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Activation of protein kinase C inhibits TRAIL-induced caspases activation, mitochondrial events and apoptosis in a human leukemic T cell line

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Abstract

TRAIL causes apoptosis in numerous types of tumor cells. However, the mechanisms regulating TRAIL-induced apoptosis remain to be elucidated. We have investigated the role of PKC in regulating TRAIL-induced mitochondrial events and apoptosis in the Jurkat T cell line. We found a caspase-dependent decline in mitochondrial membrane potential and translocation of cytochrome *c* from mitochondria into the cytosol in response to TRAIL. Both these events were prevented by PKC activation. Moreover, PKC activation considerably reduced the activation of caspases, PARP cleavage and apoptosis when induced upon TRAIL treatment. MAPK activation was involved in the mechanism of PKC-mediated inhibition of TRAIL-induced cytochrome *c* release from mitochondria. Furthermore, inhibition of the MAPK pathway partially reversed the PKC-mediated inhibition of TRAIL-induced apoptosis. Besides, PKC activation may also inhibit the TRAIL-induced apoptosis through a MAPK-independent mechanism. Altogether, these results indicate a negative role of PKC in the regulation of apoptotic signals generated upon TRAIL receptor activation. *Cell Death and Differentiation* (2001) **8,** 172–181.

Keywords: TRAIL; apoptosis; Jurkat; mitochondria; PKC; MAPK

Abbreviations: PKC, Protein kinase C; TNF, tumor necrosis factor, TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; MAPK, mitogen-activated protein kinase; DISC, death-inducing signaling complex; PDBu, phorbol-12,13-dibutyrate; PARP, poly(ADP-ribose)polymerase; cFLIP, cellular FADD-like interleukin 1-converting enzyme-inhibitory protein; IAPs, inhibitor-of-apoptosis proteins; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; CHX, cycloheximide; ψm, membrane potential

Introduction

Apoptosis is a cell death process that is required for integrity and homeostasis of multicellular organisms and is also important in pathological situations.[1,2] The past decade has witnessed an enormous progress in our knowledge of the pathway for the execution of apoptotic cell death. Several members of the tumor necrosis factor (TNF) family have so far been reported to play an important role in the induction of apoptosis.[3] TRAIL (APO-2 ligand), a recently discovered member of the TNF family, is a type II transmembrane protein which provokes apoptosis mainly in tumor cells.[4] TRAIL also functions as an apoptosis inducer in activation-induced cell death, immune privilege, T cell-mediated cytotoxicity and autoimmunity.[5–9] TRAIL mRNA is constitutively present in many tissues unlike the restricted expression of CD95L, another pro-apoptotic member of the TNF family.[10,11] TRAIL has four specific receptors, namely TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4, of which TRAIL-R1, -R2 and -R4 are type I transmembrane proteins whereas -R3 is a glycosylphosphatidyl inositol linked protein. The mRNAs of TRAIL-R1, -R2 and -R4 are as frequently expressed as that of TRAIL whereas that of TRAIL-R3 is rather restricted. TRAIL-R1 and -R2 are 'death receptors', with cytoplasmic death domains that trigger apoptotic cell death, whereas TRAIL-R3 and TRAIL-R4 are 'decoy receptors' which are unable to transduce death signals and could inhibit TRAIL-induced apoptosis.[12–20]

Although rapid advances in our understanding of the biology of CD95-mediated cell death have unveiled exciting new perspectives in this area of research, progress in understanding TRAIL-induced apoptosis has been rather modest thus far, and often contradictory results have been reported.[12–15,21–25] However, more recent data have demonstrated that TRAIL receptors recruit the adapter protein FADD for caspase-8 activation.[26–28] Apical caspase processing is followed by activation of BID, processing of caspase-9, -3 and -7, cleavage of the nuclear enzyme poly(ADP-ribose)polymerase (PARP) and apoptotic cell death.[29–31]

There is emerging evidence for the importance of signals from mitochondria during the effector phase of apoptotic cell death.[32] Results of several investigations have suggested that apoptosis-inducing agents like CD95L and TNF-α can trigger the uncoupling of electron transport from ATP production, with the resultant decrease of membrane potential (ψm),[33] which can be attributed to the opening of a mitochondrial permeability transition (PT) pore.[32] Through this PT pore, cytochrome *c* is released into the cytosol[34,35] and together with Apaf-1 and procaspase-9 induce the activation of caspase-9, leading to activation of caspase-3 and downstream apoptotic events.[36–38] However, other

investigations have revealed that mitochondrial cytochrome *c* release could also occur independently of mitochondrial PT.[39] The role of mitochondria in death receptor-mediated apoptosis has largely been examined in the case of CD95-mediated apoptosis,[40] although more recent research has also addressed the involvement of mitochondria-related events in TRAIL-induced apoptosis.[41,42]

Recent results have provided substantial evidences that serine/threonine PKC upon activation by phorbol ester could inhibit apoptosis triggered by different inducers,[43–45] including CD95 ligation.[46–49] It is known that PKC activates the MAPK pathway.[50–52] Nevertheless, several independent lines of evidence indicate that PKC prevents CD95-mediated apoptosis by both MAPK pathway-dependent[53–55] and -independent[55] mechanisms. However, the regulation of TRAIL-induced apoptosis seems to be more complex than that induced by CD95L.[41,42]

The findings outlined above prompted us to study the role of PKC, if any, in regulating TRAIL-induced apoptosis. We hereby report that treatment of human Jurkat T cells with a PKC activator markedly interferes with caspase activation, mitochondrial events and apoptosis induced by TRAIL. In this work, we have also addressed the question as to the role played by the MAPK pathway in mediating this PKC-induced survival mechanism.

