H9c2 cardiac myoblasts undergo apoptosis in a model of ischemia consisting of serum deprivation and hypoxia: inhibition by PMA

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Abstract Cardiac myocytes undergo apoptosis under condition of ischemia. Little is known, however, about the molecular pathways that mediate this response. We show that serum deprivation and hypoxia, components of ischemia in vivo, resulted in apoptosis of rat ventricular myoblast cells H9c2. Hypoxia alone did not induce significant apoptosis for at least 48 h, but largely increased the proapoptotic action of serum deprivation. H9c2 cells apoptosis is evidenced by an increase in terminal (TdT)-mediated dUTP nick end-labeling-positive nuclei and by activation of caspases 3, 6, 7 and 9, and loss of mitochondrial functions. In this model of simulated ischemia, represented by serum deprivation plus hypoxia, cardiomyoblasts apoptosis was associated with a p53-independent Bax accumulation and with a down-regulation of Bcl-xL, whereas the levels of cIAP-1, cIAP-2 and X-IAP proteins did not change. Phorbol-12-myristate-13-acetate significantly reduced the induction of apoptosis, inhibiting caspase 3 cleavage, Bax accumulation, Bcl-xL down-regulation as well as restoring cell viability.

Key words: Ischemia; H9c2; Caspase; Apoptosis; Bcl-2 family proteins; Phorbol-12-myristate-13-acetate

1. Introduction

Programmed cell death, or apoptosis, is a major form of cell death that is used to remove excess, damaged or infected cells throughout life. It is either an essential component of normal development or a response to pathological disease states. Apoptotic signaling varies from cell to cell, and different apoptosis inducers may trigger distinct signaling pathways [1]. A common and critical event in the execution phase of apoptosis is the activation of a family of aspartate-specific proteases termed caspases [2,3], which participate in a cascade where initiator caspases activate effector caspases and ultimately cleave a set of proteins, causing disassembly of the cell. Caspases are synthesized as proenzymes that are activated by proteolytic processing [4]. Activation of caspases may be regulated directly or indirectly by members of Bcl-2 family proteins [5,6]. Caspase activity can be induced by the proapoptotic Bax and inhibited by the anti-apoptotic Bcl-2 or Bcl-xL.

Apoptosis in the heart is a necessary mechanism for normal remodelling and morphogenesis and is important in the development of cardiac failure during injury due to ischemia/reperfusion and myocardial infarction [7]. Recent studies have indicated that apoptotic death occurs in cardiac cells exposed to a variety of damaging stimuli both in vitro and in the intact heart in vivo [8–11]. Thus myocyte apoptosis is induced in response to ischemia, as well as during tissue reperfusion in rodents, rabbits and humans [9,12,13]. Despite the convincing evidence that apoptosis occurs, the mechanism and signaling pathway(s) activated by hypoxic/ischemic stimuli resulting in cardiac cell apoptosis are poorly understood. Caspases and Bcl-2 family proteins are involved in apoptotic cell death in cardiomyocytes [14–16], but nothing is known about molecules able to block the death of ischemic cardiomyocytes.

In the present study we report that H9c2 rat ventricular myoblasts undergo apoptosis in conditions of simulated ischemia, represented by serum withdrawal plus hypoxia, that is prevented by phorbol-12-myristate-13-acetate (PMA). The protective effect of PMA correlates with a marked down-regulation of the proapoptotic protein Bax and with an up-regulation of the anti-apoptotic protein Bcl-xL.

2. Materials and methods

2.1. Materials

Chemicals and biochemicals, Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin), DAPI (4,6-diamidino-2-phenylindole) and Hoechst 33342 were from Sigma. Anti-caspase 6 and 7, X-IAP, cIAP-1, cIAP-2 antibodies were purchased from New England Biolabs; anti-caspase 3, Bax and p53 antibodies were obtained from Santa Cruz; anti-Bcl-xL antibody from Trevigen. PMA, Gö6850 and Ac-IETD-AMC (Ac-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin) were from Alexis Biochemicals and JC-1 (5,5′,6,6′-tetrachloro-

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Abbreviations: PMA, phorbol-12-myristate-13-acetate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Ac-IETD-AMC, Ac-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin; Ac-LEHD-AFC, Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; z-DEVD-cmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethylketone; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; TUNEL, terminal (TdT)-mediated dUTP nick end-labeling; $\Delta\Psi_m$, mitochondrial membrane potential; PKC, protein kinase C; IAPs, inhibitor of apoptosis proteins; ARC, apoptosis repressor with caspase recruitment domain; DAPI, 4,6-diamidino-2-phenylindole

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1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) from Molecular Probes. z-DEVD-cmk (benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethylketone) and Ac-LEHD-AFC (Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin) were purchased from Calbiochem.

