1210 cells. A 24-hr treatment with PD98059 [21] or LY294002 [22], which inhibit p44/42 MAPK kinase (MEK1/2) and PI3K, respectively, did not increase caspase activity (Fig. 1A), indicating that these pathways are not required for survival of L1210 cells under normal growing conditions. The adenylate cyclase inhibitors 2′,5′-dideoxyadenosine and MDL-12,330A were also ineffective (not shown). In contrast, ODQ, an inhibitor of sGC, elicited a marked enhancement of caspase activity at 100 μM (Fig. 1A). This increase was reduced by about 40% by co-treatment with Br-cGMP, a cell-permeant cGMP analogue. It should be noted that L-NMMA, a NOS inhibitor, was not effective, and we failed to detect NOS isoforms in L1210 extracts by Western blot. Thus, it is unlikely that L1210 cells express *NOS* genes under these experimental conditions. Moreover, addition of SNAP, an NO donor, increased caspase activity somewhat,

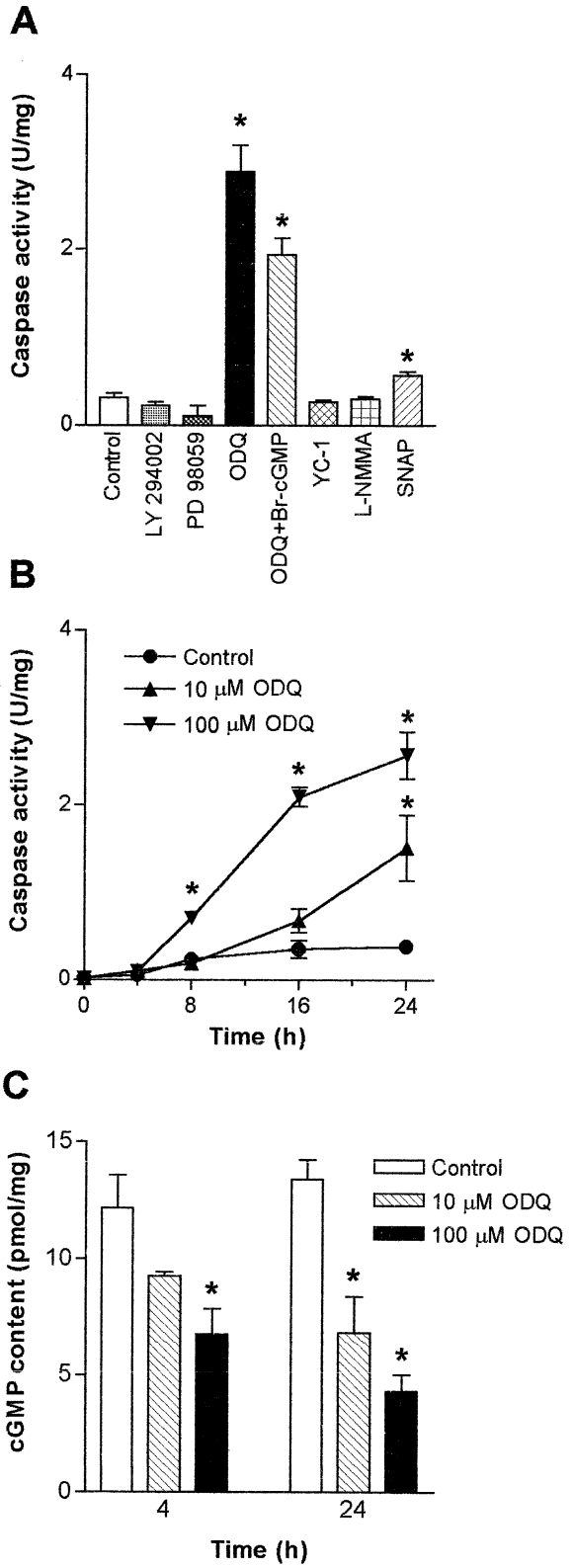

Fig. 1. ODQ induces caspase activation and reduces cGMP content in L1210 cells. (A) L1210 cells were treated with the indicated compounds. After 24 hr, cells were harvested and assayed for caspase activity. (B) L1210 cells were treated with 10 or 100 μM ODQ, harvested at the time indicated, and assayed for caspase activity. (C) L1210 cells were treated with 10 or 100 μM ODQ and, after 4 or 24 hr, harvested and assayed for cGMP content. Results represent means ± SEM (N = 3), *$P < 0.05$ vs control.

