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Ouabain exacerbates activation-induced cell death in human peripheral blood lymphocytes

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ABSTRACT

Lymphocytes activated by mitogenic lectins display changes in transmembrane potential, an elevation in the cytoplasmic Ca^{2+} concentrations, proliferation and/or activation induced cell death. Low concentrations of ouabain (an inhibitor of Na^{+},K^{+}-ATPase) suppress mitogen-induced proliferation and increases cell death. To understand the mechanisms involved, a number of parameters were analyzed using fluorescent probes and flow cytometry. The addition of 100nM ouabain to cultures of peripheral blood lymphocytes activated with 5μg/ml phytohemagglutinin (PHA) did not modify the increased expression of the Fas receptor or its ligand FasL induced by the mitogen. However, treatment with ouabain potentiated apoptosis induced by an anti-Fas agonist antibody. A synergy between ouabain and PHA was also observed with regard to plasma membrane depolarization. PHA *per se* did not induce dissipation of mitochondrial membrane potential but when cells were also exposed to ouabain a marked depolarization could be observed, and this was a late event. It is possible that the inhibitory effect of ouabain on activated peripheral blood lymphocytes involves the potentiation of some of the steps of the apoptotic process and reflects an exacerbation of the mechanism of activation-induced cell death.

Key words: lymphocytes, ouabain, PHA, apoptosis, membrane depolarization.

INTRODUCTION

Ouabain is a cardiotonic steroid that has been widely used, as well as other cardiac glycosides, in the clinic. The known mode of action of ouabain is through the inhibition of the Na^{+},K^{+} pump. The Na^{+},K^{+}-ATPase is associated to the plasma membrane and is responsible for the maintenance, in the interior of the cells, of elevated levels of K^{+} and low concentration of Na^{+} typical of the majority of mammalian cells. The pump uses the energy of hydrolysis of one ATP molecule to transport to the exterior of the cell three ions of Na^{+} in exchange of the entry of two K^{+} ions (Skou 1990).

Quastel and Kaplan (1968) were the first to describe the inhibition by ouabain of lymphocyte proliferation induced by PHA. Since then, a number of workers, including ourselves, has described the inhibitory effects of ouabain on lymphocyte proliferation induced by various stimuli such as mitogens (Szamel et al. 1981, Moraes et al. 1989, Olej et al. 1998), phorbol ester (Olej et al. 1994, Brodie et al. 1995), CD3 (Szamel et al. 1995), Interleukin-2 (Redondo et al. 1986) and calcium ionophore (Jensen et al. 1977).

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Very early signals such as the expression of *c-myc* (Olej et al. 1998) and CD69 (Pires et al. 1997) are increased in activated lymphocytes exposed to ouabain suggesting that these lymphocytes are originally responsive to stimulation. One explanation for this lack of proliferation in parallel with an apparent response to the stimulus could result from the fact that activated lymphocytes were following the apoptotic pathway as opposed to the proliferative pathway. Anner et al. (1994) showed that peripheral blood lymphocytes underwent cell death when exposed to the endogenous isoform of ouabain produced in the hypothalamus. Similarly, our group also described apoptosis in activated PBL exposed to commercial ouabain (Olej et al. 1998). Similarly, it has been reported that Jurkat T cells (a human lymphocyte cell line) underwent apoptosis after exposure to the drug (Orlov et al. 1999).

Lymphocytes are subjected to cell death checkpoints at many points during their lifespan. The process of activation-induced cell death (AICD) is a physiologic mechanism occurring at the end of immune responses essential to maintain the homeostasis of the immune system. Overexpression of the molecules Fas receptor and FasL is seen in stimulated lymphocytes and activation of Fas receptor by its ligand triggers the death program (Nagata and Goldstein 1995, Martin and Green 1995). Lack of this mechanism leads to autoimmunity and to the failure of limiting the expansion of an immune response by eliminating effector cells that are not longer needed (Lynch et al. 1995). The process of AICD involves reduction in cell size; activation of the caspase machinery via caspase 8 which in turn activates caspase 3 to initiate the degradation of a variety of substrates; increased intracellular calcium levels and activation of nucleases, leading to DNA degradation (Kaufmann and Hengartner 2001).

The expression of FasL is preceded and regulated by an increase of the transcription factor c-Myc (Genestier et al. 1999, Kasibhatla et al. 2000, Brunner et al. 2000). Our previous work (Olej et al. 1998) has shown that PHA-activated lymphocytes exposed to ouabain undergo apoptosis, and despite the fact that c-*myc* mRNA is increased in ouabain treated PHA-activated lymphocytes the expression of FasL in these cells was not investigated. On the other hand, the function of c-Myc in relation to Fas expression is controversial (de Alboran et al. 2001, Wang et al. 1998), and there are evidences that the transcription factor NFAT participate in the regulation of Fas expression in activated lymphocytes (Latinis et al. 1997).