Results

Protein Kinase C activation prevents TRAIL-induced apoptosis

Activation of apoptotic TRAIL receptors by TRAIL has important implications for the selective elimination of tumor cells and the use of TRAIL as a therapeutic agent is a matter of growing interest.[56] On the other hand, TRAIL could also play a role in activation-induced cell death of T lymphocytes.[7] In this respect, it has been previously shown that Jurkat T lymphocytes are very sensitive to TRAIL-induced apoptosis.[6,26,28] Jurkat cells mainly express mRNA (Figure 3c) and protein[22,26,28] for TRAIL-R2, though we also detected a significant amount of mRNA for TRAIL-R1 (Figure 3c) and -R4 (results not shown).

Activation of the serine/threonine PKC elicits survival signals in many different systems, including CD95-induced apoptosis.[43–49] Since the regulation of TRAIL-mediated cell death appears to be more complex than that induced by CD95L,[41,42] we were interested on investigating whether PKC activation could modulate TRAIL-mediated apoptosis. Results shown in Figure 1a indicate that in Jurkat cells, TRAIL-induced chromatin condensation was abrogated in presence of the PKC activator PDBu. In order to quantify this effect, we determined by FACS analysis the effect of PKC activation on the induction of hypodiploid apoptotic cells in TRAIL-treated Jurkat cells. As shown in Figure 1b (left panel), in cultures of Jurkat cells treated with TRAIL for 15 h there was a marked induction of apoptosis as compared to the control cultures. In contrast, a considerable reduction in the percentage of apoptotic cells in the cultures was observed when Jurkat cells were incubated with TRAIL in the presence of PKC activator (Figure 1b, left panel). This inhibition was similar to that observed in the presence of a non-cytotoxic dose (50 μM) of Z-VAD-fmk (Figure 1b, right panel), a broad spectrum caspase inhibitor.[57] Furthermore, PDBu continued to exert its apoptosis-inhibitory action till our experimental period of 48 h (Figure 1c).

One of the key biochemical events associated with apoptosis is the cleavage by caspases of the 116-113 kDa nuclear enzyme PARP within the bipartite nuclear location signal to produce two fragments of 85 and 29 kDa.[58] By Western blot analysis, we determined the cleavage of PARP in Jurkat cells incubated with TRAIL for 8 h. The 85 kDa fragment of PARP cleavage was clearly visualized in TRAIL-treated cells (Figure 2a). However, PARP cleavage was significantly diminished in the presence of PKC activator in TRAIL-treated cells (Figure 2a). These results suggested that PKC was playing a negative role in apoptotic signals generated upon ligation of TRAIL receptors which lead to the activation of execution caspases.

During apoptosis, caspases are activated by cleavage of their inactive native forms. To investigate the step in TRAIL-induced apoptosis that is affected by PKC, we analyzed the processing of caspase-8, the most apical caspase activated in both CD95[59,60] and TRAIL-induced apoptosis.[26–28,31] Processing of caspase-8 upon TRAIL receptor ligation has been recently described in different cells.[26–28,30,31] In TRAIL-treated Jurkat cells, we detected both the 55 and 53 kDa inactive pro-forms corresponding to caspase-8a and -8b as well as the 43 and 41 kDa intermediate products corresponding to cleavage of both caspase-8a and -8b between the large and small subunits (Figure 2b). This cleavage process finally results in the release of the large 18 kDa subunit and the assembly of the active caspase.[61] Interestingly, when co-stimulated with PKC activator, a markedly reduced cleavage of caspase-8 was observed in TRAIL-treated Jurkat cells (Figure 2b). These results suggested that PKC activation could be blocking TRAIL-induced apoptosis at the initiator caspase-8 level. However, as caspase-8 can also be activated through a feedback loop involving mitochondria,[62] we could not exclude the possibility that the inhibition of caspase-8 observed in our studies was a secondary effect of a block in a TRAIL-induced, mitochondria-regulated pathway.

PKC-mediated inhibition of TRAIL-induced apoptosis is protein synthesis-independent and is still observed after delayed addition of PDBu

Resistance of cells to death receptor-induced apoptosis has been associated with the presence of short-lived inhibitory proteins which can be removed by protein synthesis inhibitors.[63–65] To ascertain whether PDBu-induced resistance to TRAIL-mediated apoptosis was due to the induction of such inhibitors, we examined by RT–PCR the mRNA levels of cFLIP and IAPs in Jurkat cells treated with the PKC activator. As shown in Figure 3a, Jurkat cells express a certain amount of mRNA for cFLIP and X-IAP, but these levels were not up-regulated following an 8 h treatment with PDBu. Jurkat cells did not express hIAP1 mRNA and was not