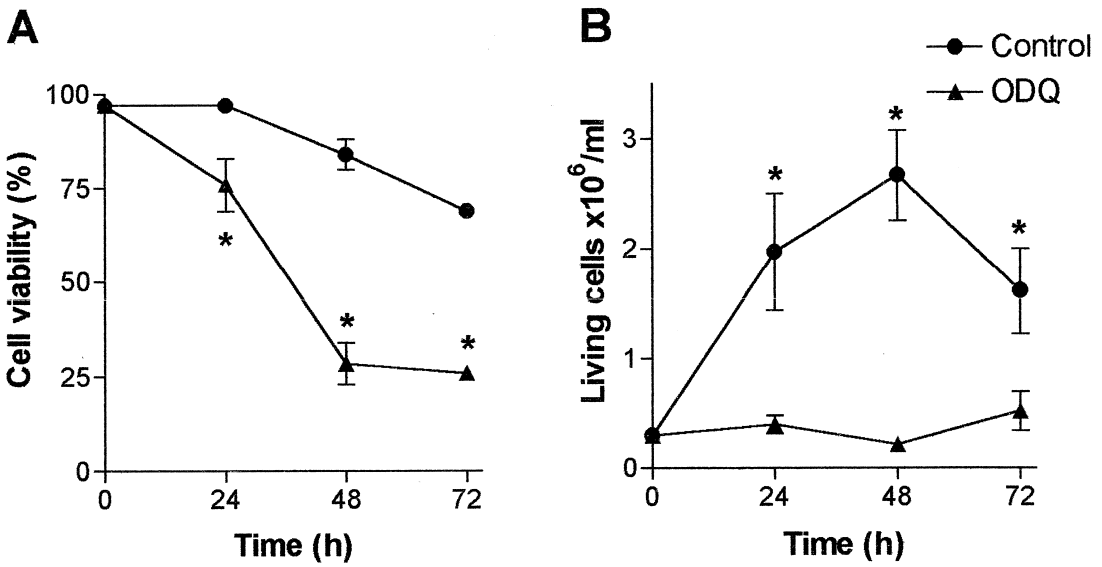

Fig. 2. Effect of ODQ on cell viability and growth. (A) L1210 were treated with 100 μM ODQ and, at the time indicated after treatment, cell viability was assessed by trypan blue exclusion. (B) L1210 were treated with 100 μM ODQ and counted at the time indicated. Results represent means ± SEM (N = 3), *$P < 0.05$ vs control.

but YC-1, an sGC activator that acts independently of NO [23], did not affect caspase activation.

The time-course and dose-dependence of caspase activation following ODQ is depicted in Fig. 1B. After 100 μM ODQ, a progressive increase in the enzyme activity was observed, starting from 8 hr. ODQ at 10 μM elicited a delayed and less marked induction of caspase activity. Interestingly, in this concentration range, ODQ provoked a dose-dependent reduction in the content of cGMP (Fig. 1C). In particular, the cGMP level fell to 51% and 32% of control following a 24-hr treatment with 10 and 100 μM ODQ, respectively. The residual content of cGMP may be due to corpuscolate GC activity, which has been shown to be insensitive to ODQ [14]. Altogether, these results suggest that basal sGC activity, but not NOS activity, may be required for survival of L1210 cells. Indeed, the caspase activation evoked by ODQ was accompanied by a significant reduction in cell viability (Fig. 2A) and block of cell growth (Fig. 2B). This reduced cell viability could reflect a high turnover and therefore an increased number of dead cells. This would also correspond with an unchanged number of living cells.

3.2. Effect of ODQ on caspase processing and Bcl-2 family members

Activation of members of the caspase family involves proteolytic cleavage of the proenzyme into smaller subunits [1]. Fig. 3 shows that ODQ caused the processing of caspases-9 and -3, an initiator and an effector caspase respectively, as evidenced by the appearance or increase of the proteolytic fragments. Moreover, ODQ induced the release of cytochrome *c* into cytosol, a critical event for the activation of procaspase-9 [13]. Since Bcl-2 family proteins

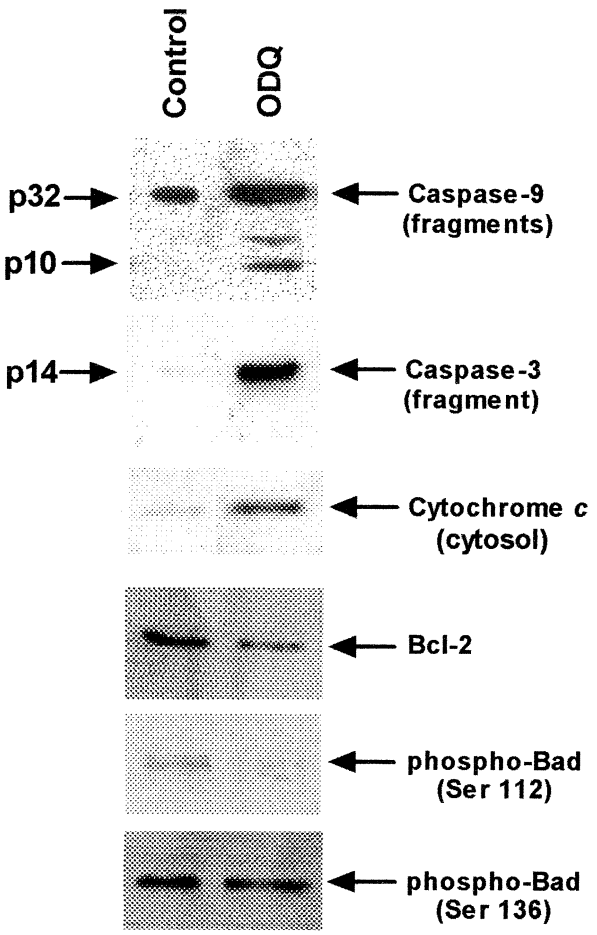

Fig. 3. Effect of ODQ on caspase-9 and -3 processing, cytochrome *c* release, Bcl-2 content, and Bad phosphorylation. L1210 cells were incubated with 100 μM ODQ and harvested after 16 hr (in the case of caspase-9, caspase-3, and cytochrome *c*) or 4 hr (in the case of Bcl-2 and Bad). Cell extracts were analyzed by Western blotting by using specific antibodies as described in Materials and Methods.