2.2. Cell culture and simulated ischemia

The H9c2 embryonal rat heart-derived cells [17] (obtained from European Collection of Cell Cultures) were cultured in Dulbecco's modified Eagle's medium (DMEM, Celbio) supplemented with 10% heat-inactivated fetal calf serum (FCS). Subconfluent cells (70–80%) were subcultured 1:4. The cells were trypsinized, plated (10 000–12 000 cells/cm^2) in 60-mm dishes for caspase activity assay or in 100-mm dishes for immunoblotting and grown for 24 h before apoptotic treatments. Simulated ischemia was achieved by culturing the cells in serum-deficient DMEM in an anaerobic workstation (BugBox, Jouan, France) saturated with 5% CO_2/95% N_2 at 37°C for the indicated time periods. The number of dead cells was determined by trypan blue exclusion by counting cells with a Burker hemocytometer and was calculated as the ratio of stained dead cells to the total amount of cells.

2.3. Determination of caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrates Ac-DEVD-AMC for caspase 3 (6, 7), Ac-IETD-AMC for caspase 8 and Ac-LEHD-AFC for caspase 9, during a 15-min incubation at 37°C, as detailed elsewhere [18]. Caspase activity is expressed as U/mg protein, where 1 U is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per min in the standard conditions described. Caspase activity is indicated as DEVDase activity, since it includes the activity of all caspase enzymes that cleave the substrate Ac-DEVD-AMC (mainly effector caspases 3, 6, 7).

2.4. Mitochondrial reductase activity

Cellular reductase activity of live cultured cardiomyoblasts was determined by measuring the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid) [19] by mitochondrial reductases, using the MTT assay kit from Boehringer Mannheim.

2.5. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was assessed using a fluorescent dye, the lipophilic cationic probe JC-1 [20]. H9c2 cells were seeded (10 000 cells/well) in 96-well plates and exposed to the treatments. At the end of incubation, cardiac myocytes were incubated in DMEM containing 3 μM JC-1 for 30 min at room temperature in the dark. After staining, medium was removed, cells were washed twice with PBS, resuspended in a total volume of 200 μl PBS and analyzed in a multilabel counter (Victor^2, Perkin Elmer). Collapse of $\Delta\Psi_m$ was examined for each sample using 535 nm (FL1) versus 590 nm (FL2) fluorescence. JC-1 forms aggregates in cells, which leads to high values in FL2 fluorescence, indicating a normal mitochondrial potential. Loss of $\Delta\Psi_m$ leads to reduction in FL2 fluorescence (aggregate state of JC-1) and a concomitant increase in FL1 fluorescence (monomeric state of JC-1). Data were expressed as ratio of FL1/FL2 fluorescence. As positive control for loss of $\Delta\Psi_m$, cells were treated with 10 μM valinomycin for 20 min at 37°C.

2.6. Western blotting

The cellular proteins were extracted in lysis buffer (20 mM HEPES, pH 7.0, 5 mM dithiothreitol (DTT), 2 mM EDTA, 0.1% 3-[(3-cholaminodopropyl)dimethylammonio]-1-propanesulfonate, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, pepstatin and leupeptin), diluted 1:1 with Laemmli buffer, then denaturated by boiling. Aliquots corresponding to 25 μg (Bax), 50 μg (caspase 3) or 80 μg (caspases 6 and 7, p53, X-IAP, cIAP-1, cIAP-2, Bcl-xL) protein were analyzed by SDS–PAGE (10% or 15% gel). After blotting, the nitrocellulose membrane was blocked with 5% non-fat dry milk for 1 h, washed with Tris-buffered saline and probed overnight at 4°C (caspases 6 and 7, X-IAP, cIAP-1, cIAP-2, Bcl-xL) or at room temperature for 60 min (caspase 3, Bax, p53) with the specific primary antibody. After further washing, the membrane was incubated for 30 min with the secondary antibody (horseradish peroxidase-conjugated). After washing, immunoreactive bands were visualized with an enhanced chemiluminescence kit (Amersham). Quantitative analysis of immunoblotted bands was performed by means densitometry (QuantiScan, Biosoft).

