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Fas mediated apoptosis of human Jurkat T-cells: intracellular events and potentiation by redox-active α-lipoic acid

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Abstract

Activation of caspases is required in Fas receptor mediated apoptosis. Maintenance of a reducing environment inside the cell has been suggested to be necessary for caspase activity during apoptosis. We explored the possibility to potentiate Fas mediated killing of tumor cells by α-lipoic acid (LA), a redox-active drug and nutrient that is intracellularly reduced to a potent reductant dihydrolipoic acid. Treatment of cells with 100 μM LA for 72 h markedly potentiated Fas-mediated apoptosis of leukemic Jurkat cells but not that of peripheral blood lymphocytes from healthy humans. In Jurkat, Fas activation was followed by rapid loss of cell thiols, decreased mitochondrial membrane potential, increased $[Ca^{2+}]_i$ and increased PKC activity; all these responses were potentiated in LA pretreated cells. PKCδ played an important role in mediating the effect of LA on Fas-mediated cell death. In response to Fas activation LA treatment potentiated caspase 3 activation by over 100%. The ability of LA to potentiate Fas mediated killing of leukemic cells was abrogated by a caspase 3 inhibitor suggesting that increased caspase 3 activity in LA-treated Fas-activated cells played an important role in potentiating cell death. This work provides first evidence showing that inducible caspase 3 activity may be pharmacologically up-regulated by reducing agents such as dihydrolipoic acid.

Keywords: antioxidant; calcium; caspase; cell death; protein kinase c; signal transduction

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium ion concentration; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CH11, agonistic Fas IgM Ab; DHLA, dihydrolipoate; D-PBS, Dulbecco's PBS, pH 7.4; Fas+, Fas bearing; FasL, Fas ligand; ICE, interleukin-1b converting enzyme; IMDM, Isocove's modified Dulbecco medium; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide; LA, α-lipoic acid or 6,8, thioctic acid; MBB, monobromobimane; PI, propidium iodide; PKC, protein kinase C; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT mediated nick end labeling

Introduction

The transmembrane Fas Ag is a member of the tumor necrosis factor/nerve growth factor receptor family which can trigger apoptosis. Interaction between Fas-Fas ligand (FasL)[2] transduces apoptotic signals in sensitive target cells. This pathway to induce programmed cell death has been suggested to be of potential use in cancer treatment. Treatment with anti-Fas Ab has been shown to suppress the growth of Fas bearing (Fas+) tumor cells.[1] Also, malignant glioma cells are susceptible to Fas mediated apoptosis triggered by agonistic Ab.[2] The killing of myelogenous leukemia cells by the Fas/FasL pathway has the remarkable potential of serving as a novel and effective approach for leukemia immunotherapy.[3] Proliferation of vascular smooth muscle cells in response to injury plays a central role in the pathogenesis of vascular disorders. FasL gene transfer to the wall of blood vessel induced apoptosis of Fas+ vascular smooth muscle cells and inhibited neointima formation in injured rat carotid artery.[4] Thus, Fas mediated apoptosis is expected to have marked therapeutic potential in certain disorders.

In contrast to the apoptosis triggering role of reactive oxygen species observed in several cell systems [5,6] superoxide anion functions as a natural inhibitor of Fas mediated cell death.[7] Hydrogen peroxide has been also observed to delay Fas mediated apoptosis.[8] Nitric oxide, another reactive species, is also known to inhibit Fas mediated apoptosis.[9] Activation of cysteine death proteases, caspases, is required for commitment to Fas mediated apoptosis.[10] Recent studies have hypothesized that maintenance of a reducing environment inside the cell is necessary for caspase activity during apoptosis.[8] Consistently, chemical agents that block or cause oxidation of intracellular thiols have been shown to inhibit Fas mediated apoptosis.[11,12] There is no report, however, showing that increased reducing environment of the cell could facilitate Fas mediated apoptosis.

Intracellular thiols are known to play a central role in the regulation of redox sensitive signal transduction.[13,14] α-Lipoic acid (LA), also known as thioctic acid (1,2 dithiolane-3-pentanoic acid), is a sulfur containing antioxidant that is widely used as a dietary supplement and as a drug to treat diabetic complications.[15] Trace amounts of LA naturally occurring in human tissues are present in α-keto acid dehydrogenase complexes where it is covalently bound to a protein-lysyl residue.[16] Exogenously supple-

mented LA is readily taken up by a variety of cells and tissues where it is rapidly reduced by NADH or NADPH dependent enzymes to dihydrolipoate (6,8 dithiooctanoic acid, DHLA).[17,18] Significant accumulation of intracellular DHLA in LA treated Jurkat cells has been previously shown in several studies.[18–20] DHLA is a strong reductant with a two electron reduction potential of -0.32 V.[21] It is known to regenerate major physiological antioxidants of lipid and aqueous phases such as vitamin E, ascorbate and glutathione.[16] A remarkable ability of LA to enhance the content of reduced thiols in Jurkat T-cells and to thus alter intracellular thiol redox status has been found.[22] Because LA is a commonly used nutritional supplement and a clinical drug that is known to result in the intracellular generation of the potent reducing agent DHLA, we sought to investigate whether Fas mediated apoptosis in leukemic Jurkat cells could be potentiated by treatment with LA. Jurkat cell has been the most widely used model to study Fas-induced apoptosis and caspase 3 was first described in this cell line.[8,10,23–26] The possible effect of LA on Fas Ag activated peripheral blood lymphocytes (PBL) isolated from healthy human was also examined.

Results

Potentiation of Fas mediated apoptosis in leukemic Jurkat T-cells by α-lipoic acid

Following activation of Jurkat T-cells by the agonist Ab CH11 rapid externalization of membrane phosphatidylserine was observed. In cells pretreated with 100 μM LA for 72 h membrane phosphatidylserine externalization was markedly accelerated (Figure 1). Loss of cell viability following CH11 activation of Jurkat T-cells was a delayed response that followed membrane phosphatidylserine externalization. Until the 2 h time point following treatment of cells with CH11 no loss of cell viability was observed as studied by the propidium iodide exclusion assay. A remarkable loss of cell viability was noted in cells that were activated for 4 h. Consistent with the potentiating effect of LA on CH11 induced membrane

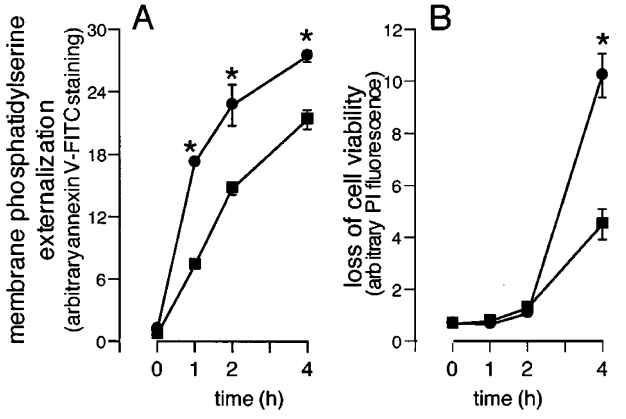

Figure 1 Potentiation of Fas mediated apoptosis in Jurkat cells by LA treatment. (**A**) Time course of membrane phosphatidylserine externalization; (**B**) loss of cell viability as determined by the inability of cells to exclude propidium iodide (PI). Cells were either not (squares) or treated (circles) with 100 μM LA for 72 h. Cells in culture were either not (0 h) or treated with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. Mean fluorescence values obtained from at least 10 000 cells are plotted. Data are mean $\pm$ S.D. Effect of LA treatment compared to the corresponding LA non-treated value: $^*P<0.001$