The present work attempted to investigate the expression of Fas and Fas-L in mitogen activated human peripheral lymphocytes exposed to ouabain, a condition known to predispose these cells to apoptosis.

MATERIALS AND METHODS

Preparation of Peripheral Blood Lymphocytes

PBL were obtained by fractionating heparinized blood from healthy volunteers on ficoll-hypaque (hystopaque, Sigma-Aldrich) density gradient centrifugation. Briefly, 4ml of blood was layered on 3ml of hystopaque and spun down for 30 min at $400\times$ g. The PBL fraction was washed twice with 0.9% NaCl (7 min at $250\times$ g). Cell pellets were then resuspended at 10^6 cells/ml in RPMI 1640 (Sigma-Aldrich), supplemented with 5×10^{-5} M β-mercaptoethanol, 25mM Hepes, pH adjusted to 7.4 with NaOH, 60mg/l penicillin, 100mg/l streptomycin (all purchased from Sigma-Aldrich) and 10% fetal calf serum (FCS) (Gibco), inactivated at 56°C for 1h. The cell number was adjusted at 10^6 cells/ml and used in the specific assays.

Treatment of Cells with PHA and Ouabain

PBL at 10^6 cells/ml in supplemented RPMI medium with 10% FCS were incubated with 5μg/ml PHA (Sigma-Aldrich) in 96-well culture plates in a 5% CO_2 atmosphere at 37°C for 6, 24 and 72h, in the presence or absence of a freshly prepared solution of ouabain (100nM or 100μM) (Sigma-Aldrich) in RPMI medium. After this, cells were used for specific assays.

Analysis of FAS (CD95 or APO-1) and FAS Ligand (FasL or CD95L) Expression

Cells (10^6/ml) incubated for 6, 24, and 72h with or without PHA and/or 100nM ouabain, were spun down and the pellet was incubated with 1μg(2μl) of anti-human FAS-PE (clone number DX2) (BD Pharmingen™) or anti-human FAS-L (clone number NOK-1) (BD Pharmingen™), for 30 min at 4°C, washed twice in chilled PBS and resuspended in PBS containing 1% formaldehyde. For FasL analysis, after primary incubation, cells were spun down and incubation with a secondary antibody goat anti-mouse IgG-FITC human adsorbed (BD Pharmingen™) Analysis for surface immunofluorescence was performed on a flow cytometer.

Measurement of Plasma Membrane Potential with Oxonol – DiBAC$_4$(3)

Acute changes in the plasma membrane potential were measured by flow cytometry using bis-(1,3-dibutilbarbiturate) trimethine oxonol – DiBAC$_4$ (3) (Molecular Probes). Cells were incubated for 6 and 24h with or without PHA and/or 100nM or 100μM ouabain. Oxonol was prepared according to manufacturers instructions and diluted in RPMI to be used at a final concentration of 150nM. The incubation time with oxonol was 30 min at 37°C and 5% CO_2. As a positive control, we used 50mM KCl in the last 10 min of incubation. Analysis for surface immunofluorescence was performed on a flow cytometer.

Measurement of Mitochondrial Mass with Mitotracker Green

To investigate mitochondrial mass, we used the fluorescent probe mitotracker green FM (MTG) (Molecular Probes) which is a cell-permeant mitochondrion-selective dye that passively diffuses across the plasma membrane and accumulates in active mitochondria. PBL at 10^6 cells/ml were incubated for 6 and 24h with or without PHA and/or 100nM ouabain. The dye was diluted in DMSO, stored at −20°C (1mM) and used at a final concentration of 50nM in supplemented RPMI. The incubation time with MTG was 30 min. at 37°C and 5% CO_2. Samples were analyzed by flow cytometry.

Evaluation of Mitochondrial Membrane Potential with 3, 3-dihexyloxacarbocyanine iodide-DiOC$_6$(3)

To evaluate the mitochondrial membrane potential ($\Delta\Psi$m), we used 3,3-dihexyloxacarbocyanine iodide [DiOC$_6$(3)] dye (Sigma-Aldrich). Cells were incubated for 6 and 24h with or without PHA and/or 100nM ouabain. The dye was diluted in RPMI and used at a final concentration of 5nM. Cells were incubated with DiOC$_6$(3) for 40 min at 37°C and 5% CO_2. As a positive control, 50μM of the protonophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich) was used in the last 20 min of incubation. DiOC$_6$(3) and FCCP were diluted in ethanol and stored at −20°C (1mM). Samples were analyzed using a flow cytometer.