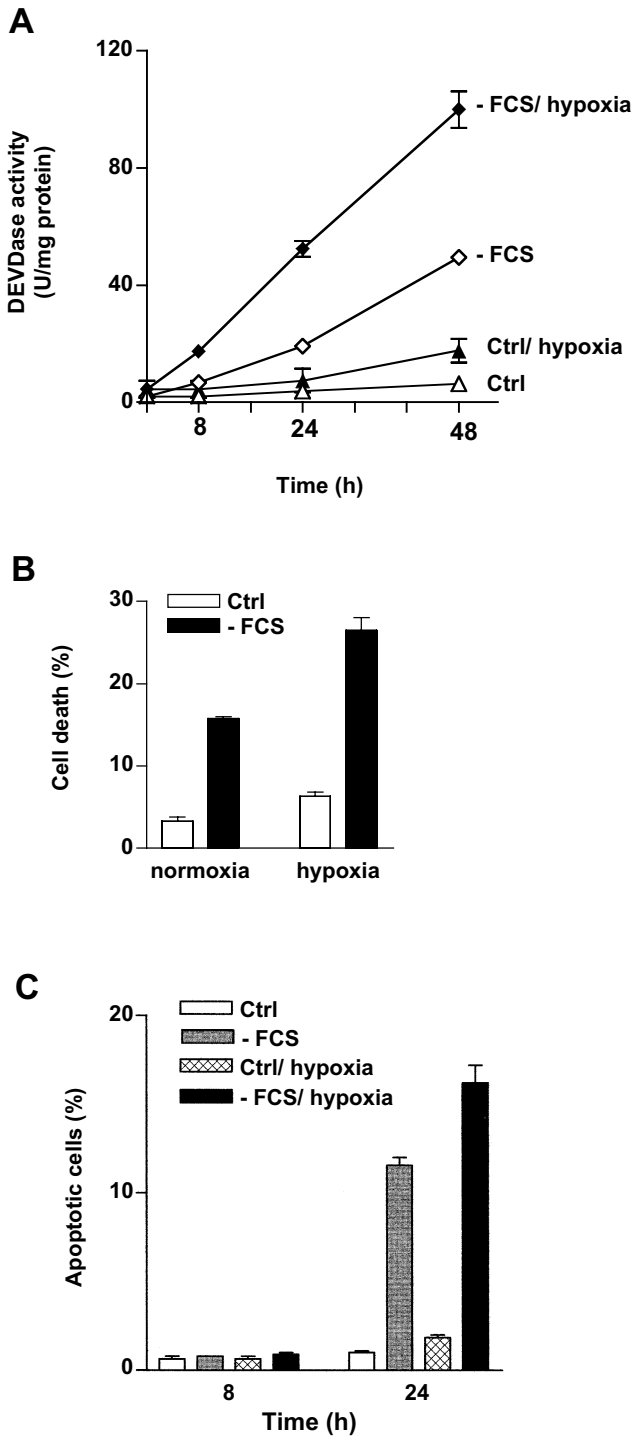

Fig. 1. Deprivation of serum and oxygen induces apoptosis in H9c2 cells. A: H9c2 cells were incubated in standard DMEM (Ctrl) or in serum-deprived DMEM (-FCS), either in normoxic or in hypoxic conditions for the indicated times. Cells were then harvested and assayed for caspase activity. B: H9c2 cells were incubated in standard DMEM (Ctrl) or in serum-deprived DMEM (-FCS), either in normoxic or in hypoxic conditions for 72 h. At the end of the incubation, cell death was assessed by trypan blue exclusion. C: H9c2 cells were incubated in standard DMEM (Ctrl) or in serum-deprived DMEM (-FCS), either in normoxic or in hypoxic conditions for the indicated times. At the end of each time point, quantitative assay of apoptotic cardiomyoblasts (TUNEL assay) was performed, as described in Section 2. In all panels, the results represent means ± S.E.M. of three determinations.