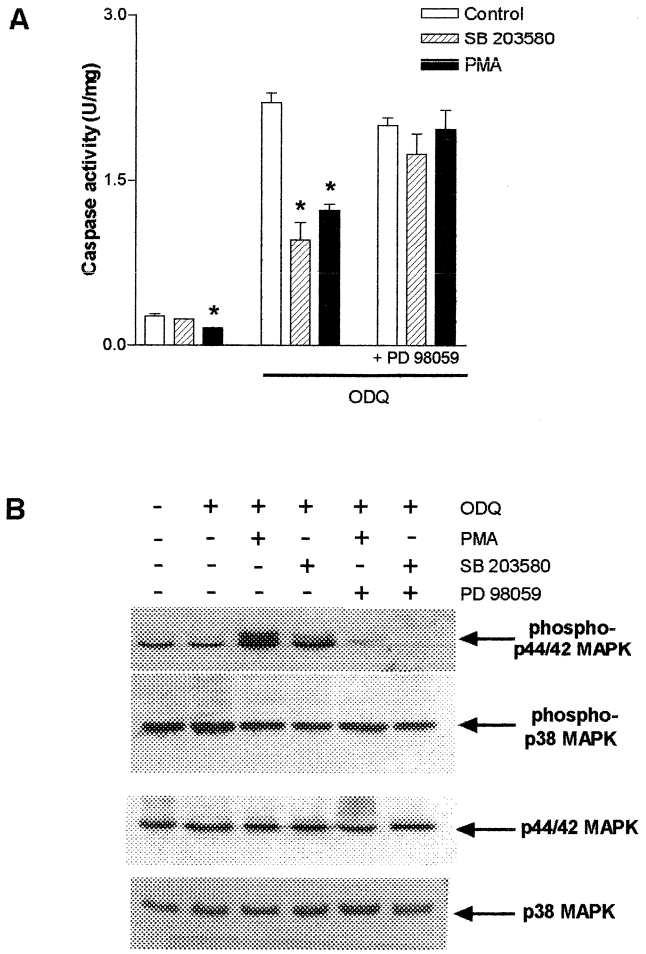

Fig. 4. PMA and SB203580 reduce ODQ-induced caspase activation and stimulate p44/42 MAPK phosphorylation. (A) L1210 cells were incubated for 24 hr in the presence of PD98059, SB203580, PMA, and ODQ (100 μM) as indicated, and assayed for caspase activity. Results represent means ± SEM (N = 3), *$P < 0.05$ vs corresponding control. (B) L1210 cells were incubated in the presence of the indicated compounds for 4 hr and analyzed for the amount of phosphorylated or total p44/42 and p38 MAPKs by Western blotting. The small differences in band intensity between phospho-p38 MAPK samples were not reproducible.

play a critical role in the decision of the cell to die or survive by acting at multiple levels with a prompt impact on cytochrome *c* release and caspase activation [13], we investigated the effect of ODQ on some members of this protein family (Fig. 3). We observed a reduction in Bcl-2 content, already evident after 4 hr of treatment. Bcl-2 appeared to be associated with the mitochondrial fraction. The amount of phosphorylated Bad (at Ser 112) was also reduced, whereas phosphorylation of Bad at Ser 136 was not (Fig. 3). We could not observe any significant variation in the level and translocation to mitochondria of Bax and Bid, which have been implicated in some models of apoptosis [13]. In addition, we failed to show detectable amounts of Bik and Bim, two other proapoptotic family members, by Western blot analysis (not shown).

3.3. Reduction of ODQ-induced caspase activation by stimulation of p44/42 MAPK

PMA, a phorbol ester and activator of classical and novel protein kinase C isoforms, and SB203580, a p38 MAPK inhibitor, have been reported to modulate apoptosis in different models [7–9]. The possible involvement of p44/42 and p38 MAPKs in the ODQ-induced apoptosis of L1210 cells was evaluated by testing these pharmacological agents on caspase activation. Fig. 4A shows that both PMA and SB203580 significantly reduced the induction of caspase activity by ODQ and that their effect was prevented by the simultaneous addition of the p44/42 MAPK kinase inhibitor PD98059. Furthermore, Western blot analysis (Fig. 4B) showed that PMA and, even if less effectively, SB203580, caused p44/42 MAPK activation in the presence of ODQ, as evidenced by an increase in the amount of phosphorylated forms. PMA and SB203580 increased p44/42 MAPK activation even when given alone (not shown), whereas ODQ alone was not effective (Fig. 4B). As expected, p44/42 MAPK activation following PMA or SB203580 was strongly inhibited by PD98059. Instead, the phosphorylation of p38 MAPK was not markedly affected by these drugs. It should be noted that phosphorylation of MAPKs was monitored after 4 hr of treatment, i.e. when caspase activity was not significantly affected by ODQ (Fig. 1B). Furthermore, the total amount of p44/42 and p38 MAPK was not modified by these agents at this time (Fig. 4B).