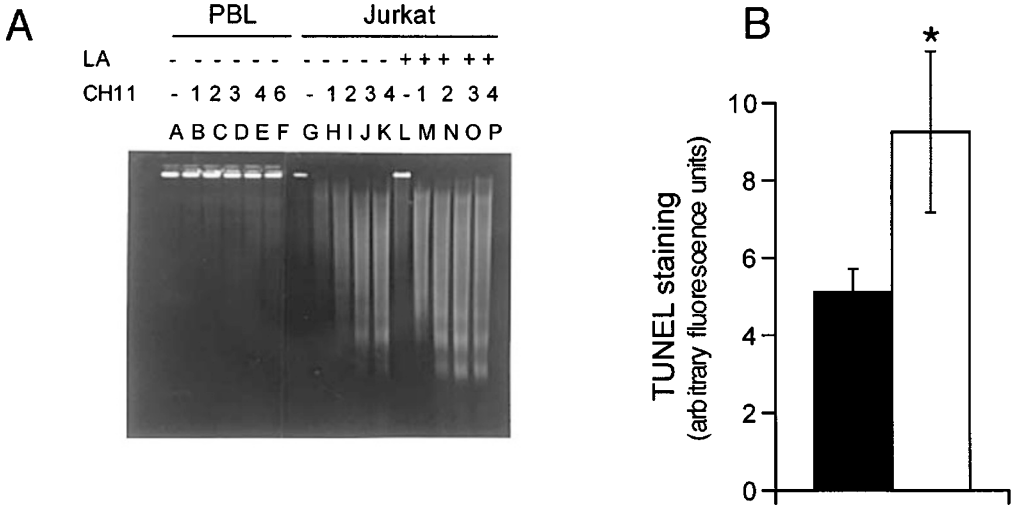

Figure 2 Potentiation of Fas mediated DNA fragmentation in Jurkat cells by LA treatment. (**A**) The effect of CH11 (200 ng/ml) treatment on DNA integrity of peripheral blood lymphocytes (lanes A–F) and Jurkat cells (lanes G–P). LA, 100 μM, 72 h before CH11 treatment. The duration of CH11 treatment is indicated in hours. (**B**) Quantitative analysis of the effect of LA treatment on Fas mediated apoptosis in Jurkat cells. Cells were either not (solid bar) or treated (open bar) with 100 μM LA for 72 h. After this, cells were treated with 200 ng/ml of the agonistic Ab CH11 for 4 h. DNA fragmentation was quantitated by terminal deoxynucleotidyl transferase mediated nick end labeling. Mean $\pm$ S.D. of arbitrary mean fluorescence units are plotted. $^*P<0.001$ compared to the corresponding LA non-treated cells

phosphatidylserine externalization, LA treated Jurkat cells were more susceptible to Fas receptor activation dependent death compared to cells that were not treated with LA (Figure 1). A dose (25 – 100 μM) dependent effect of LA on potentiating Fas mediated Jurkat cell death was observed (not shown).

Under the conditions used in this study the agonist Ab that activates the Fas receptor, CH11, did not trigger apoptosis in PBL. This was confirmed by phosphatidylserine staining, propidium iodide dependent cell viability assay (not shown) as well as DNA integrity assay on agarose gel. Also, LA alone or in combination with CH11 did not influence DNA integrity in PBL (Figure 2A). However, in Jurkat cells CH11 treatment resulted in rapid loss of DNA integrity and laddering. The kinetics of this apoptosis response was clearly accelerated in cells that were pretreated with LA for 72 h (Figure 2A). The potentiating effect of LA on CH11 induced DNA fragmentation in Jurkat cells was quantitatively confirmed using the TUNEL assay (Figure 2B) as well as by the flow cytometric determination of DNA fragmentation (Figure 3).

Fas receptor expression in lymphocytes freshly isolated from healthy human and acute leukemic Jurkat cells

Immunostaining for the presence of Fas receptor showed that while approximately 35% of PBL stained positive almost 100% of Jurkat cells contain the receptor (Figure 4A). When PBL and Jurkat cells were stained to study the abundance of the receptor, it was observed that the density of Fas receptor on the Jurkat cells was much higher than that in PBL. LA treatment to PBL or Jurkat did not influence the expression of Fas (Figure 4B).

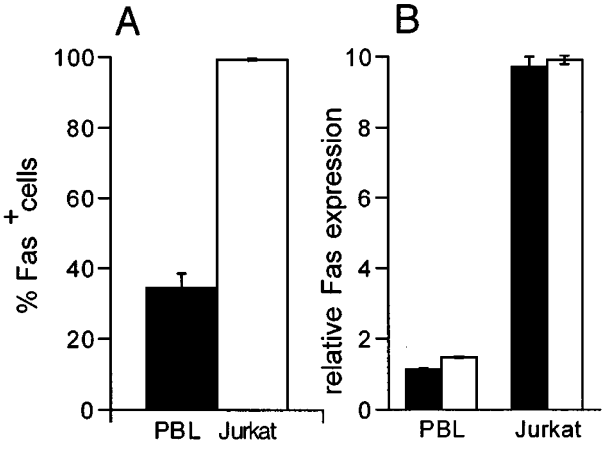

Figure 4 Expression of Fas Ag in peripheral blood lymphocytes (PBL) and Jurkat cells. (**A**) Illustrates the percentage of the whole population of cells that stained positive for the presence of the Fas Ag. (**B**) Illustrates the total amount of Fas Ag present in PBL and Jurkat. Cells were either not (open) or treated (solid) with 100 μM LA for 72 h. Mean $\pm$ S.D. of arbitrary mean fluorescence units are plotted

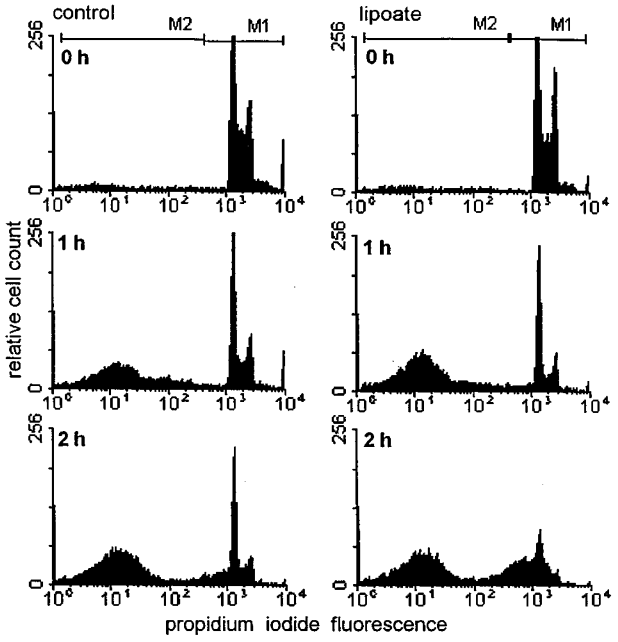

Figure 3 Flow cytometric determination of DNA fragmentation in Jurkat cells. Cells were either not (left column) or treated (right column) with 100 μM LA (lipoate) for 72 h. After this, cells were either not (top row) or treated (middle and bottom row) with 200 ng/ml CH11 for the indicated time interval. Propidium iodide fluorescence was collected from permeabilized cells. Intact DNA (region M1) and fragmented DNA (region M2) signals are shown in the histogram