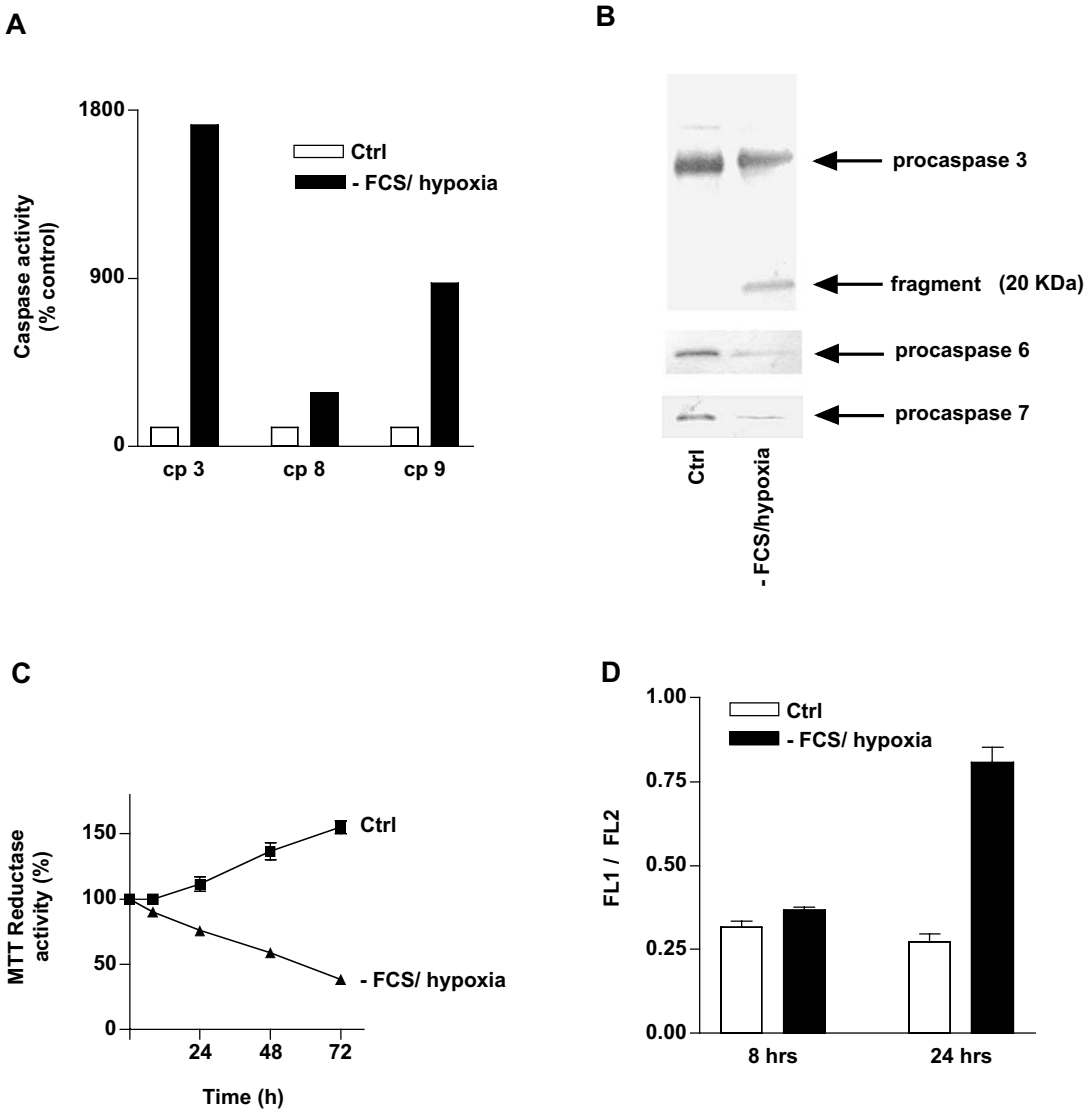

Fig. 2. Caspase activity and mitochondrial dysfunction in H9c2 cells exposed to simulated ischemia. A: H9c2 cells were incubated in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia) for 24 h. Cells were then harvested and assayed for the activity of the caspases 3, 8 and 9 as described in Section 2. Results are expressed as percentage of the caspase activity measured in control cells, taken as 100%. The panel reports the result obtained in one experiment, representative of three. B: H9c2 cells were incubated in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia) and harvested after 24 h. Cell extracts were analyzed for caspase 3, 6 and 7 processing by Western blotting. Results are representative of three independent experiments. C: H9c2 cells were incubated in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia) for 8, 24, 48 and 72 h. At the end of incubation, mitochondrial viability was assessed by the MTT reduction assay. Results represent means ± S.E.M. ($N = 4$), expressed as percentage of control, taken as 100%. D: H9c2 cells were incubated for 8 and 24 h in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia). At the end of incubation, $\Delta\Psi_m$ was assessed as described in Section 2. As a positive control of $\Delta\Psi_m$ collapse, H9c2 cells were treated with 10 μM of valinomycin (not shown). Data are means ± S.E.M. ($N = 6$) of results obtained in one experiment, and representative of three.