3.4. Effect of cGMP-elevating agents on caspase activation induced by various apoptotic stimuli

In order to assess whether sGC can modulate apoptosis induced by other stimuli, L1210 cells were serum-deprived or treated with spermine in the presence of the sGC activator YC-1 or the cell-permeant cGMP analogue Br-cGMP. Caspase activity was induced by serum deprivation of growing L1210 cells within 1 day (Fig. 5A). Fig. 5B shows that both YC-1 and Br-cGMP reduced caspase activation. The simultaneous treatment with PD98059 could not prevent the YC-1 effect on caspase activity. PMA was able to reduce the increase in caspase activity elicited by serum deprivation, and this effect was partially inhibited by PD98059. We have recently shown that exogenously added spermine causes caspase activation of leukemia cells and release of cytochrome *c* from mitochondria both in intact cells [16] and in a cell-free model of apoptosis [20]. The time-course of caspase activation following 5 mM spermine in L1210 cells is reported in Fig. 5C, and shows a high caspase activity after 16 hr. The addition of Br-cGMP, YC-1, or PMA even reduced caspase activation induced by a 16-hr exposure of the cells to spermine (Fig. 5D), and again PD98059 inhibited the effect of PMA significantly, but not that of YC-1. In accordance with these results, Fig. 5E shows that YC-1, differently from

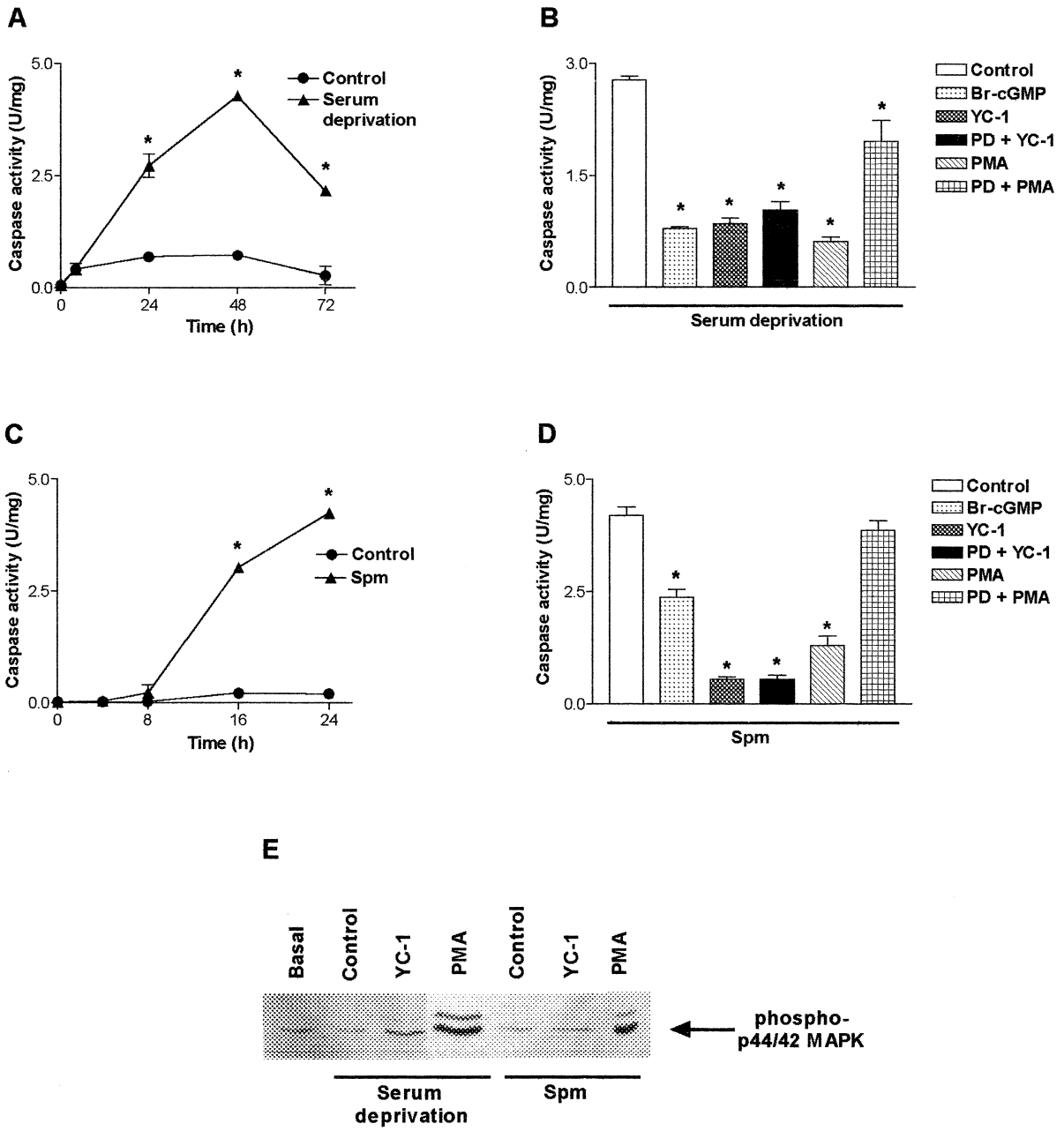

Fig. 5. cGMP-elevating agents and PMA reduce caspase activation induced by serum deprivation or spermine treatment. (A) L1210 cells were shifted to a serum-free medium and assayed for caspase activity at the time indicated. (B) L1210 cells were shifted to a serum-free medium and immediately incubated in the presence of 8-Br-cGMP, YC-1, PD98059 (PD), and PMA as indicated. After 24 hr, cells were harvested and assayed for caspase activity. (C) L1210 cells were treated with 5 mM spermine (Spm) and assayed for caspase activity at the time indicated. (D) L1210 cells were incubated with 5 mM spermine in the presence of the indicated compounds. After 16 hr, cells were harvested and assayed for caspase activity. Results represent means ± SEM (N = 3), $^*P < 0.05$ vs control. (E) L1210 cells were incubated in a serum-free medium or with 5 mM spermine (Spm) in the presence of the indicated compounds. After 4 hr, cells were harvested and the amount of phosphorylated p44/42 MAPK was evaluated by Western blotting.