Measurement of Apoptosis by Annexin Binding and anti-Fas

Annexin V conjugated to fluorescein isothiocyanate (FITC) (TACS-Annexin kit – FITC, RD systems) was used to determine changes in phosphatidylserine symmetry. 10^6 cells/ml were incubated for 6 or 24h with or without PHA and 100nM ouabain. At the last 2h of incubation, 1μg of anti-FAS-PE antibody (clone number DX2) (BD Pharmingen™) was added. Cells were spun down and incubated for 30 min at 4°C with a secondary antibody rat anti-mouse Kappa-PE (clone number X36) (BD Pharmingen™). After this time, cells were spun down again and incubated for 15 min at room temperature in the presence or absence of 1μl Annexin V-FITC (diluted according to the manufacturer instructions). Samples were analyzed by flow cytometer FAS-PE versus Annexin V-FITC.

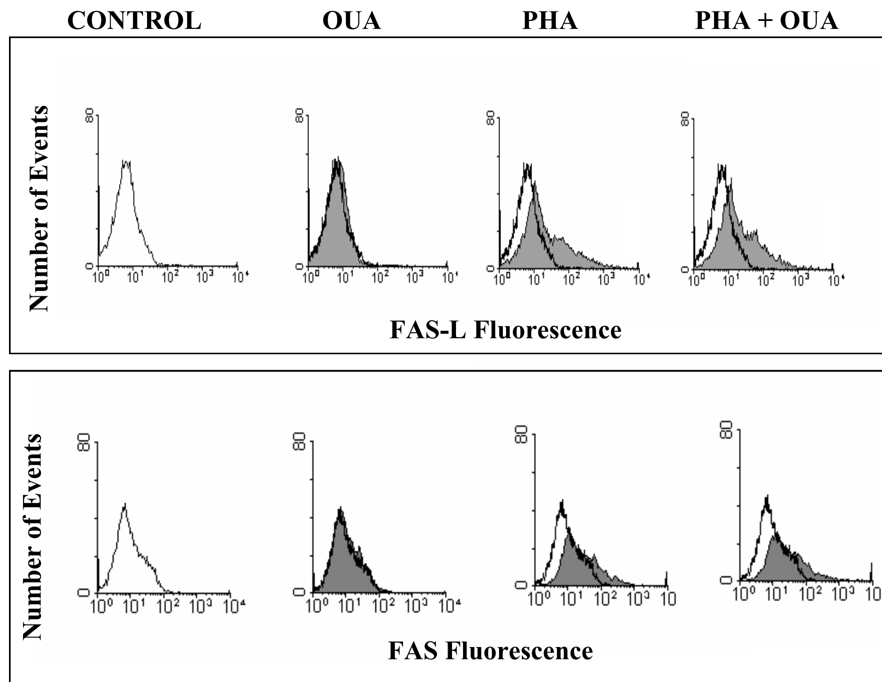

Fig. 1 – FasL and Fas expression in PBL cultured for 6h with or without 5μg/ml PHA and/or 100nM ouabain. The black line profile indicates control (untreated cells) and the gray histograms indicate cells exposed PHA and/or OUA. The figure is representative of five experiments of FasL and representative of thirteen of Fas.

Analysis by Flow Cytometry

10^6 cells were incubated for the indicated time and cell size and complexity analyzed by flow cytometry using light scatter parameters: forward scattering (FSC) and side scattering (SSC). For analysis using fluorescent probes, samples were kept on ice up to the moment of the analysis. The final volume of incubation was 300μl. All samples were analyzed using a FACScalibur flow cytometer. The fluorescence was excited with an argon laser (excitation wavelength 488nm) and analyzed in FL-1 (wavelength 530$\pm$30nm – Annexin V, FasL, MTG, $DiOC_6(3)$ and oxonol) or in FL-2 (wavelength 585nm – CD95PE). At least 10,000 events were acquired. A life-gate based on light scatter parameters (forward and side scatter) and staining with 1μg/ml PI was done. Dead cells, aggregates and debris were excluded. Data were recorded on a Macintosh computer using Cell Quest Software and statistically analyzed using the WinMDI version 2.8 software.

Statistical Analysis

Results were reported as mean $\pm$ SE. Data were analyzed statistically by the Student's t-test and differences between means were consider to be significant when $P < 0.05$.

RESULTS

Effect of Ouabain on Fas and FasL Expression by PHA-activated Lymphocytes

Mitogen activated cells show an increase in Fas expression. It has been reported that stimulation of these receptors induce the activation of Cl^- channels in lymphoid Jurkat cells (Szabò et al. 1998, Lepple-Wienhues et al. 1998). In our experiments an early rise of Fas expression could be observed within 6h of incubation on PHA-activated lymphocytes. The addition of 100nM ouabain to cells exposed to PHA did not significantly affect Fas or FasL expression. After 24h of incubation, the levels of Fas expression reached similar levels to those at

TABLE I

Percentage of cells expressing Fas receptors after incubation with PHA and 100nM ouabain (OUA).