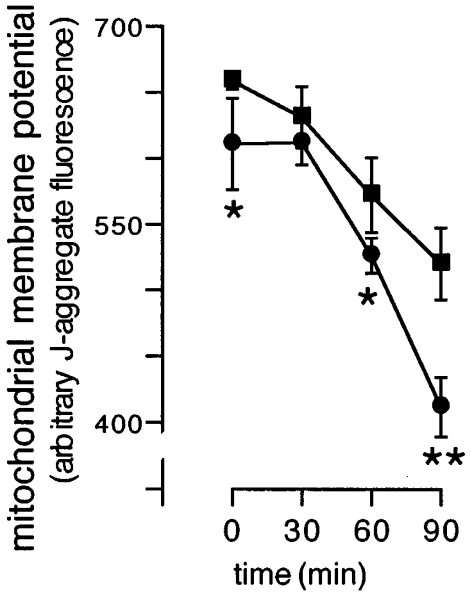

Figure 5 Mitochondrial membrane depolarization following activation of the Fas receptor in Jurkat cells. Cells were either not (square) or treated (circle) with 100 μM LA for 72 h. After this treatment, cells were treated or not (0 min) with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. Mitochondrial membrane potential was measured on the basis of J-aggregate formation in JC-1 loaded cells. LA treatment accelerated CH11 induced loss of membrane potential. $^{*}P<0.05$ and $^{**}P<0.001$ compared to corresponding LA non-treated cells

Mitochondrial function

Loss of mitochondrial membrane potential as indicated by J-aggregate formation was one of the earliest intracellular changes following CH11 induced Fas receptor activation. Within 1 h of such activation a marked loss of mitochondrial membrane potential was observed. Cells pretreated with 100 μM LA for 72 h showed a significantly lower mitochondrial function. Following 90 min of Fas-receptor activation, mitochondrial function was significantly lower in LA-treated cells compared to that in non-treated control cells (Figure 5).

Intracellular Ca^{2+} changes

In CH11 induced Fas activated Jurkat cells, the loss of mitochondrial function was associated with increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$). Figure 6A illustrates graphic representation of results obtained from indo-1 loaded cells. The X-axis represents cell size, and the Y-axis represents unbound/bound indo-1 ratio. A downward deflection of the signal along the Y-axis reflects increased $[Ca^{2+}]_i$; and a left-ward shift of the signal along the X-axis represents cell shrinking, a characteristic feature of apoptosis. Using this form of measurement we were able to directly relate changes in $[Ca^{2+}]_i$ with apoptosis. Figure 6Aa shows that under resting conditions the $[Ca^{2+}]_i$ is maintained at a relatively low level. After activation of cells with CH11, a clear downward shift of the signal is observed (Figure 6Ab) suggesting increasing $[Ca^{2+}]_i$ 60 min after activation. This effect is more remarkable 90 min after activation (Figure 6Ac) where elevation of $[Ca^{2+}]_i$ is followed by cell shrinking. The effect of CH11 on $[Ca^{2+}]_i$ of LA treated cells is shown (Figure 6Ac and f). LA treatment alone did not cause any significant change in baseline $[Ca^{2+}]_i$ (Figure 6Ad). However, CH11 induced increase in $[Ca^{2+}]_i$ was accelerated in LA treated cells compared to LA non-treated cells (compare Figure 6Ab *vs* Ae and Ac *vs* Af). This is more clearly evident from Figure 6B where the data have been quantitatively plotted. Figure 6Ae and Af clearly show a right-angle effect indicating that Fas activation induced cell shrinkage is preceded by elevation of $[Ca^{2+}]_i$.

Protein kinase C (PKC) activation

Elevated $[Ca^{2+}]_i$ in CH11 activated Jurkat cells was accompanied with marked activation of cellular PKC (Figure 7A). CH11 induced PKC activation was markedly enhanced in cells that were treated with LA. Rottlerin, a PKCδ inhibitor at the concentration used,[31] significantly inhibited CH11 induced loss of cell viability. In PKCδ inhibited cells, the ability of LA to potentiate Fas receptor mediated cell death was markedly diminished (Figure 7B).