PMA, did not stimulate p44/42 MAPK phosphorylation markedly either in serum-deprived or spermine-treated cells after 4 hr of treatment. PD98059 abolished p44/42 MAPK phosphorylation following YC-1 or PMA (not shown). Finally, as shown in Fig. 6, YC-1 was able to afford some protection against other known apoptotic stimuli, i.e. chelerythrine, staurosporine, anisomycin, etoposide, or ceramide, as judged by the reduction in caspase activation, although to a various degree. In L1210-DR cells, osmotic shock deregulated polyamine uptake allowing intracellular accumulation of spermine, even when added at low concentrations; under these conditions, addition of micromolar spermine is enough to trigger caspase activation [16]. Fig. 6 shows that YC-1

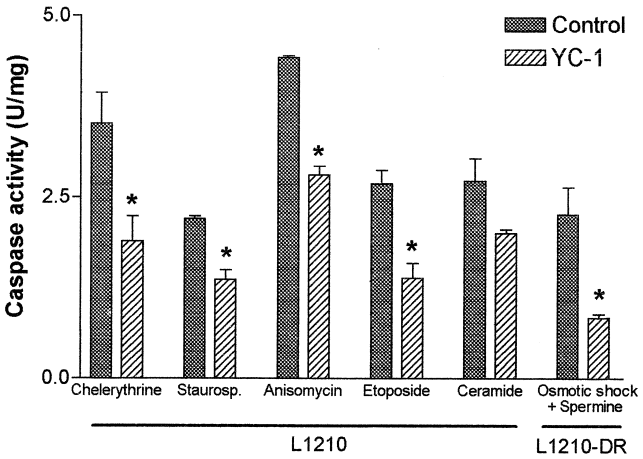

Fig. 6. YC-1 reduces caspase activation induced by various apoptotic stimuli. L1210 cells were treated with various apoptotic stimuli in the presence or absence of YC-1. After 4-hr incubation (in the case of chelerythrine and staurosporine) or 20-hr incubation (in the case of anisomycin, etoposide, and ceramide), cells were harvested and assayed for caspase activity. L1210-DR cells were incubated under hypo-osmotic conditions for 24 hr and then further incubated with 100 μM spermine for 8 hr in the presence or absence of YC-1 before assaying for caspase activity. Results represent means ± SEM (N = 3), $*P < 0.05$ vs control.

was even effective in reducing caspase activation elicited in these cells by combined osmotic shock and 100 μM spermine.

4. Discussion

The intracellular signaling pathways involved in survival of leukemia cells remain poorly understood. According to studies performed with a large variety of cell types, signaling proteins that may convey death/survival signals include MAPKs, PI3K, NOS, sGC, and adenylate cyclase [3–11]. By treating proliferating L1210 leukemia cells with specific inhibitors of these enzymes, we identified ODQ, an sGC inhibitor, as an agent able to induce a marked increase in caspase activity and loss of cell viability.

The proapoptotic effect of ODQ, which correlated with a decrease in the cGMP level, suggests that sGC is important for the survival of leukemia L1210 cells, although other mechanisms, not related to sGC inhibition, may not be ruled out by the present study. However, the involvement of sGC in the modulation of apoptosis of L1210 cells is also supported by the experiments with YC-1 and Br-cGMP, which indicate that cGMP may confer some protection versus a wide range of apoptotic stimuli. Indeed, several authors have shown that the antiapoptotic function shown by NO in some cell systems is mediated, at least partially, by an NO-dependent generation of intracellular cGMP. Such cell systems include B lymphocytes [24], embryonic motor neurons [25], hepatocytes [11], PC12 cells [26], and monocytic U937 cells [27]. However, little is known about the mechanism(s) by which cGMP prevents apoptosis signaling. Furthermore, in some cell models, such as endothelial cells [28], adult cardiomyocytes [29] or primary osteoblasts [30], cGMP may behave as a proapoptotic mediator of NO.

It has been reported that B-cell chronic lymphocytic leukemia cells express a spontaneously inducible NOS isoform displaying antiapoptotic activity [31]. However, in our model, the NOS inhibitor L-NMMA caused no effect, and no NOS isoform was detectable. In fact, SNAP, an NO donor, increased caspase activity significantly. These data are consistent with the reported lack of NOS activity in L1210 cells [32], which stop growing when co-cultured with NO-generating macrophages.

ODQ-induced caspase activity of L1210 cells was associated with the release of cytochrome *c* from mitochondria and the proteolytic processing of caspases-9 and -3, in agreement with the current view of apoptosis-related events triggered by many stimuli [13]. Many death and survival signals converge on mitochondria and are mediated through members of the Bcl-2 family, which in turn control the release of cytochrome *c* and caspase activation. In the present study, we identified the reduction in Bcl-2 content and in the phosphorylation of Bad at Ser 112 as early events potentially relevant in mediating the ODQ-induced apoptosis. Our results are in agreement with a report [24] showing that prevention of apoptosis of splenic B-lymphocytes by NO and cGMP analogues is associated with sustained Bcl-2 content, both at the mRNA and protein levels.