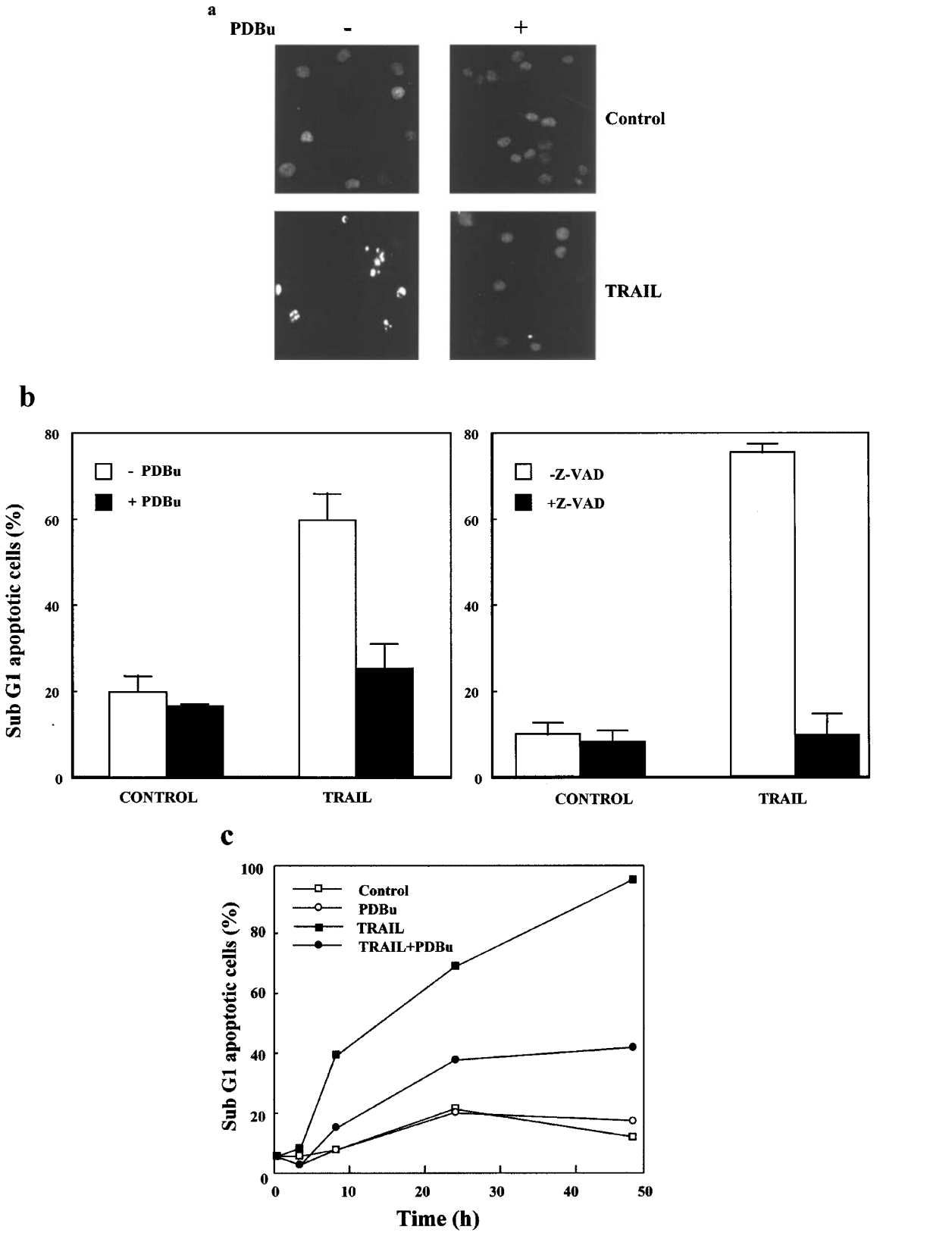

Figure 1 Treatment with PDBu inhibited TRAIL-mediated apoptosis of Jurkat cells. Jurkat cells (5×10^5/ml) were treated for 8 h with 50 ng/ml TRAIL (**a, b**), either in the absence or presence of 20 ng/ml PDBu. Other cultures (**b**, right panel) were incubated with 50 μM Z-VAD-FMK for 2 h and treated for an additional 8 h period with 50 ng/ml TRAIL. Following treatments, (**a**) chromatin condensation in nuclei and (**b**) subG1 apoptotic cells in the culture were determined by DAPI staining/ fluorescence microscopy and flow cytometry, respectively. The percentage of subG1 apoptotic cells is shown. Error bars represent S.D. from three independent experiments. In (**c**), cells were treated as indicated in the figure and apoptosis determined by flow cytometry

induced by PDBu (not shown). Furthermore, we determined the protein synthesis requirement of PDBu-induced resistance. As protein synthesis inhibitors caused apoptosis (Figure 3b), in these experiments Jurkat cells were pretreated with CHX and subsequently incubated for a short time (3 h instead of the usual 8 h incubation) in the absence or presence of TRAIL. Results shown in Figure 3b indicate that CHX-treated Jurkat cells were clearly more sensitive to TRAIL-mediated apoptosis than cells incubated without this protein synthesis inhibitor. However, when incubated in the presence of PDBu, CHX-treated cells were still markedly resistant to the apoptosis-inducing effect of TRAIL (Figure 3b).

To test whether down-regulation of the expression of pro-apoptotic TRAIL receptors or inhibition of TRAIL-mediated clustering of these receptors were involved in the inhibitory action of PDBu,[55] we carried out two different experimental approaches. First, we measured by RT–PCR the mRNA levels of TRAILR1 and TRAILR2 in cells incubated for 8 h with PDBu. Stimulation of Jurkat cells with PDBu did not alter mRNA levels of these receptors (Figure 3c). Second, we examined the effect of a delayed addition of PDBu on TRAIL-induced apoptosis. As shown in Figure 3d, TRAIL-induced apoptosis can still be markedly inhibited by PKC activation even 2 h after the addition of TRAIL. Since assembly of the DISC and activation of initiator caspase-8 in Jurkat cells are probably maximal 1 h after the addition of TRAIL,[26,28] the above results suggested that changes in receptor levels or clustering are not likely involved in the inhibitory effect of PDBu.

Caspase-dependent mitochondrial events in TRAIL-induced apoptosis and its prevention by protein kinase C activation