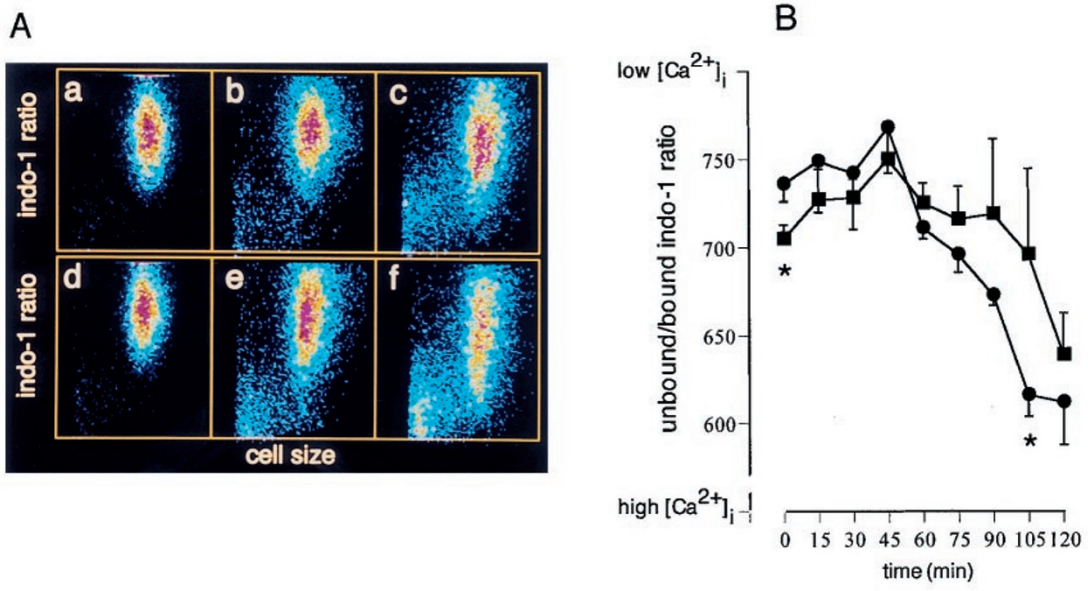

Figure 6 Increased intracellular Ca^{2+} in Fas activated Jurkat T-cells. (**A**) Dot plots showing that cell shrinking, a characteristic feature of apoptosis, is preceded by increase in intracellular Ca^{2+} in Fas activated cells. Cells were either not (**a**–**c**) or treated (**d**–**f**) with 100 μM LA for 72 h. After this treatment, cells were treated or not (**a** and **d**) with the agonistic Ab CH11 (200 ng/ml) for 1 h (**b** and **e**) or 1.5 h (**c** and **f**). In each frame, the X-axis represents forward scatter or cell size and the Y axis represents unbound/bound indo-1 ratio. A leftward shift of signal along the X-axis indicates decrease in cell size or shrinking, and a downward shift of the signal indicates increased intracellular Ca^{2+}. Experiments were carried out in the presence of 1 mM EGTA. Thus, increased intracellular Ca^{2+} is mainly contributed by Ca^{2+} mobilized from intracellular pools. The colours represent the relative cell number. From the violet to the red end of the visible spectrum colors represent progressively increasing cell densities. Intracellular Ca^{2+} was followed in indo 1 loaded cells. This illustration was prepared using the software Multiplus (Phoenix Flow Systems, San Diego, CA, USA). (**B**) Kinetics of increase in intracellular Ca^{2+} following activation of Fas. Cells were either not (square) or treated (circle) with 100 μM LA for 72 h. After this treatment, cells were treated or not (0 min) with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. Intracellular Ca^{2+} was estimated from indo-1 loaded cells as described above. Data are mean $\pm$ S.D. *$P<0.001$ compared to the corresponding LA non-treated cells

Cellular thiols

Almost concurrent with the loss of mitochondrial function and elevation of $[Ca^{2+}]_i$ loss of cellular thiols was observed in Fas activated Jurkat cells (Figure 8). Remarkable loss of total reduced protein thiol was a rapid response that reached a maximum within 80 min of Fas activation (Figure 8A). Activation of Fas also resulted in loss of GSH from the cells. Treatment of Jurkat cells with LA for 72 h increased cell GSH content. However, Fas activation dependent loss of GSH from

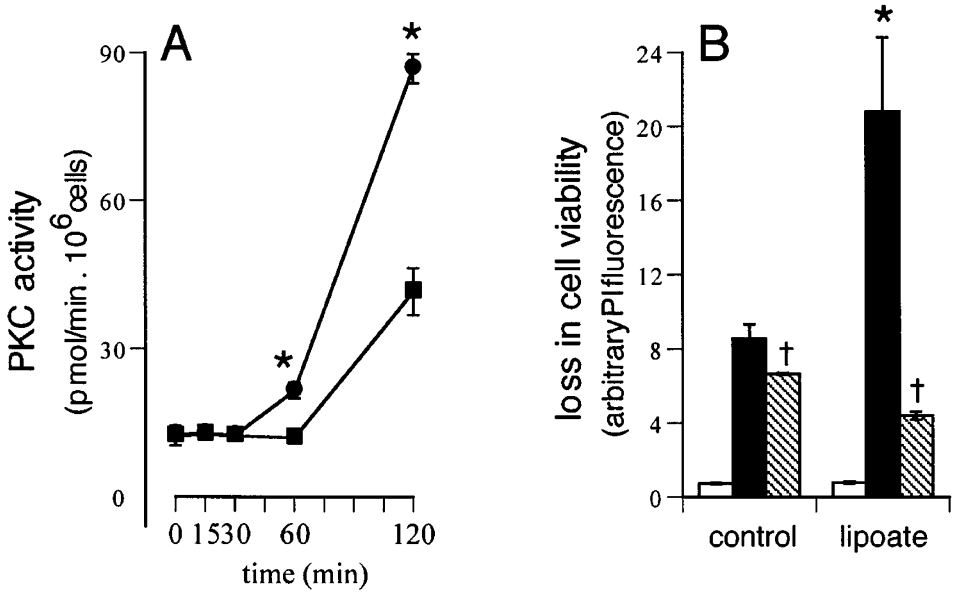

Figure 7 Protein kinase C (PKC) activity and its role in Fas mediated apoptosis of Jurkat cells. (**A**) Activation of PKC in Fas activated cells. Cells were either not (square) or treated (circle) with 100 μM LA for 72 h. After this treatment, cells were treated or not (0 min) with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. PKC activity was measured from permeabilized cells. *$P<0.001$ compared to LA non-treated cells. (**B**) The potentiating effect of LA on Fas mediated cell death was abrogated by inhibition of PKCδ activity. Cells were either not (control) or treated (lipoate) with 100 μM LA for 72 h. After this treatment, cells were treated or not (open bar) with the agonistic Ab CH11 (200 ng/ml) for 4 h in the absence (solid bar) or presence (hatched bar) of 10 μM of rottlerin, a PKCδ inhibitor with IC_{50} 3–6 μM. Rottlerin was added to cells 15 min before CH11 treatment. Loss of cell viability was determined on the basis propidium iodide staining of non-permeabilized cells. Mean fluorescence values are plotted. Data are mean $\pm$ S.D. *$P<0.001$ compared to the corresponding LA non-treated CH11 treated cells. †$P<0.001$ compared to the corresponding rottlerin non-treated CH11 treated cells

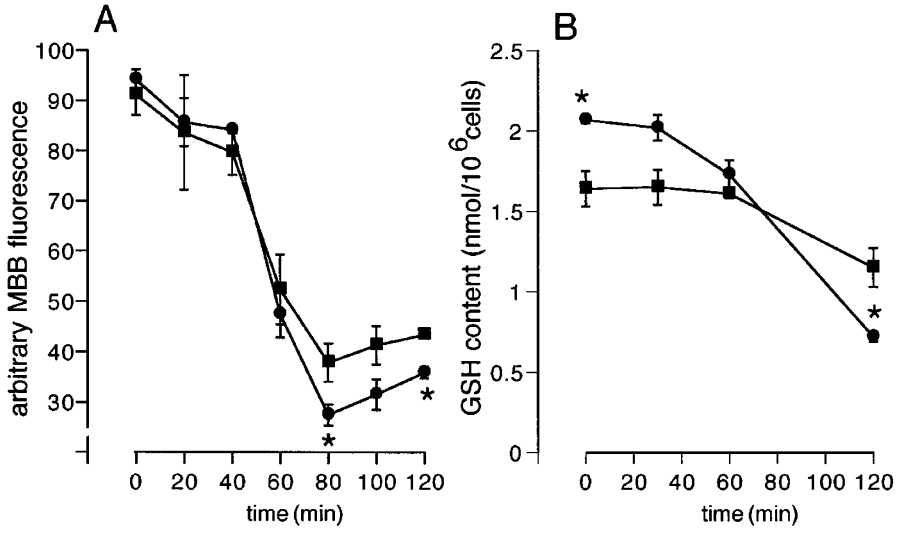

Figure 8 Loss of cellular thiols during Fas mediated apoptosis. Cells were either not (square) or treated (circle) with 100 μM LA for 72 h. After this treatment, cells were treated or not (0 min) with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. (**A**) Loss of total protein thiols as determined by monobromobimane staining. (**B**) Loss of cellular GSH. Data are mean $\pm$ S.D. *$P<0.001$ compared to the corresponding LA non-treated cells

LA treated cells was faster compared to that from LA non-treated cells (Figure 8B).