It is known that phosphorylation of Bad at serines 112 and 136 results in sequestration of Bad in cytosol and neutralization of its proapoptotic activity [13,33]. This event may be critical for the action of survival signals through activation of specific kinases. Phosphorylation of Bad at Ser 136 appears to be under the control of the PI3K/Akt pathway [33]. However, experiments with the specific PI3K inhibitor LY294002 suggest that this pathway is not highly relevant for the survival of L1210 cells. Accordingly, Li *et al.* [34] have recently concluded that cGMP inhibits apoptosis in hepatocytes via a PI3K/Akt-independent mechanism.

On the other hand, phosphorylation of Bad at Ser 112 may be dependent on the p44/42 MAPK pathway [6] or on PKA [33]. We have shown here that PMA protected L1210 cells from ODQ and that this effect appeared largely mediated by p44/42 MAPK activation, being blocked by PD98059. Indeed, it has been reported that in some cell systems, such as vascular endothelial growth factor-stimulated endothelial cells [35] or cytokine-activated mesangial cells [36], cGMP may mediate activation of p44/42 MAPK by NO. However, it does not seem likely that cGMP protective effects in L1210 cells are mediated by p44/42 MAPK. In fact, ODQ did not significantly affect p44/42 MAPK phosphorylation, and the inhibitor of p44/42 MAPK pathway PD98059 did not prevent the YC-1 protective action or provoke apoptosis when given alone. Moreover, the PMA rescue effect was apparent with other apoptotic

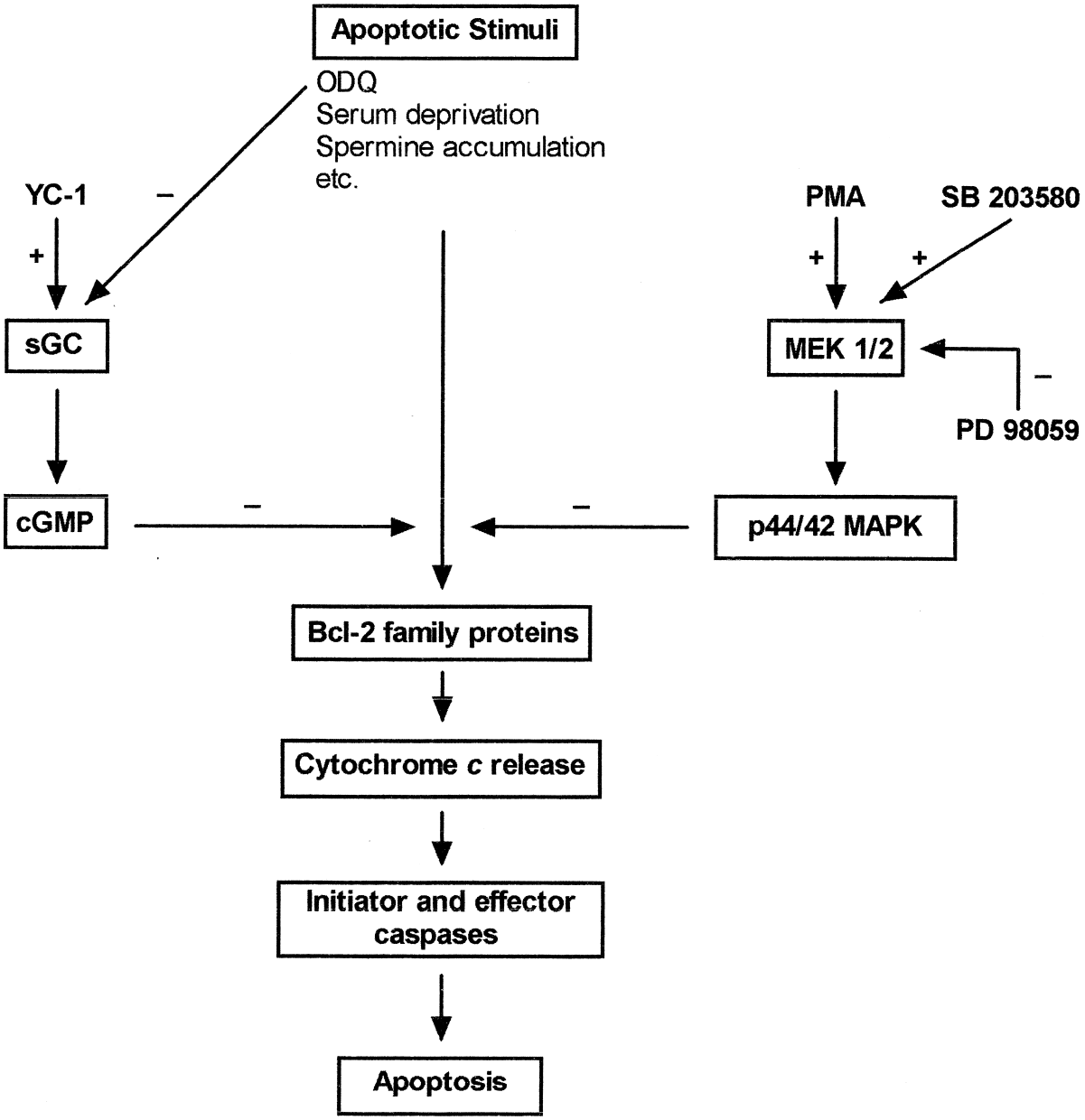

Fig. 7. Proposed pathways involved in the control of caspase activation and survival of L1210 cells on the basis of the modulators used in this study. An arrow does not necessarily denote a single step. In particular, the effect of PMA on the p44/42 MAPK pathway is probably mediated by protein kinase C and that of SB203580 may be mediated or not by p38 MAPK as discussed in the text.