It has recently become evident that mitochondria is involved in CD95-mediated apoptosis in CD95 type II cells.[40] Loss of mitochondrial transmembrane potential (ψm) and translocation of mitochondrial cytochrome *c* into the cytosol have been observed early after apoptosis induction.[35] These mitochondrial changes induced by CD95 ligation in Jurkat cells are caspase-dependent.[66] Recent reports have described similar findings in TRAIL-treated cells, although the role of mitochondria in TRAIL-induced apoptosis remains controversial.[41,42,67] To determine whether PKC activation was affecting mitochondrial signalling in TRAIL-induced apoptosis, we have examined mitochondrial membrane potential as well as the presence of cytochrome *c* in the cytosol of TRAILR-stimulated cells. Similar to recently reported findings,[41,42,67] we found that the number of cells with a decreased mitochondrial membrane potential markedly increased when the cells were incubated with TRAIL for 8 h (Figure 4a). The fall in membrane potential was completely prevented in presence of the caspases inhibitor Z-VAD-fmk (Figure 4a, right panel). Likewise, TRAIL induced the release of cytochrome *c* into the cytosol of Jurkat cells (Figure 4b, upper panels), and this effect was also inhibited by Z-VAD-fmk (Figure 4b, right panel). These results confirmed recent works[41,42,67] and provided compelling evidence for the involvement of caspases upstream of mitochondrial changes induced by TRAIL. To further investigate the role of PKC activation as a protecting event of TRAIL-induced apoptosis, we studied the effect of PDBu on both TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release. Results of these experiments indicated (Figure 4a, left panel) that the reduction in ψm induced by TRAIL in Jurkat cells was checked considerably in cells co-stimulated with PDBu. Similarly, when we determined the sub-cellular localization of cytochrome *c* in TRAIL-treated Jurkat cells, we observed that the marked translocation of cytochrome *c* from mitochondria into the cytosol induced by TRAIL was almost completely prevented by co-treatment with PDBu (Figure 4b, left panels). Further evidence of the inhibitory action of PKC in the mitochondria-operated pathway activated upon TRAIL receptor ligation, was obtained when we examined the activation of caspase-9. Once released from mitochondria, cytochrome *c* will bind to Apaf-1, an event that triggers oligomerization of Apaf-1/cytochrome *c* in complexes that activate procaspase-9.[68] Figure 4c illustrates that TRAIL activated the processing of caspase-9 in Jurkat cells and this effect was considerably inhibited by PKC activation.

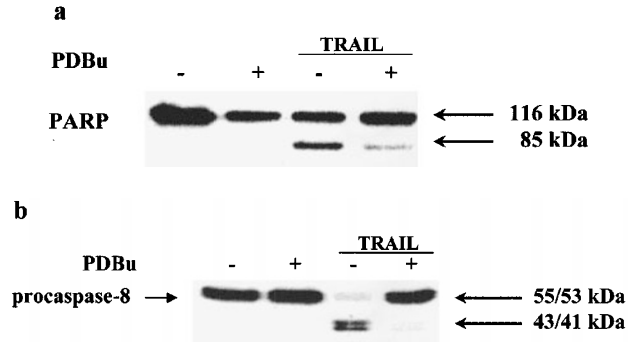

Figure 2 Prevention of TRAIL-induced caspase activation by a PKC activator. Jurkat cells were treated with 50 ng/ml TRAIL for 8 h in the absence or presence of 20 ng/ml PDBu. Cell lysates were prepared and PARP cleavage (**a**) or caspase-8 processing (**b**) were determined by Western blot analysis. The 116 kDa PARP and its 85 kDa cleavage product are indicated by arrows. Caspase-8 activation was detected by the appearance of intermediate cleavage products of 43/41 kDa from 55/53 kDa native forms, indicated by arrows

Activation of the MAPK pathway is partially required for PKC-mediated prevention of TRAIL-induced apoptosis

Activation of PKC in cells results in the stimulation of the MAPK pathway.[50–52] The MAPK pathway plays a survival role and inhibits CD95-mediated apoptosis.[53–55] Based on these results, we decided to evaluate the role of MAPK activation in PKC-induced prevention of TRAIL-mediated cytochrome *c* release and apoptosis by using PD 098059, a specific inhibitor of MEK-1 activation.[69] Figure 5a shows that PKC activation prevented in a dose-dependent manner the release of cytochrome *c* into the cytosol. Maximal inhibition was achieved at PDBu doses between 10 and 20 ng/ml. Interestingly, TRAIL-induced release of cytochrome *c* from mitochondria was not prevented by PKC activation when cells were incubated in the presence of PD 098059 (Figure 5a). In