Caspase 3 activation

Activation of the Fas receptor by treatment of cells with the agonistic Ab CH11 resulted in rapid increase in the activity of the cysteine protease caspase 3 (Figure 9A). A remarkable observation was that pretreatment of Jurkat cells with LA markedly potentiated CH11 induced caspase activation. LA treatment doubled CH11 induced caspase activation that was observed after 1 or 2 h of Fas receptor activation (Figure 9A). These activity results were confirmed by data from Western blots showing enhanced CH11 induced cleavage of the

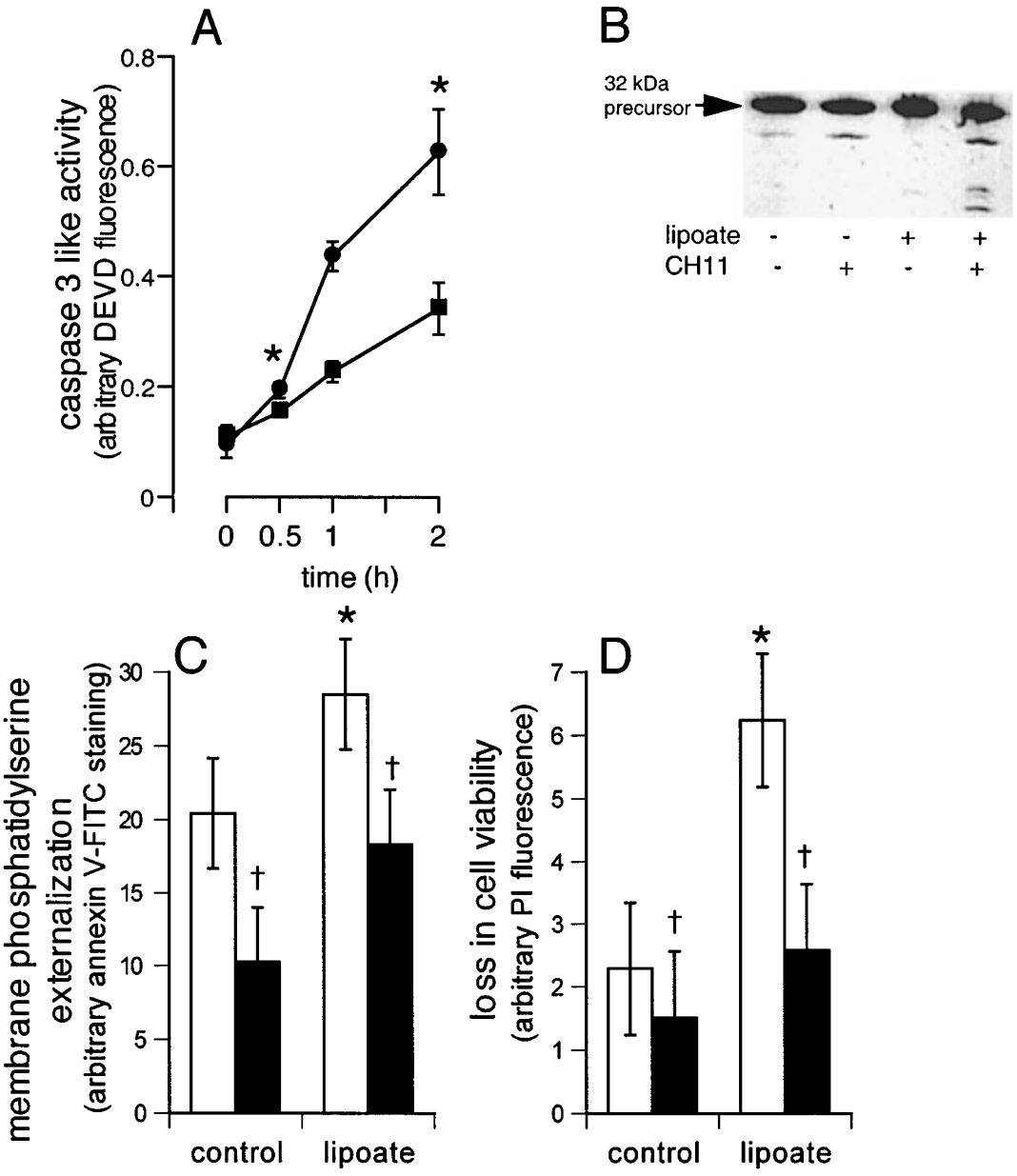

Figure 9 Role of caspase 3 in Fas mediated apoptosis and its potentiation by α-lipoic acid. (**A**) Jurkat cells were either not (square) or treated (circle) with 100 μM LA for 72 h. After this treatment, cells were treated or not (0 min) with the agonistic Ab CH11 (200 ng/ml) for the indicated duration. *$P<0.001$ compared to the corresponding LA non-treated cells. (**B**) CH11 induced activation of caspase 3 protein as observed by cleavage of the native protein in Jurkat cells. LA treatment (lipoate; 100 μM LA, 72 h) potentiated CH11 induced (200 ng/ml, 1 h) caspase 3 cleavage. (**C**) Treatment of Jurkat cells with 25 μM of caspase-3 inhibitor II (solid bars) inhibited membrane phosphatidylserine externalization in both LA non-treated and LA treated cells. (**D**) Caspase-3 inhibitor II inhibited CH11 induced loss of Jurkat cell viability. This effect was more pronounced in LA treated cells. In the presence of caspase-3 inhibitor II the potentiating effect of LA on Fas mediated apoptosis was completely abrogated. Loss of cell viability was determined on the basis propidium iodide staining of non-permeabilized cells. In line and bar graphs shown data are mean $\pm$ S.D. *$P<0.001$ compared to the corresponding LA non-treated cells; †$P<0.01$ compared to the corresponding caspase-3 inhibitor II non-treated cells