Time (h) / Treatment	6 (13)	25 (4)	72 (3)
CONTROL	34.4 ± 4.4	43.5 ± 8.7	55.7 ± 0.9
OUA	36.2 ± 4.1	44.8 ± 10.2	56.4 ± 2.0
PHA	$55.8 \pm 4.5^{\#}$	$78.6 \pm 7.1^{\Psi}$	$89.5 \pm 3.9^{\#}$
PHA + OUA	$62.7 \pm 5.3^{*}$	$88.3 \pm 4.9^{*}$	$80.3 \pm 3.6^{*}$

Results represent mean ± standard error (SE). Number of experiments are indicated between brackets. $^{\#}P < 0.01$ and $^{\Psi}P < 0.05$ both compared to control group. $^{*}P > 0.05$ compared to PHA group (Student's t-test).

72h and no difference could be observed between PHA-activated lymphocytes and activated cultures that received ouabain (Fig. 1 and Table I). FasL was only studied at 6h and similar to what was seen with Fas, no difference could be observed between PHA-activated lymphocytes and activated cultures that received ouabain (Fig. 1 and Table II).

TABLE II

Percentage of cells expressing FasL receptors after incubation with PHA and 100nM ouabain (OUA).

Time / Treatment	6h (5)
CONTROL	7.5 ± 1.6
OUA	9.1 ± 2.8
PHA	$38.8 \pm 5.3^{\#}$
PHA + OUA	$41.6 \pm 4.1^{*}$

Results represent mean ± standard error (SE). Number of experiments are indicated between brackets. $^{\#}P < 0.01$ compared to control group. $^{*}P > 0.05$ compared to PHA group (Student's t-test).

Effect of Ouabain on anti-Fas-induced Apoptosis

It has been demonstrated that ouabain is capable of potentiating some of the effects observed during anti-Fas-induced apoptosis in Jurkat lymphocytes (Bortner et al. 2001, Nobel et al. 2000). In the present work the addition of anti-Fas to PHA-activated lymphocytes cultured in the presence of ouabain, resulted in an increased amount of apoptotic cells. Furthermore, the majority of dead cells were CD95 positive cells (Fig. 2).

Effect of Ouabain on Plasma Membrane Depolarization of PHA-activated Lymphocytes

It has been described (Kiefer et al. 1980) that mitogens are capable of inducing plasma membrane depolarization, although the role played by such a process during activation is not fully understood. In other systems, prevention of depolarization using a K^+ channel blocker inhibits subsequent apoptosis (Maeno et al. 2000, Dallaporta et al. 1999). Being ouabain an inhibitor of Na^+,K^+ATPase it could be exerting its effect through plasma membrane depolarization. In our hands, the effect produced by ouabain was dose dependent; 100nM was incapable of inducing plasma membrane depolarization, while