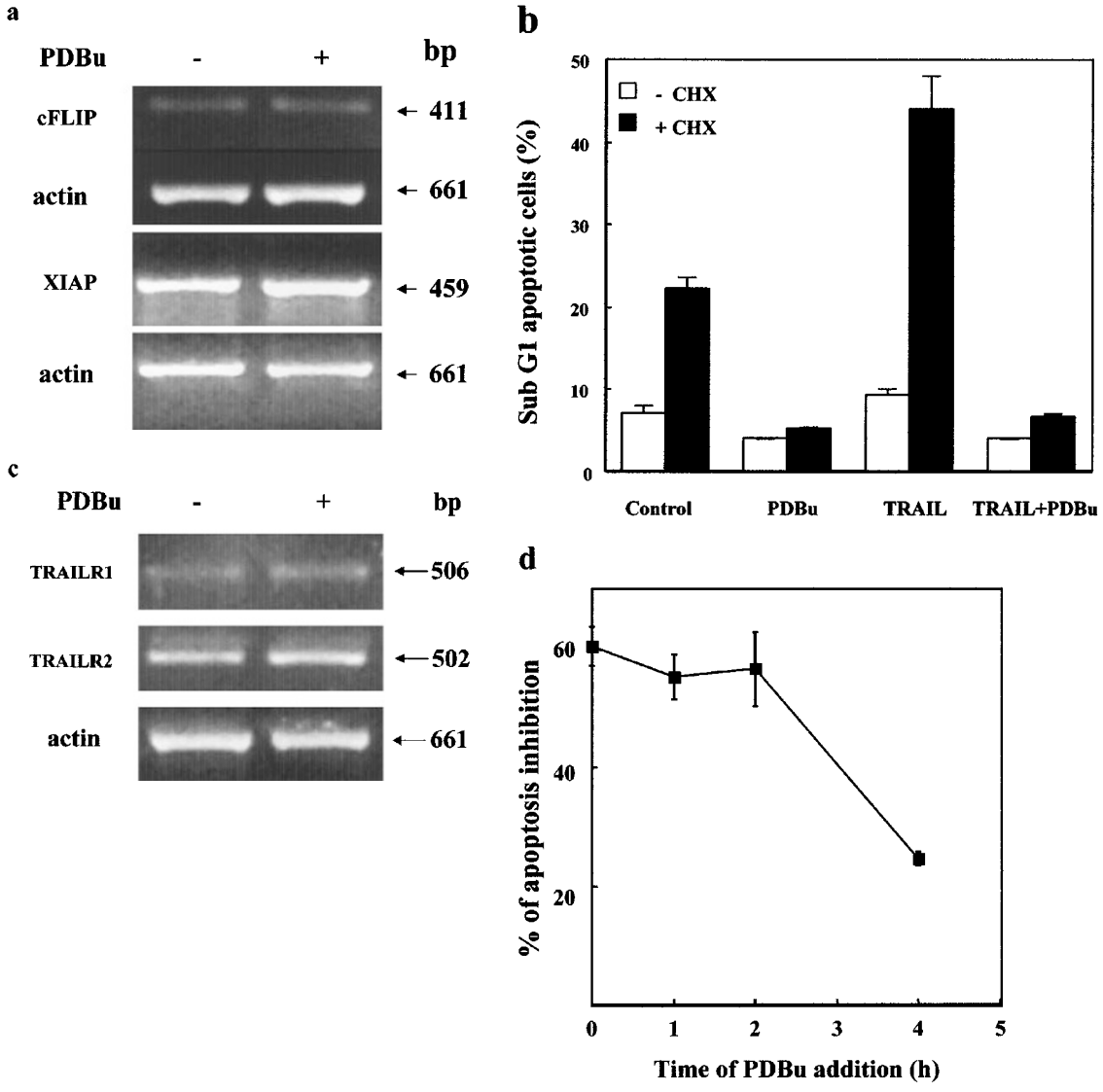

Figure 3 Role of caspase inhibitors and receptor clustering in PDBu-induced inhibition of TRAIL-mediated apoptosis. Total mRNA was prepared from cells incubated for 8 h with or without PDBu (20 ng/ml) and RT – PCR was performed as described under Materials and Methods (**a, c**). In (**b**) Jurkat cells were pre-treated with or without CHX (0.5 μg/ml) for 30 min followed by the addition of nothing (Control), PDBu (20 ng/ml), TRAIL (50 ng/ml) or PDBu+TRAIL. Cultures were further incubated for 3 h and apoptosis was assessed by flow cytometry. Error bars represent S.D. from three independent experiments. (**d**) Cells were treated with or without TRAIL (50 ng/ml) from time 0 h. One single addition of PDBu (20 ng/ml) was performed at the times indicated in the figure and apoptosis determined 6 h after the addition of TRAIL. Error bars represent S.D. from three independent experiments

the presence of the MEK1 inhibitor, the activation of the MAPK pathway by PDBu, was substantially abolished (Figure 5b). We next examined whether PD 098059 was able to prevent PKC-mediated inhibition of TRAIL-induced caspase-3 activation and apoptosis in Jurkat cells. Results shown in Figure 5c indicate that the MEK1 inhibitor only slightly reduced the PDBu-induced inhibition of TRAIL-mediated caspase-3 activation and attenuated the inhibitory effect of PKC activation on TRAIL-induced apoptosis (Figure 5d). However, prevention by PD 098059 of PKC-mediated inhibition of TRAIL-induced apoptosis was never complete, suggesting a possible existence of a MAPK-independent and PKC-dependent mechanism that may have also participated in inhibition of TRAIL receptor-mediated cell death.

Discussion

Receptors for death ligands are expressed in many tumor cells which can therefore be killed by the appropriate ligands.[4] These observations offer the possibility of using death ligands as anti-tumor agents. However, in systemic anti-tumor treatments, severe toxic effects have been observed with TNF-α and CD95 ligand. TNF-α causes a lethal inflamatory response[70] and CD95L produces lethal liver damage.[71] In contrast, repeated systemic exposure of non-human primates to elevated doses of TRAIL does not produce significant changes in clinical parameters.[56,72] These *in vivo* results are in agreement with data obtained *in vitro* indicating that TRAIL is not cytotoxic toward a variety of cultured normal cells.[56] All