stimuli, such as serum deprivation and spermine. In summary, these results suggest that p44/42 MAPK is not required for survival of L1210 cells under normal growth conditions, although stimulation of p44/42 MAPK may protect against various apoptotic stimuli.

p38 MAPK has also been implicated in the mediation or modulation of apoptotic signals, mainly on the basis of the effects of p38 MAPK inhibitors such as SB203580. In particular, it has been reported that exposure of HL-60 cells to sodium nitroprusside, an NO donor, induces p38 MAPK activation and apoptosis, which is inhibited by p38 MAPK inhibitors [9]. Analogously, SB203580 reduced the apoptotic action of ODQ in the present study. However, even the SB203580 effect may be largely mediated by p44/42 MAPK activation, inhibited as it is by PD98059. We and others have recently reported that p38 MAPK inhibitors can stimulate p44/42 MAPK in ECV304 and HepG2 cells, respectively [19,37]. This may contribute to an understanding of the results of Birkenkamp *et al*. [8], who showed that SB203580 repressed the apoptotic process of erythroleukemia TF-1 cells induced by growth factor deprivation and that PD98059 counteracted this action. However, it remains to be established whether the p44/42 MAPK-activating effect of SB203580 is consequent to p38 MAPK inhibition,

which would imply the occurrence of a cross-talk between p38 and p44/42 MAPKs, as suggested by Singh *et al*. in HepG2 cells [37]. Alternatively, a direct effect of SB203580 on some component of the p44/42 MAPK cascade may be hypothesized. Indeed, Hall-Jackson *et al*. [38] have reported how SB203580, while inhibiting c-Raf *in vitro*, activates c-Raf in intact Swiss 3T3 fibroblasts and human 293 cells in a p38 MAPK-independent way, thus suggesting the possibility of a downstream effect on the p44/42 MAPK cascade in some cell systems.

Quite recently, PKA activation was proposed as a mechanism involved in the cGMP-mediated protection of hepatocytes against apoptosis, since 5 μM KT5720, a specific PKA inhibitor, blocked the cGMP-dependent suppression of cell death [34]. However, a 24-hr treatment of L1210 cells with KT5720 (5–10 μM) could not mimic the ODQ action in that it did not induce caspase activity (not shown), suggesting that PKA is not implicated in ODQ-induced apoptosis.

In conclusion, the present paper hints at a relevant role for sGC in the survival of L1210 leukemia cells under either basal growing conditions or following apoptotic stimuli. In addition, stimulation of p44/42 MAPK may counteract the action of apoptotic stimuli, possibly through a pathway largely independent of cGMP. In Fig. 7 is depicted a simplified overview of some stimuli, downstream pathways, and pharmacological modulators discussed in this study. Pharmacological agents able to modify the activities of the enzymes involved in these pathways may prove useful in controling the growth of leukemia cells for therapeutic purposes.

Acknowledgment

This work was supported by grants from Italian MURST (ex 40% and 60%) and from the University of Bologna (Progetti Pluriennali di Ricerca e.f. 2000).

References

[1] Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–6.
[2] Meinhardt G, Wendtner CM, Hallek M. Molecular pathogenesis of chronic lymphocytic leukemia: factors and signaling pathways regulating cell growth and survival. J Mol Med 1999;77:282–93.
[3] Kolb JP. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia 2000;14:1685–94.
[4] Mateo V, Lagneaux L, Bron D, Biron G, Armant M, Delespesse G, Sarfati M. CD47 ligation induces caspase-independent cell death in chronic lymphocytic leukaemia. Nat Med 1999;5:1277–84.
[5] Dent P, Jarvis WD, Birrer MJ, Fisher PB, Schmidt-Ullrich RK, Grant S. The roles of signaling by the p42/p44 mitogen-activated protein (MAP) kinase pathway; a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity. Leukemia 1998;12:1843–50.
[6] Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 1999;286:1358–62.
[7] Berra E, Diaz-Meco MT, Moscat J. The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway. J Biol Chem 1998;273:10792–7.
[8] Birkenkamp KU, Dokter WH, Esselink MT, Jonk LJ, Kruijer W, Vellenga E. A dual function for p38 MAP kinase in hemopoietic cells: involvement in apoptosis and cell activation. Leukemia 1999; 13:1037–45.
[9] Jun CD, Pae HO, Kwak HJ, Yoo JC, Choi BM, Oh CD, Chun JS, Paik SG, Park YH, Chung HT. Modulation of nitric oxide-induced apoptotic death of HL-60 cells by protein kinase C and protein kinase A through mitogen-activated protein kinases and CPP32-like protease pathways. Cell Immunol 1999;194:36–46.
[10] Stefanelli C, Pignatti C, Tantini B, Stanic I, Bonavita F, Muscari C, Guarnieri C, Clo C, Caldarera CM. Nitric oxide can function as either a killer molecule or an antiapoptotic effector in cardiomyocytes. Biochim Biophys Acta 1999;1450:406–13.
[11] Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 1997;272:31138–48.
[12] Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood 1998;92:2484–94.
[13] Desagher S, Martinou JC. Mitochondria as the central point of apoptosis. Trends Cell Biol 2000;10:369–77.
[14] Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one. Mol Pharmacol 1995;48:184–8.
[15] Flamigni F, Facchini A, Capanni C, Stefanelli C, Tantini B, Caldarera CM. p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells. Biochem J 1999;341:363–9.
[16] Stefanelli C, Bonavita F, Stanic I, Mignani M, Facchini A, Pignatti C, Flamigni F, Caldarera CM. Spermine causes caspase activation in leukemia cells. FEBS Lett 1998;437:233–6.
[17] Stefanelli C, Bonavita F, Stanic I, Pignatti C, Farruggia G, Masotti L, Guarnieri C, Caldarera CM. Inhibition of etoposide-induced apoptosis with peptide aldehyde inhibitors of proteasome. Biochem J 1998; 332:661–5.
[18] Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstom PA, Roy S, Vaillancourt JP, Chapman KT, Nichols DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 1997;272:17907–11.
[19] Flamigni F, Facchini A, Giordano E, Tantini B, Stefanelli C. Signaling pathways leading to the induction of ornithine decarboxylase: opposite effects of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors. Biochem Pharmacol 2001;61:25–32.
[20] Stefanelli C, Bonavita F, Stanic I, Pignatti C, Flamigni F, Guarnieri C, Caldarera CM. Spermine triggers the activation of caspase-3 in a cell-free model of apoptosis. FEBS Lett 1999;451:95–8.
[21] Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. J Biol Chem 1995;270:27489–94.
[22] Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). J Biol Chem 1994;269:5241–8.
[23] Ko F-N, Wu C-C, Kuo S-C, Lee F-Y, Teng CM. YC-1, a novel activator of platelet guanylate cyclase. Blood 1994;84:4226–33.
[24] Genaro AM, Hortelano S, Alvarez A, Martinez C, Bosca L. Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. J Clin Invest 1995;95:1884–90.