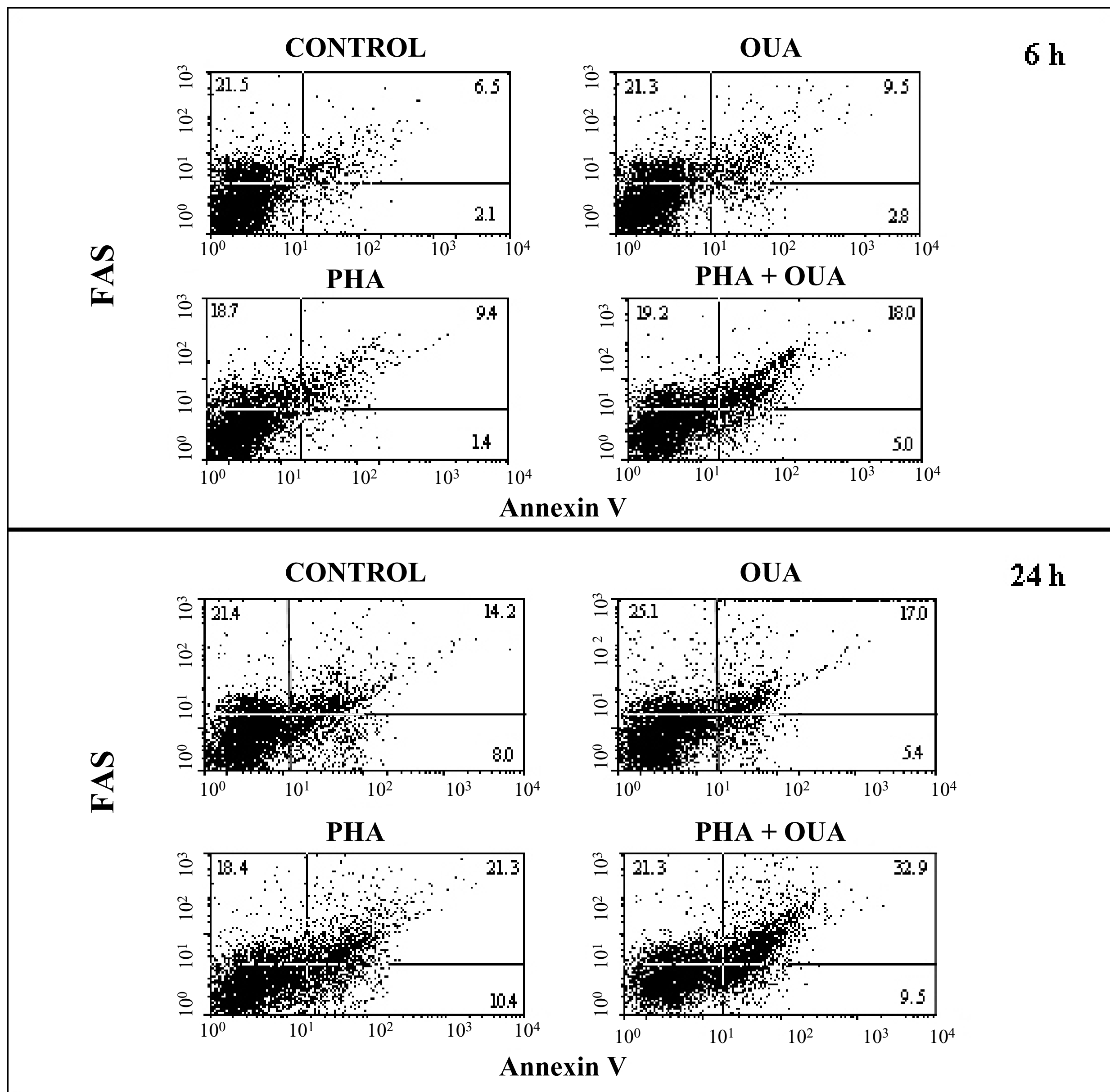

Fig. 2 – Apoptosis induction by anti-Fas antibody following 5μg/ml PHA and 100nM ouabain treatment. Lymphocytes were treated with 5μg/ml PHA and/or 100nM ouabain for 6 or 24h. After that period, cells were exposed to anti-Fas antibody for 2h. Apoptotic cells were measured using Annexin V. The sum of the percentage of cells in the right hand side indicate the total annexin V positive cells induced by anti-fas The quadrants show Annexin V versus Fas fluorescence. This figure is representative of four experiments.

100μM ouabain promoted depolarization. However, both 100nM and 100μM were capable of augmenting the degree of depolarization observed in the presence of PHA (Fig. 3 and Table III). PBL from 6h cultures always presented a smaller second peak that increased when PHA was used and was not formed by macrophages or dead cells measured by propidium iodide incorporation (data not shown).

Effect of Ouabain on Mitochondrial Mass and Mitochondria Membrane Potential

Mitochondrial mass was assessed using mitotracker green dye. As early as 6h post-PHA stimulus there was a 30.3% increase of mitochondrial mass in activated PBL and at 24h this increase was 50%. The addition of 100nM ouabain slightly inhibited