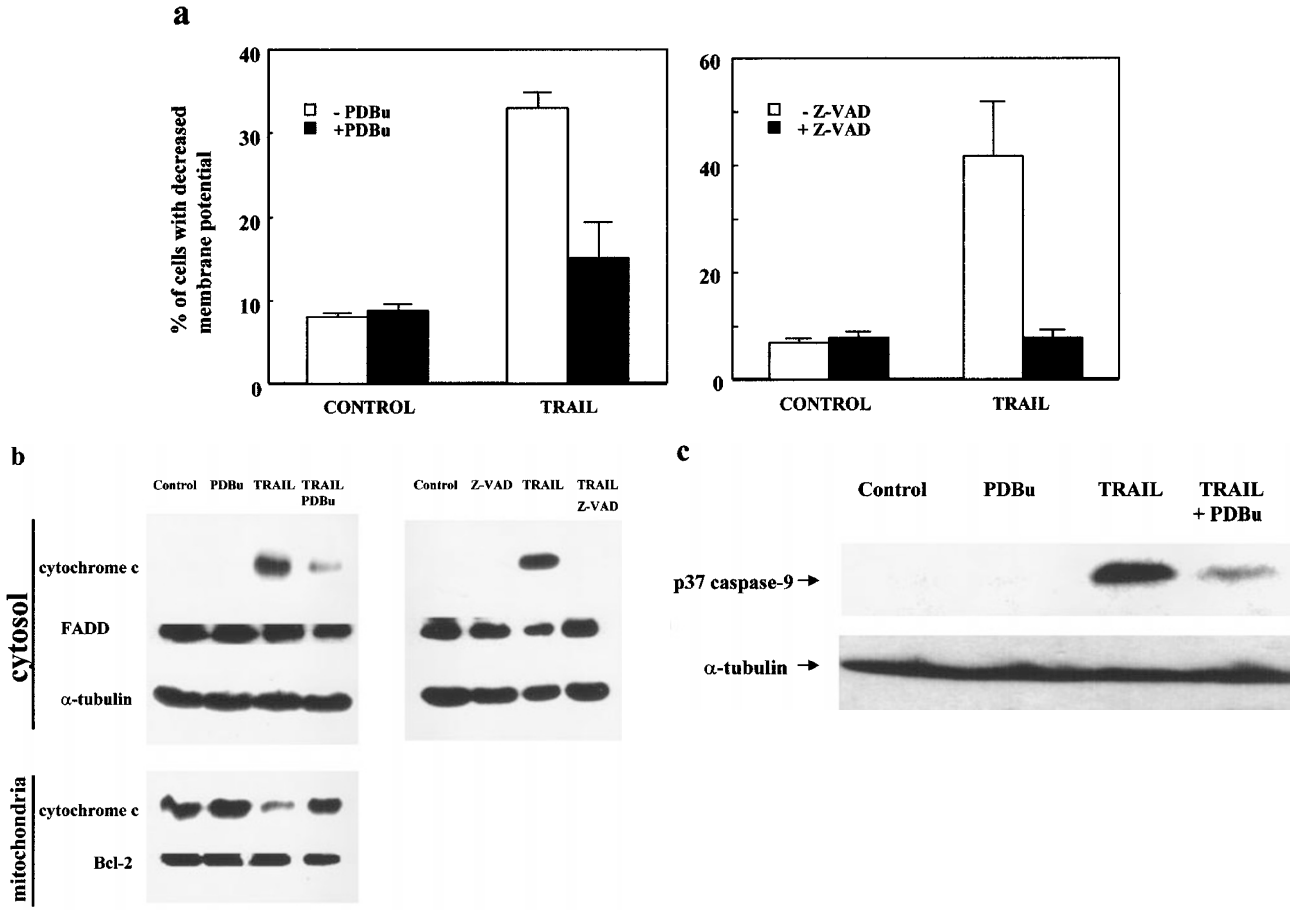

Figure 4 TRAIL-induced mitochondrial changes were abrogated by PKC activation. Jurkat cells were treated with 50 ng/ml TRAIL for 8 h in the absence or presence of 20 ng/ml PDBu (**a, b**, left panels). (**a**) ψm was analyzed by flow cytometry as described under Materials and Methods using the fluorochrome $DIOC_6(3)$. (**b**) Cells were fractionated into membrane (including mitochondria) and cytosolic fractions as described under Material and Methods. Levels of cytochrome *c* in each fraction were determined by immunoblot analysis. Z-VAD-FMK (50 μM) was added to other cultures of Jurkat cells (**a, b**, right panels). After 2 h of incubation, cells were further incubated for 8 h with 50 ng/ml of TRAIL. After this incubation, (**a**) ψm was determined by flow cytometry and (**b**) levels of cytochrome *c* in the cytosolic fraction were determined by immunoblot analysis. (**a**) Error bars represent S.D. from three independent experiments. (**b**) Cytosolic blots were probed for α-tubulin as a control for protein loading. Blots were also probed for cytosolic (FADD) and mitochondrial (Bcl-2) markers to determine the purity of the subcellular fractions. Bcl-2 protein was undetectable in the cytosolic samples and the membrane fractions were only minimally contaminated with cytosolic proteins (not shown). (**c**) Cells were treated for 8 h with or without TRAIL in the presence or absence of PDBu. Caspase-9 activation was assessed by the generation of the 37-kDa fragment of cleaved procaspase-9, α-tubulin serves as a control for protein loading. Data of a representative experiment are presented in **b** and **c**

these observations point out to a possible use of TRAIL as a new anti-tumor agent in human malignancies.[72]

Despite the potential of TRAIL as a useful tool in anti-cancer therapy, very little is known about the mechanisms regulating the sensitivity to TRAIL-induced apoptosis. It has been suggested that the expression of decoy receptors TRAIL-R3 and -R4, may restrain the sensitivity of cells to TRAIL.[12–20] However, analyses of TRAIL receptor expression in a number of human tumor cell lines have indicated no correlation between TRAIL sensitivity and TRAIL-R3/R4 mRNA expression.[73] Our results conclusively indicate for the first time that PKC activity could be an important regulator of apoptotic signals emerging from TRAIL receptors. Recent data have demonstrated the existence of two types of cells (I and II) in terms of the apoptosis signalling mechanism downstream of CD95 activation.[40] Although mitochondrial parameters are affected in both cell types upon CD95 ligation at the cell surface, overexpression of bcl-2 only inhibits CD95-induced apoptosis in type II cells,[40] indicating the existence of a mitochondria-mediated CD95-induced mechanism of apoptosis in these cells. Jurkat cells have been previously classified as CD95 type II cells.[62] In this respect, we have demonstrated that the induction by TRAIL of a caspase-dependent mitochondrial apoptotic pathway is severely impaired when cells are incubated with a PKC activator. Our results therefore raise the question whether the TRAIL mediated apoptosis in Jurkat cells is essentially dependent on the observed mitochondrial changes. In this respect, it has been demonstrated that ectopic expression of bcl-2 inhibits TRAIL-induced apoptosis in certain cells.[74] However, more recent work have reported that Bcl-2 overexpression does not inhibit TRAIL-induced cytochrome *c* release[41] and apoptosis.[41,42] Whether these discrepancies reflect different levels of expression of transfected Bcl-2 is not known. Our data indicate that the inhibitory action of PDBu in