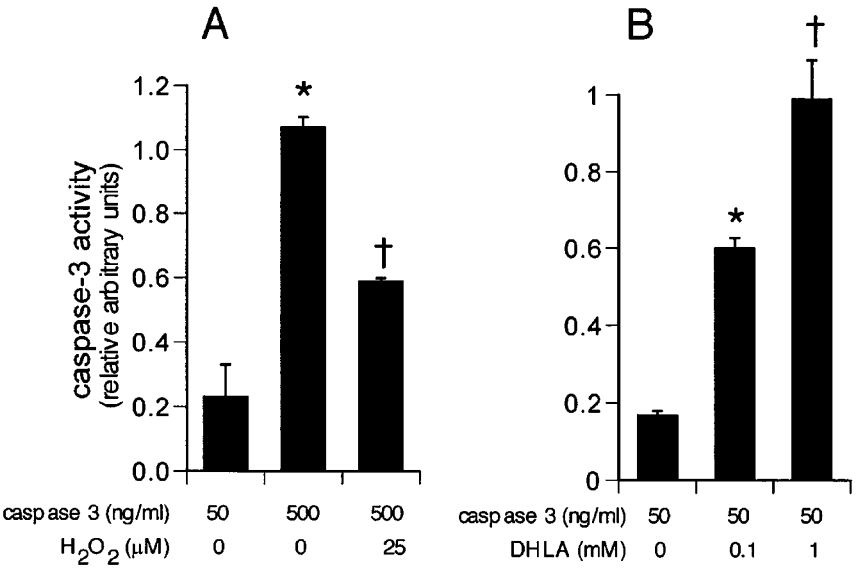

Figure 10 Dihydrolipoate potentiates the activity of purified active recombinant human caspase 3. (**A**) Dose-dependent activity of active recombinant human caspase 3 and inhibition of activity in the presence of hydrogen peroxide. Activity of the protein (at the indicated concentrations) was measured in the absence or presence of 25 μM hydrogen peroxide in the assay mixture. *$P<0.001$ higher compared to the activity of 50 ng/ml protein; †$P<0.001$ lower compared to the activity of 500 ng/ml protein. (**B**) Activity of 50 ng/ml of caspase 3 was measured in the absence or presence of dihydrolipoate (DHLA) as indicated in the figure. *$P<0.001$ higher compared to the activity of 50 ng/ml protein; †$P<0.001$ higher compared to the activity of 50 ng/ml protein in the presence of 100 μM DHLA

caspase 3 protein in LA treated cells compared to LA non-treated cells (Figure 9B). In cells that were treated with a caspase 3 inhibitor, CH11 induced apoptosis as indicated by externalization of membrane phosphatidylserine and loss of cell viability was significantly inhibited. In these inhibitor treated cells, the potentiating effect of LA on Fas mediated apoptosis was markedly diminished (Figure 9C and D).

To test whether LA or DHLA may directly influence the activity of caspase 3 the activity of 50 ng/ml of the recombinant active enzyme was studied. When used at adequate concentration this enzyme was observed to have high activity that was inhibited in the presence of an oxidant, hydrogen peroxide (Figure 10A). DHLA markedly stimulated the activity of recombinant caspase 3 (Figure 10B). However, the presence of LA in the enzyme activity assay mixture did not influence caspase 3 activity (not shown).

Discussion

Potentiation of Fas mediated cell death

Signal transduction pathways leading to apoptosis have been of outstanding interest in biotechnology mostly because successful apoptotic agents could in principle treat diseases like cancer with greater specificity and few side effects.[32] Therapeutic potential of the Fas/FasL system has been evident in several studies.[1–3,33] We observed that the agonistic anti-Fas Ab did not induce apoptosis in PBL isolated from healthy human although 30% cells tested Fas+. Comparing results on the presence (Figure 4A) and abundance (Figure 4B) of Fas receptors in Jurkat and PBL it is evident that the density of Fas receptor in PBL was many-fold lower. Previously it has been shown that a critical level of expression of Fas/APO-1 is a prerequisite for induction of apoptosis.[34] LA showed a remarkable ability to potentiate Fas mediated cell death in leukemic Jurkat cells, but not in healthy PBL. Previously chemotherapeutic agents such as doxurubicin and vincristine, and the alkaloid taxol have been shown to facilitate Fas mediated cell death.[2,33] Doxorubicin, vincristine and taxol are anti-tumor drugs that are used for cancer therapy. At high concentrations these agents *per se* are toxic to cells. In contrast, LA is a safe nutrient mostly known for its ability to bolster cellular glutathione levels, alter intracellular redox state and help protect against diabetic complications.[15,17,22] This work presents first evidence showing that the redox active agent, LA, may potentiate Fas mediated death in leukemic Jurkat cells. This observation is consistent with a previous report showing that reactive oxygen species such as superoxide anion functions as a natural inhibitor of Fas mediated cell death [7] since DHLA is known to quench superoxide anions.[16]

Redox regulation of caspase activity

Because the potentiating effect of LA treatment on Fas mediated apoptosis was observed in one of the earliest markers of apoptosis, externalization of membrane phosphatidyl serine, it might be suspected that LA regulates one or more early intracellular events. Expression of the Fas receptor was not influenced by LA treatment suggesting that LA may have influenced intracellular events signaling for apoptosis. An early event in Fas mediated apoptosis that was strikingly influenced by LA treatment was the activation of the cysteine death protease CPP32 or caspase 3. The potentiating effect of LA treatment on Fas mediated apoptosis of Jurkat cells was markedly decreased by a caspase 3 inhibitor indicating that

indeed increased caspase 3 activity in LA-treated Fas-activated cells played a significant role in potentiating cell death. Caspases are known to play a central role in Fas mediated apoptosis of Jurkat cells.[10] Fas sequentially activates caspase-like proteases that lead to nuclear damage.[35] It has been suggested that the regulation of caspase activity may be of significance in pathologies where manipulation of apoptosis is expected to have therapeutic significance.[10]

The putative active site of caspase 3 contains a cysteine residue.[23] In several signaling proteins active site cysteine residues have been shown to be a redox sensitive site.[13,14] Indeed caspase 3 has been shown to be redox sensitive in a number of studies. This information, however, has been derived from studies where oxidants and thiol-blocking agents down-regulated caspase 3 activity.[8,9,11,12] Previously it has been reported that low concentration of hydrogen peroxide inhibits caspase activity in Jurkat cells.[8] Consistently we observed that the activity of purified caspase 3 protein was inhibited by hydrogen peroxide. This study establishes the other side of the same coin showing that indeed caspase 3 activity may be also potentiated by intracellular reducing agents such as DHLA. This is the first study showing that inducible caspase 3 activity may be up-regulated pharmacologically.

In this work LA, a commonly used nutritional supplement and clinical drug, was used as a tool to manipulate cellular redox status. LA is rapidly taken up by cells and reduced enzymatically to DHLA.[16–18] The DHLA/LA redox couple has a strong reducing power with -0.32 V as the reduction potential. Reduction potential of this redox couple is stronger than all other endogenous redox couples such as GSH/GSSG or NADH/NAD^+.[21] The ability of DHLA to reduce protein thiols e.g. thioredoxin has been reported.[14,16] Thus, the use of LA provides a valid model to test the effect of an uniquely potent intracellular reducing agent DHLA on caspase activation. Previously it has been hypothesized, but not established, that the maintenance of a reducing environment inside the cell is necessary to allow for adequate caspase-activity during apoptosis.[8] Our results showing potentiated activity response of caspase following Fas activation of LA treated cells lend firm support to this hypothesis and reveal the possibility that intracellular caspase activity in Fas activated cells may be up-regulated by a potent intracellular reducing agent. This contention is further supported by our observation showing that the activity of active recombinant caspase 3 is markedly upregulated in the presence of DHLA. In Jurkat cells phosphatidylserine externalization following Fas activation is dependent on death protease activation.[25] Thus, accelerated externalization of phosphatidylserine in LA-treated Fas-activated cells may be because of higher death protease activity in these cells compared to the corresponding LA non-treated cells.