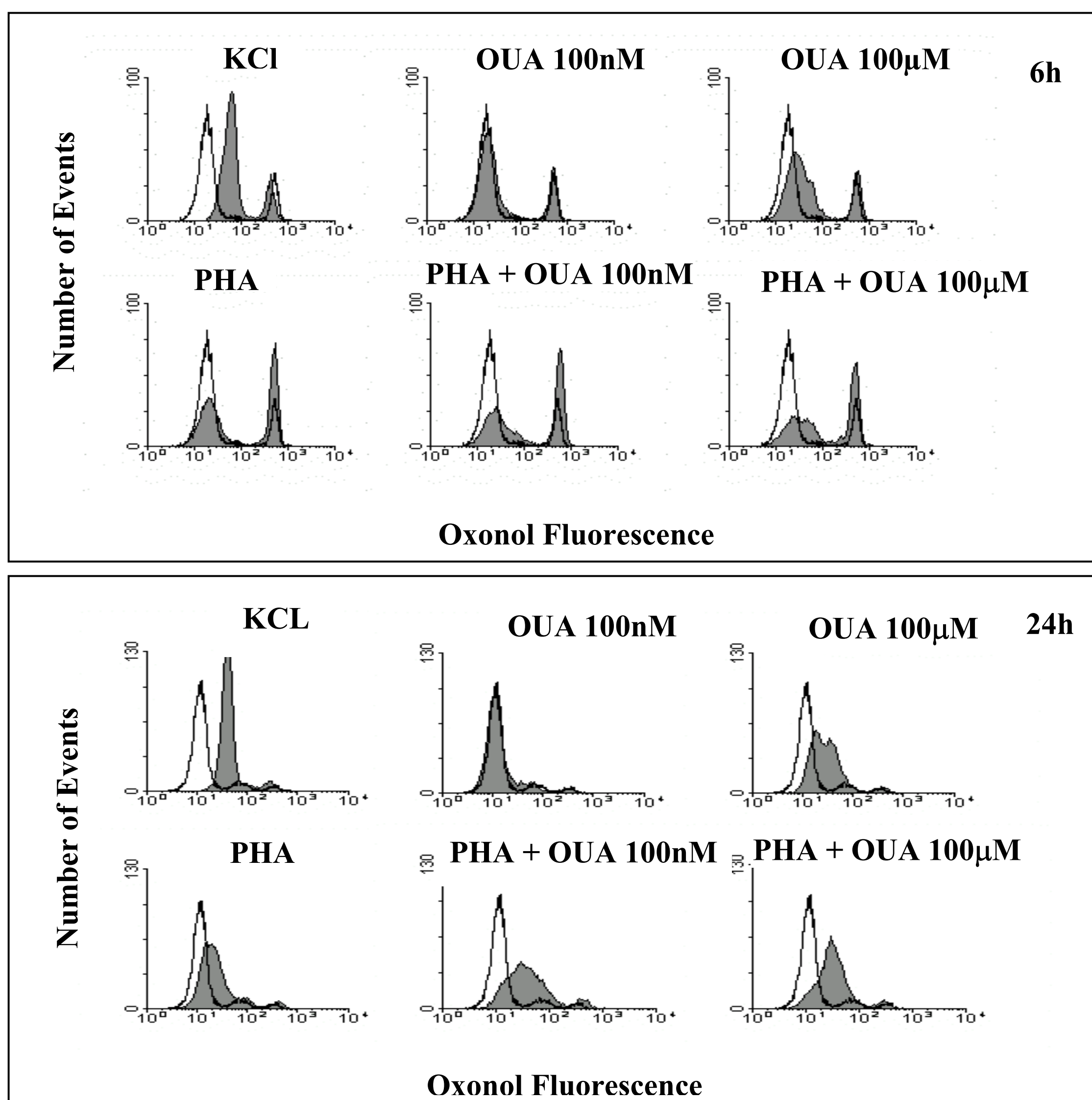

Fig. 3 – Plasma membrane depolarization measured by oxonol fluorescence after 6 or 24h culture. PBL were exposed to 5μg/ml PHA and/or ouabain (OUA). KCl^- was used as a positive control. The black line profile indicates control (untreated cells) and the gray histograms indicate cells exposed PHA and/or OUA. The figure is representative of three experiments incubated for 6h or 24h. Changes to the right (increased fluorescence) indicate depolarization.

the increase seen at 6h and after 24h no increase in mitochondrial mass was observed in cultures stimulated by PHA in the presence of ouabain (Fig. 4A).

Disruption of mitochondrial membrane potential is a characteristic of many apoptotic cells. Using the cationic lipophilic dye $DiOC_6(3)$ it was found that by 6h, 100nM ouabain by itself did not induce mitochondrial membrane depolarization. The same occurred with 100μM ouabain (data not shown). Lymphocytes exposed to PHA showed an increase in the fluorescence level of $DiOC_6(3)$ compatible with the increase of mitochondrial mass, suggesting that this result might not represent hyperpolarization of mitochondrial membrane potential. After 24h of culture, PHA also induced an increase in $DiOC_6(3)$ fluorescence, but the combination PHA + ouabain led to a clear disruption of the mitochondrial membrane potential (Fig. 4B and Table IV).

TABLE III

Plasma membrane potential following incubation with PHA and ouabain.

TREATMENT	6h	1	2	3	24h	4	5	6
CONTROL		37.6	7.4	18.1		16.1	33.0	16.9
100nM OUA		49.4	9.6	17.5		25.8	48.2	16.4
100μM OUA		65.2	9.8	17.3		33.9	68.2	33.9
5μg/ml PHA		98.6	17.3	30.9		43.3	92.2	30.6
PHA + 100nM OUA		135.2	37.4	44.3		56.9	132.0	48.4
PHA + 100μM OUA		131.2	51.1	55.4		66.5	105.6	37.7

Results are represented as the median fluorescence intensity of oxonol of three different experiments. The numbers 1, 2, and 3 correspond to different experiments with incubation time of 6h. The numbers 4, 5 and 6 correspond to different experiments with incubation time of 24h.

TABLE IV

Mitochondrial membrane potential following incubation with PHA and ouabain.

TREATMENT	6h	1	2	3	24h	4	5	6
CONTROL								
Depolarization		6*	9	13		15	3	6
Normal		88	87	84		88	94	89
Hyperpolarization		6	4	3		6	3	5
OUA								
Depolarization		5	13	3		5	3	9
Normal		91	81	94		86	93	90
Hyperpolarization		4	6	3		8	4	1
PHA								
Depolarization		8	17	6		8	3	9
Normal		75	68	73		73	55	78
Hyperpolarization		17	15	21		18	42	13
PHA + OUA								
Depolarization		12	32	9		30	42	15
Normal		80	60	77		67	54	75
Hyperpolarization		8	8	14		3	4	10

* Results are expressed as percentage of cells showing an increase (Hyperpolarization) or decrease (Depolarization) in DiOC6(3) fluorescence, or no changes in membrane potential (Normal). The numbers 1, 2, and 3 correspond to different experiments with incubation time of 6h. The numbers 4, 5 and 6 correspond to different experiments with incubation time of 24h.