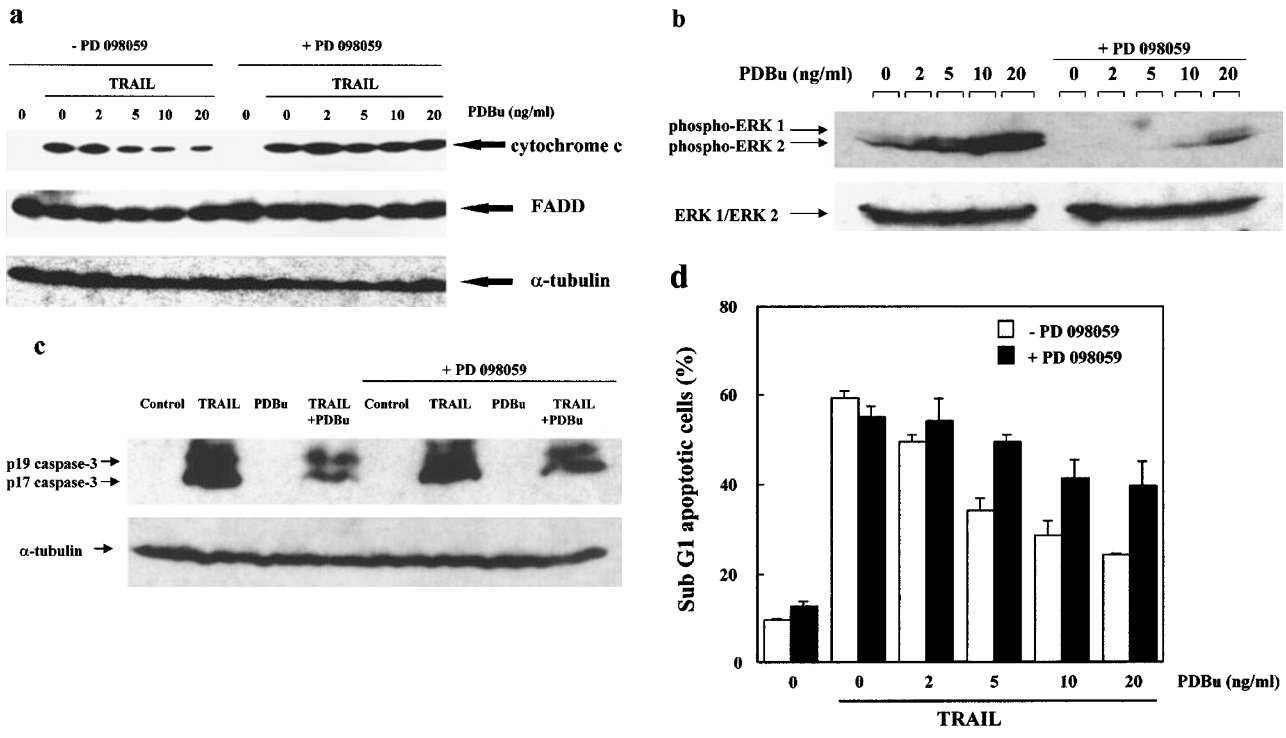

Figure 5 PKC, prevented TRAIL-induced cytochrome *c* release and partially inhibited apoptosis through activation of MAPK pathway. Jurkat cells were pre-incubated with or without the MEK-1 inhibitor PD 098059 (50 μM) for 1 h. After this incubation, cells were further incubated for 8 h (**a, c**) or 15 h (**d**), with or without TRAIL (50 ng/ml) in the absence or presence of different concentrations of PDBu (**a, d**) or 20 ng/ml PDBu (**c**). Cytochrome *c* release, caspase-3 activation and hypodiploid cells were determined as described under Materials and Methods. Bcl-2 protein was undetectable in the cytosolic samples of (**a**) (**b**) Cells were pre-incubated in absence or presence of PD 098059 (50 μM) for 1 h and subsequently treated with or without different concentrations of PDBu for 30 min. ERK1/2 activation was assessed with phospho-ERK1/2 mAb. Equal loading of protein was confirmed with ERK1/2 mAb. In (**d**) error bars represent S.D. from three different experiments

TRAIL-mediated apoptosis does not involve any change in the expression of TRAIL death receptors mRNA in Jurkat cells. Nevertheless, we can not completely exclude the possible loss of TRAIL apoptotic receptors at the protein level. Besides, PKC activation through phosphorylation of DISC components, may also inhibit a non-mitochondrial mechanism of apoptosis induced by TRAIL, as reported for CD95.[55,75] Work in this direction is currently in progress in our laboratory. However, our data on the delayed addition of PDBu to TRAIL-treated cells indicate that a step downstream of DISC formation as well as the initiator caspase-8 activation by TRAIL is negatively regulated by PKC activation. The initiator caspase-8 activation by TRAIL is a primary event[26–28] that may subsequently lead to BID cleavage[31] and the stimulation of mitochondrial events.[41,42] We are currently investigating BID cleavage and its translocation to mitochondria in TRAIL-treated cells and its possible regulation by PKC.

The signalling mechanism that results from PKC activation which inhibits the TRAIL-induced apoptosis, has been addressed in our work. As a consequence of PKC activation, p21 ras and the protein kinases c-raf, MEK1 and ERK1/2 become sequentially activated.[50–52] Activation of the MAPK pathway prevents apoptosis and promotes cell survival.[76] More recently, activation of this pathway has also been found to repress apoptosis triggered by CD95 activation.[53–55] Our present observations indicate for the first time, that activation by PKC of the MAPK pathway plays a crucial role in the abrogation of TRAIL-induced cytochrome *c* release in Jurkat cells. It however remains to be resolved as to how the activation of the MAPK pathway results in the resistance to TRAIL-mediated cytochrome *c* release. Recent data have indicated that the expression of intracellular apoptosis inhibitors such as FLIP, a protein with homology to caspase-8 that lacks catalytic activity,[77] could be responsible for resistance to TRAIL-induced apoptosis in melanoma cells.[30] However, more recent results show no correlation between FLIP expression and resistance to TRAIL in these tumor cells.[78] Although MAPK stimulation in Jurkat T cells with concanavalin A has recently been reported to induce FLIP,[54] we however have found no changes in FLIP (L/S) or IAPs mRNA expression when Jurkat cells are treated with PDBu. These results support our data with CHX in which we show that the PDBu-mediated inhibitory action is protein synthesis-independent.