[25] Estevez AG, Spear N, Thompson JA, Cornwell TL, Radi R, Barbeito L, Beckman JS. Nitric oxide-dependent production of cGMP supports the survival of rat embryonic motor neurons cultured with brain-derived neurotrophic factor. J Neurosci 1998;18:3708–14.
[26] Farinelli SE, Park DS, Greene LA. Nitric oxide delays the death of trophic factor-deprived PC12 cells and sympathetic neurons by a cGMP-mediated mechanism. J Neurosci 1996;16:2325–34.
[27] De Nadai C, Sestili P, Cantoni O, Lievremont JP, Sciorati C, Barsacchi R, Moncada S, Meldolesi J, Clementi E. Nitric oxide inhibits tumor necrosis factor-alpha-induced apoptosis by reducing the generation of ceramide. Proc Natl Acad Sci USA 2000;97:5480–5.
[28] Suenobu N, Scichiri M, Iwashina M, Marumo F, Hirata Y. Natriuretic peptides and nitric oxide induce endothelial apoptosis via a cGMP-dependent mechanism. Arterioscler Thromb Vasc Biol 1999; 19: 140–6.
[29] Taimor G, Hofstaetter B, Piper HM. Apoptosis induction by nitric oxide in adult cardiomyocytes via cGMP-signaling and its impairment after simulated ischemia. Cardiovasc Res 2000;45:588–94.
[30] Mancini L, Moradi-Bidhendi N, Becherini L, Martinetti V, MacIntyre I. The biphasic effects of nitric oxide in primary rat osteoblasts are cGMP dependent. Biochem Biophys Res Commun 2000;274:477–81.
[31] Zhao H, Dugas N, Mathiot C, Delmer A, Dugas B, Sigaux F, Kolb JP. B-cell chronic lymphocytic leukemia cells express a functional inducible nitric oxide synthase displaying anti-apoptotic activity. Blood 1998;92:1031–43.
[32] Drapier J-C, Pella C, Henry Y. Generation of EPR-detectable nitrosyl–iron complexes in tumor target cells cocultured with activated macrophages. J Biol Chem 1991;266:10162–7.
[33] Downward J. How BAD phosphorylation is good for survival. Nat Cell Biol 1999;1:E33–5.
[34] Li J, Yang S, Billiar TR. Cyclic nucleotides suppress tumor necrosis factor α-mediated apoptosis by inhibiting caspase activation and cytochrome *c* release in primary hepatocytes via a mechanism independent of Akt activation. J Biol Chem 2000;275:13026–34.
[35] Parenti A, Morbidelli L, Cui X-L, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase 1/2 activation in postcapillary endothelium. J Biol Chem 1998; 273:4220–6.
[36] Callsen D, Pfeilschifter J, Brune B. Rapid and delayed p42/p44 mitogen-activated protein kinase activation by nitric oxide: the role of cyclic GMP and tyrosine phosphatase inhibition. J Immunol 1998; 161:4852–8.
[37] Singh RP, Dhawan P, Golden C, Kapoor GS, Mehta KD. One-way cross-talk between p38 MAPK and p42/44 MAPK. Inhibition of p38 MAPK induces low density lipoprotein receptor expression through activation of the p42/44 MAPK cascade. J Biol Chem 1999;274: 19583–600.
[38] Hall-Jackson C, Goedert M, Hedge P, Cohen P. Effect of SB 203580 on the activity of c-Raf *in vitro* and *in vivo*. Oncogene 1999;18:2047–54.