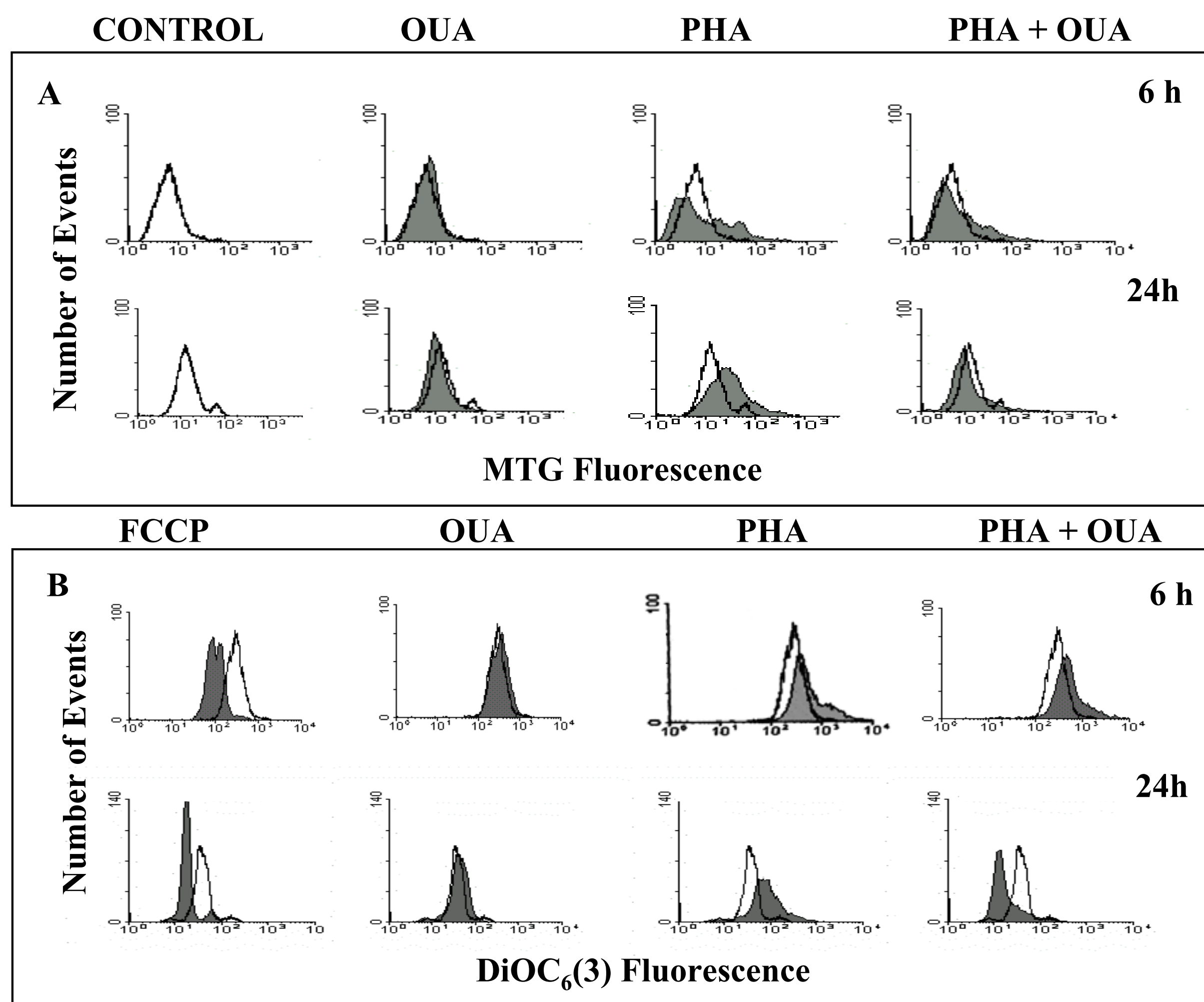

Fig. 4 – Mitochondrial mass and membrane potential in PBL treated with PHA and/or ouabain. The black line profile indicates control and the gray histograms indicate cells exposed to PHA and/or OUA for 6h or 24h. The auto-fluorescence profile of cells treated with PHA or OUA was not different from that seen with untreated cells. (A) Cells were incubated with 50nM mitotraker green (MTG) to observe mitochondrial mass. (B) Mitochondrial membrane depolarization was measured with $DiOC_6(3)$ after cells were cultured for 6 or 24h. 50μM FCCP was used as a positive control and changes to the left (decreased fluorescence) indicate depolarization. Fig. 4A is representative of four experiments. Fig. 4B is representative of three experiments. PHA = 5μg/ml, OUA = 100nM ouabain.