Loss of mitochondrial membrane potential and increase in $[Ca^{2+}]_i$

Fas receptor activation was followed by rapid loss of mitochondrial membrane potential. LA may have caused a more significant loss of mitochondrial function 90 min after Fas-activation via increased caspase 3 activity. Death protease activity has been shown to contribute to events leading to the loss of mitochondrial function in Fas activated Jurkat cells.[24] Caspase activity also disrupts mitochondrial barrier function.[36] Following Fas receptor activation, activity of caspase 3 in LA treated cells was markedly higher than that in LA non-treated cells. Loss of mitochondrial membrane potential in Fas activated Jurkat cells was accompanied by a marked increase in $[Ca^{2+}]_i$. Our results show that after Fas activation cell shrinking is preceded by increased $[Ca^{2+}]_i$ suggesting a possible role of $[Ca^{2+}]_i$ in this type of apoptosis. Ca^{2+} is released from functionally compromised mitochondria.[37] Other sources such as Ca^{2+} release from sarcoendoplasmic reticulum pool may not be ruled out, however. After 2 h of Fas activation, $[Ca^{2+}]_i$ may increase to 500 nM–1 μM.[38] At such a concentration range, $[Ca^{2+}]_i$ may cause caspase-3 activation.[39] Thus, loss of mitochondrial function may be visualized as a part of a vicious cycle set on by caspase 3 activity, followed by increased $[Ca^{2+}]_i$, which in turn further potentiates the activity of caspase-3.

Involvement of protein kinase C activity

Our results show that increased $[Ca^{2+}]_i$ in Fas activated Jurkat cells is associated with marked activation of PKC. Activation of PKC in Fas activated cells was markedly potentiated in response to LA treatment. Because LA treatment also potentiated Fas mediated apoptosis these results suggest that Fas mediated PKC activation may have contributed to the apoptosis process. To verify this hypothesis we sought to test whether PKC activity inhibition prevents Fas mediated apoptosis. Because most inhibitors of PKC activity are toxic our intent was to find an inhibitor that would be safely tolerated by cells. Rottlerin, a PKCδ inhibitor (IC_{50} 3–6 μM), matched this criteria. Our observation that rottlerin significantly inhibited Fas mediated apoptosis in LA non-treated cells suggests that PKCδ activity may be involved in mediating Fas mediated apoptosis in Jurkat cells. This contention is firmly supported by our observation in LA treated cells showing that the potentiating effect of LA on Fas mediated apoptosis of Jurkat cells is completely abrogated by rottlerin. Previous studies have shown that PKCδ is activated by CPP32 or caspase 3 at the onset of apoptosis induced by anti-Fas Ab. Such proteolytic activation of PKCδ is known to contribute to phenotypic changes associated with apoptosis.[40] Also, transfection of recombinant protein coding for the catalytic fragment of PKCδ results in the apoptotic morphology of cells and nuclei supporting that PKCδ activity is indeed involved in mediating Fas mediated apoptosis.[26]

In summary, this study provides first evidence showing that inducible caspase 3 activity may be pharmacologically up-regulated by intracellular reducing agents such as DHLA. Both mitochondria and PKCδ are targets of caspase 3 activity. In cells where the Fas receptor is activated, the loss of mitochondrial function is a part of a vicious cycle set on by caspase 3 activity, followed by increased $[Ca^{2+}]_i$, which in turn further potentiates the activity of caspase-3 leading to cell death.

Materials and Methods

Cell culture

Jurkat T-cell culture Human acute leukemic Jurkat T-cells (clone E6-1, American Type Culture Collection, ATCC, Bethesa, MD, USA) were grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 110 mg/L sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco, USA). Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C.

Isolation and culture of human peripheral blood lymphocytes Blood drawn from healthy male volunteers was collected in heparinized tubes. PBL were isolated by a standard density gradient separation on Ficoll-Hypaque (Pharmacia, Sweden) as described previously.[22] PBL were seeded at 1×10^6 cells/ml in RPMI 1640 culture medium containing 10% heat inactivated FCS and maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C.

Cell treatment and induction of apoptosis

Cells were resuspended in standard culture medium and seeded at a density of 1×10^6 cells/ml. Aqueous stock solution of LA (racemate mixture, ASTA Medica, Frankfurt, Germany) was prepared fresh for each experiment. As indicated in figure legends, cells were pretreated or not with 100 μM LA for 72 h. Apoptosis was induced by treating the cells in culture with 200 ng/ml of an agonistic anti-human Fas IgM Ab (clone CH11), Immunotech, Cedex, France). Stock solutions of caspase-3 inhibitor II (Calbiochem, La Jolla, CA, USA) and PKC-δ inhibitor, Rottlerin (Calbiochem, La Jolla, CA, USA), were prepared in DMSO.

Flow cytometric analyses

All flow cytometric assays were carried out using either XL or EPICS Elite (Coulter Corporation, Miami, FL, USA) instrument. The forward scatter and side scatter properties of cells were used to establish size gates and exclude cellular debris. In each sample at least 10 000 gated cells were examined.

Membrane phosphatidylserine externalization

To detect phosphatidyl serine externalization on the outer leaflet of plasma membrane, the cells were stained with annexin V coupled to FITC (Clonetech Inc., Palo Alto, CA, USA). Cells with FITC-conjugated annexin V were excited using a 488 nm argon ion laser and emission of FITC was recorded at 525 nm using a flow cytometer.

Cell viability

Plasma membrane integrity of all cells were determined flow cytometrically using the non-permeant DNA intercalating dye propidium iodide (PI, Molecular Probes, Eugene, OR, USA) that is generally excluded by viable cells. A 15 mW powered argon ion laser was used for excitation at 488 nm and PI fluorescence emission signal was collected at 575 nm. PI fluorescence values were used to estimate loss of cell viability.

Determination of DNA fragmentation in agarose gels

Cells (10×10^6) were suspended in 5 μl distilled water containing 50 mg/ml RNAse A. The suspension was incubated at room temperature for 20 min. After this, 5 μl of loading buffer (40% sucrose, 0.25% bromophenol blue) was added to the cell suspension. The sample was then electrophoresed through a digestion gel (0.8% agarose gel prepared with TBE [90 mM Tris, 2 mM EDTA and 90 mM boric acid, pH 8.4], 2% sodium dodecyl sulfate and 25 mg/ml proteinase K) followed by separation of DNA in 1.8% agarose gel. Electrophoresis was carried out in TBE at 20 V. Ethidium bromide stained DNA was visualized under UV illumination.

Flow cytometric determination of DNA fragmentation

This measurement was done using permeabilized cells as previously described [27] with some minor modifications. In brief, cells were washed with D-PBS and fixed for 20 min on ice by resuspending the pellet in 1% paraformaldehyde in D-PBS. The fixed cells were then centrifuged ($600 \times g$, for 5 min) and gently resuspended in a hypotonic propidium iodide solution (50 mg/ml in 0.1% w/v sodium citrate and 0.1% v/v Triton X-100). The suspension was incubated at 4°C in dark for 12–14 h before being analyzed by a flow cytometer. A 15 mV powered argon ion laser was used for excitation at 488 nm and emission was measured at 575 nm.