A very recent report has described that resistance of certain cells to CD95-mediated apoptosis can be reversed by the use of PD 098059.[79] Literature abounds in evidences of increased MAPK activities in tumor cells.[80,81] Hence it may not sound unreasonable to assume that TRAIL resistant tumors may also have a constitutively active MAPK pathway. If this assumption stands out to be valid, our results may have important clinical implications in cancer therapy since the treatment of these TRAIL-resistant tumors with inhibitors of the MAPK

pathway could sensitise these cells to soluble TRAIL. However, reversal of PKC-mediated inhibition of cytochrome c release from mitochondria by PD 098059 does not completely abolish the apoptosis inhibitory effect of PKC activation. These data may indicate that a MAPK-independent mechanism downstream of cytochrome *c* release is also activated by PKC, which attenuates TRAIL receptor-mediated caspase-3 activation and apoptosis. In this respect, PKC-dependent activation of mitochondrial Raf-1 has been recently described to induce an anti-apoptotic effect.[82] On the other hand, it has been described that cytochrome *c*-induced proteolytic processing of pro-caspase-9 is defective in cytosolic extracts from cells expressing active Akt,[83] a molecular target of the anti-apoptotic activity of PKC in certain cells.[84] These apart, an alternative explanation points out that a caspases cascade pathway independent of mitochondria[42] is also blocked by PKC activation. We are currently exploring these possibilities in order to get more insight into the mechanism underlying the PKC-dependent and MAPK-independent abrogation of TRAIL-induced apoptosis.

Materials and Methods

Materials

RPMI 1640 medium and FBS were obtained from GIBCO Europe. PDBu, CHX and DAPI (4′,6′-diamidino-2-phenylindole) were purchased from Sigma (Poole, UK). Recombinant human TRAIL was obtained from Prepro Tech EC LTD (London, England). The inhibitor of MEK1 activation, PD 098059, was purchased from Calbiochem-Novabiochem GmbH (Band Soden, Germany). Mouse anti-human caspase-8 mAb was purchased from Cell Diagnostica (Münster, Germany). Rabbit polyclonal antiserum against PARP was purchased from Boehringer Mannheim (Germany). Mouse phospho-ERK1/2 mAb recognising activated ERK1/2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse ERK1/2 mAb was purchased from Zymed (San Francisco, CA, USA). Mouse anticytochrome *c* mAb was obtained from Pharmingen (San Diego, CA, USA). Mouse anti-BCL-2 mAb was from DAKO (Denmark) Mouse anti-FADD mAb was from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal antibodies to caspase-9 p37 fragment and caspase-3 p17 subunit were obtained from New England BioLabs Inc. (Beverly, MA, USA). Monoclonal antibody to alpha-tubulin was purchased from Sigma (Poole, UK). Caspases inhibitor Z-VAD-FMK was from Enzyme System Inc. (Dublin, CA, USA). $DiOC_6(3)$ was purchased from Molecular Probes (Eugene, OR, USA).

Cell culture

Cells of the human leukemic T cell line Jurkat were maintained in culture in RPMI 1640 medium containing 10% FCS and 1 mM L-glutamine, at 37°C in a humidified 5% CO2/95% air incubator.

Determination of apoptotic cells

Analysis by flow cytometry of hypodiploid apoptotic cells was performed on a FACScan cytometer using the Cell Quest software (Becton Dickinson, Mountain View, CA, USA), after extraction of the degraded DNA from apoptotic cells following a recently described method.[85] Chromatin condensation was assessed after staining of cellular DNA with DAPI by viewing the cell preparations under a Zeiss Axiophot fluorescent microscope.

Mitochondrial membrane potential

Loss of mitochondrial membrane potential was assessed with $DiOC_6(3)$ as described.[86]

Cytochrome *c* release from mitochondria

For measurements of cytochrome *c* release from mitochondria, cells were lysed and cytosolic fractions were separated from mitochondria as previously described.[87] Cytosolic proteins (40 μg of protein) were mixed with Laemmli buffer and resolved on SDS-12% PAGE minigels. Cytochrome *c* was determined by Western blot analysis as described below.

Immunoblot detection of proteins

Cells (10^6) were washed with phosphate-buffered saline (PBS), resuspended in 20 μl sample buffer (50 mM Tris-HCl pH 6.8, 6 M urea, 6% 2-mercaptoethanol, 3% SDS, 0.003% bromophenol blue) and sonicated. Proteins were resolved on either 10% or 12% SDS-polyacrylamide minigels and transferred onto Immobilon membranes (Millipore). The blots were blocked with 5% milk powder in PBS/0.1% Tween-20 and then incubated with primary antibodies. Blots were stained with horseradish peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminiscence (ECL, Amersham Life Sciences, UK) according to the manufacturer's instructions.

Reverse transcription PCR

Total RNA was isolated from cells with Trizol reagent (Life Technologies, Inc. Grand Island, NY, USA) as recommended by the supplier. cDNAs were synthesized from 2 μg of total RNA using a RT – PCR kit (Perkin Elmer, Norwalk, CT, USA) with the supplied oligo d(T) primer under conditions described by the manufacturer. PCR reactions were performed using the primers and conditions already described.[5,54,88]

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