DISCUSSION

The molecules Fas and FasL are expressed in PHA-activated lymphocytes independent of their exposure to ouabain. Nevertheless, the induction of the apoptotic process using anti-Fas as an agonist, led to an increased amount of apoptotic cells in PHA-activated cultures in the presence of ouabain. In the Jurkat cell line, ouabain potentiates Fas-induced apoptosis and the death program triggered by this molecule (Bortner et al. 2001, Nobel et al. 2000). In a different system, glucocorticoid induced apoptosis of thymocytes, both spontaneous and glucocorticoid-driven apoptosis were dependent on a decrease in Na^+/K^+ ATPase activity and ouabain potentiated this effect (Mann et al. 2001).

There are evidences that loss of plasma membrane potential without repolarization leads to cell shrinkage and apoptosis (Bortner et al. 2001). The activity of the Na^+/K^+ pump was not measured in the present study, but the concentration used, 10^{-7}M ouabain, did not induce plasma membrane depolarization *per se* but potentiated the depolarization pro-

duced by PHA. An increased sensitivity of activated cells to ouabain has been described before (Brodie et al. 1995).

One of the earliest evidences of apoptosis is cell shrinkage, which seems to precede caspase activation and nuclear fragmentation (Bortner et al. 1997). Stimulation of the Fas receptor leads to inhibition of K^+ uptake (Bortner et al. 2001) and activates outwardly rectifying Cl^- channels, via tyrosine kinase phosphorylation (Szàbo et al. 1998). Furthermore, by blocking volume regulatory Cl^- channels it was possible to rescue cells from apoptosis (Maeno et al. 2000, Szàbo et al. 1998). The release of K^+ from the cells, activation of chloride channels and efflux of water produce the characteristic loss of volume (Bortner and Cidlowski 1996, Bortner et al. 1997).

A central role for K^+ ions in apoptosis has been advanced. Hughes et al. (1997) described that reduced intracellular K^+ concentration is a prerequisite for apoptosis as normal concentrations of this ion inhibits caspase and nuclease activities. This reduction, however, is not sufficient to trigger the apoptotic process, an apoptotic stimuli being necessary. It is worth mentioning that ouabain was shown to increase the production of IL-1β. The enzyme capable of cleaving pro-interleukin 1β to the biologically active form is the caspase ICE (IL-1β converting enzyme) and its activation was reported to be enhanced by K^+ depletion (Perregaux and Gabel 1994, Walev et al. 1995). It is quite possible therefore, that the processes of caspase activation for the induction of apoptosis and for IL-1β production are a reflection of the same mechanism.

A different view is put forward by Dallaporta et al. (1998). According to them loss of cytosolic K^+ occurs downstream of mitochondrial depolarization, cytochrome c release and caspase activation, but they also consider this step crucial for the activation of endonucleases. In our experience, loss of mitochondrial membrane potential was clearly evident only when PHA-activated cells were exposed to ouabain and appeared to be a late event.

In conclusion, treatment of activated PBL with ouabain potentiated apoptosis induced by an anti-Fas agonist antibody. A synergy between ouabain and PHA was also observed with regard to plasma membrane depolarization in agreement with the observation that sustained plasma membrane depolarization favors apoptosis. The mitochondria pathway, resulting in depolarization and cytochrome c release, did not seem to be involved when only PHA was used. However, dissipation of mitochondrial membrane potential was observed when cells were also exposed to ouabain, and in this case it was a late event. It is possible that the inhibitory effect produced by ouabain on activated peripheral blood lymphocytes represents an exacerbation of the effects seen during AICD.

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RESUMO

Quando linfócitos são ativados por lectinas mitogênicas apresentam mudanças do potencial de membrana, elevação das concentrações citoplasmáticas de cálcio, proliferação e/ou morte celular induzida por ativação (AICD). Concentrações baixas de ouabaína (um inibidor da Na^+,K^+-ATPase) suprimem a proliferação induzida por mitógenos e aumentam a morte celular. Para entender os mecanismos envolvidos, uma série de parâmetros foram avaliados usando sondas fluorescentes e citometria de fluxo. A adição de 100nM de ouabaína para culturas de linfócitos de sangue periférico ativadas por fitohemaglutinina (PHA) não modificou o aumento de expressão do receptor Fas ou de seu ligante FasL induzida pelo mitógeno. No entanto, o tratamento com ouabaína poten-

ciou a apoptose induzida por um anticorpo anti-Fas funcionando como agonista. Um sinergismo entre ouabaína e PHA também foi observado com relação à despolarização da membrana plasmática. Com relação à membrana mitocondrial, PHA por si só não produziu despolarização, mas quando as células foram também expostas à ouabaina uma dissipação do potencial foi observado, mas isso foi um evento tardio. É possível que o efeito inibitório da ouabaína em linfócitos de sangue periférico ativados envolva a potencialização de alguns aspectos do processo apoptótico e reflita uma exacerbação do mecanismo de AICD.

Palavras-chave: linfócitos, ouabaína, PHA, apoptose, despolarização de membrana.

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