Expression of Fas Ag (CD95)

Cells were incubated with FITC labeled anti-human Fas (CD95) monoclonal Ab (Immunotech, Cedex, France) for 30 min at 4°C. Cells were then washed twice in Dulbecco's PBS, pH 7.4 (D-PBS) and finally resuspended in D-PBS. Expression of Fas was immediately determined using a flow cytometer. Appropriate isotype control was used to determine background fluorescence. Cells with FITC-conjugated antibodies were excited using a 488 nm argon ion laser and emission of FITC was recorded at 525 nm.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was measured flow cytometrically using 5,5,6,6,-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA).[28] This probe is able to selectively enter into mitochondria, exists in a monomeric form emitting at 527 nm (green fluorescence) after excitation at 490 nm. However, depending on the mitochondrial membrane potential, JC-1 is able to form J-aggregates that are associated with a large shift in emission (590 nm, red fluorescence). Thus, the color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized. For loading of JC-1, cells (1×10^6) were resuspended in 1 ml of medium and incubated with 10 μg/ml of JC-1 for 10 min at 37°C before analysis. Both red and green (not shown) fluorescence emissions were analyzed using the FL-1 and FL-2 channels of a flow cytometer.

Determination of changes in intracellular Ca^{2+}

Cells grown in 10% FCS containing medium either with or without LA were pelleted ($125 \times g$, 5 min) and resuspended in Isocove's Modified Dulbecco Medium (IMDM, Gaithersburg, MD, USA) and then activated with CH11. Calcium flux was measured in indo-1 (Molecular Probes,

Eugene, OR, USA) loaded cells as described previously.[20] Experiments were carried out either in the presence of 1 mM of the extracellular calcium chelator EGTA (Sigma, MO, USA) to follow CH11 induced mobilization of intracellular calcium reserves.

Determination of protein kinase C (PKC) activity from whole cells

Cells in culture were washed twice with D-PBS and resuspended in reaction buffer (5.2 mM $MgCl_2$, 94 mM KCl, 12.5 mM HEPES, 12.5 mM EGTA, and 8.2 mM $CaCl_2$, pH 7.4) into 200 μl portions (0.1×10^6 cells). The measurement of PKC activity was started by addition of streptolysin-O (0.3 IU, Sigma, St. Louis, MO, USA) mixed with [γ-^{32}P]ATP (300–450 c.p.m./pmol, final concentration 250 μM, DuPont, NEN, Boston, MA, USA), and 100 μM peptide substrate (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, Sigma, St. Louis, MO, USA). After 10 min incubation at 37°C, the reaction was stopped by addition of 100 μl of 25% trichloroacetic acid in 2 M acetic acid. The samples were left on ice for 10 min and then centrifuged to remove the proteins. Samples (25 μl each) were then spotted on phosphocellulose disc papers (Gibco BRL, Gaithersburg, MD, USA). The disc papers were washed twice with 1% phosphoric acid, and twice with deionized water. The total radioactivity on each disc was determined using liquid scintillation analyzer. The background phosphorylation in the absence of the substrate peptide was subtracted from all samples. To determine the specificity of results obtained for PKC, an additional measurement of the enzyme activity was done using the pseudosubstrate PKC peptide inhibitor (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn; Sigma, St. Louis, MO, USA) as reported previously.[29]

Flow cytometric determination of cellular sulfhydryls

Cellular sulfhydryls were determined flow cytometrically using the thiol probe monobromobimane (MBB, Molecular Probes, Eugene, OR, USA) as described previously.[22] MBB was dissolved in acetonitrile to obtain a 8 mM stock concentration. Cells were pelleted ($125 \times g$, $\times$ for 5 min) and resuspended in PBS (pH 7.4) at 10^6 cells/ml. MBB stock solution was added to the cell suspension such that the final concentration of the bimane reagent was 40 μM. Bimane loaded cells were excited using a 20 mW powered UV line of a Innova 90-4 argon ion laser (Coherent, Palo Alto, CA, USA) set at 350 nm in a flow cytometer. Fluorescent emission from cellular sulfhydryl reacted bimane was recorded using a 450 nm band pass filter.

HPLC determination of glutathione

Cells were pelleted ($125 \times g$, for 5 min) and deproteinized by treatment with 4% monochloroacetic acid. Following the acid treatment, the mixtures were snap-frozen in liquid nitrogen and stored at -80°C for the HPLC determination of GSH content using a coulometric detector as described previously.[18] The electrodes of the coulometric detector for GSH assays were set at following potentials: electrode 1, +0.40 V; electrode 2, +0.85 V; and guard cell, +0.90 V. GSH was separated using a C-18 column (150 mm long $\times$ 4.6 mm i.d., 5 μm pore size; Alltech, Deerfield, IL, USA) and a mobile phase consisting of 98% 50 mM NaH_2PO_4 (pH 2.7) and 2% acetonitrile. The flow rate was maintained at 1 ml/min throughout the analysis. Data were collected using a PE Nelson 900 series interface and analyzed using the software Turbochrom 3 (Perkin Elmer, San Jose, CA, USA).

CPP32 or caspase 3 like activity

Cell pellets were lysed in PBS containing 0.2% v/v Triton X-100 on ice for 10 min. The cell lysates were centrifuged at $10\,000 \times g$ for 5 min and clear supernatants were collected and placed on ice. The cell extracts (50 μg protein) were incubated with 60 μM of a fluorigenic caspase-3 substrate (Ac-DEVD-AMC, Calbiochem, La Jolla, CA, USA) in incubation buffer (5 mM dithiothreitol, 50 mM HEPES, 10% sucrose, 0.1% CHAPS, pH 7.5) for 20 min. The activity of purified active recombinant human caspase 3 protein (Pharmingen, San Diego, CA, USA) was studied using the above mentioned conditions except that the reaction mixture did not contain dithiothreitol. The reaction was halted with 10% sodium dodecylsulfate. The fluorescence was measured at 380 nm excitation and 460 nm emission.[30]

CPP32 or Caspase 3 immunoblot

For anti-caspase 3 immunoblots, cytosolic extracts of cells were separated on a 13% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose, and probed with mouse anti-CPP32 monoclonal Ab (Transduction Laboratories, Lexington, KY, USA). This was followed by proving with appropriate horseradish peroxidase coupled secondary antibodies (Transduction Laboratories, Lexington, KY, USA). Bound Ab was detected by enhanced chemiluminescence (ECL, Amersham, Cleveland, OH, USA).

Terminal deoxynucleotidyl transferase (TdT) mediated nick end labeling (TUNEL)

TUNEL assay was done using the MEBSTAIN® Apoptosis kit (Immunotech Cedex, France). Briefly, cells were washed with PBS and then fixed in 4% paraformaldehyde at 4°C for 30 min on ice. Cells were washed twice with PBS containing 0.2% BSA. Proteinase K was added to the cell pellet. Cells were incubated for 30 min at 37°C. Permeabilization of the cells was performed with 1 ml PBS containing 0.5% Tween and 0.2% BSA. Cells were incubated in TdT reaction reagent (TdT+biotin-dUTP) for 1 h at 37°C. After washing with PBS and blocking, cells were stained with avidin-FITC and analyzed using a flow cytometer.

Statistics

Data presented are mean $\pm$ S.D. or representative of at least three independent experiments. For flow cytometric measurements mean autofluorescence expressed in arbitrary units have been plotted. Difference between group means was tested by Students *t*-test. The minimum level of significance was set at $P < 0.01$.

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