2.7. DNA breaks detection by in situ end-labeling and nuclear staining

To label apoptotic nuclei, the 3′-OH end of DNA fragments were visualized by the method of terminal transferase-mediated dUTP nick end-labeling (TUNEL), using the Apoptosis Detection System fluorescence kit (Promega, WI, USA) according to the manufacturer's instructions. The nuclei of apoptotic and non-apoptotic cells were counterstained with DAPI (0.1 μg/ml). The labeled cells were analyzed by fluorescence microscopy. The percentage of apoptotic cells was calculated as the ratio of the number of TUNEL-positive cells to the total number of DAPI-stained cells, counted in three different random fields. To confirm myoblast apoptosis, nuclei were stained with the karyophilic dye Hoechst 33342 (1 μg/ml). The morphological features of apoptosis (chromatin condensation and nuclear fragmentation) were monitored by fluorescence microscopy.

3. Results

3.1. Deprivation of serum and oxygen triggers caspase activity and apoptosis in H9c2 cells

During ischemia, multiple changes contribute to cellular death. Among these are deprivation of nutrients, growth and survival factors and oxygen. To study the effects of these stimuli, the cell line H9c2, which is derived from the embryonic rat heart, was exposed to culture conditions that mimicked ischemia. We tested the ability of serum and oxygen deprivation to induce caspase activation, an early marker of

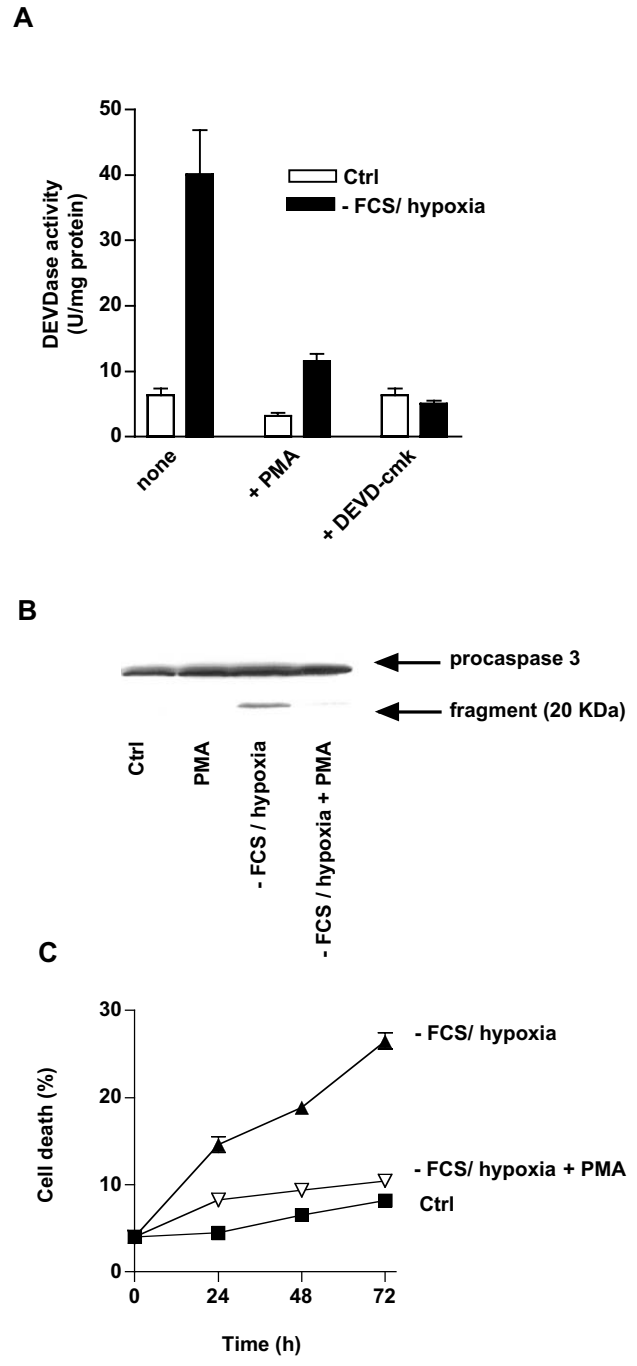

Fig. 3. PMA inhibits apoptosis in H9c2 cells exposed to simulated ischemia. A: H9c2 cells were incubated for 24 h in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia), in absence or in presence of 160 nM PMA or 100 μM z-DEVD-cmk. At the end of the incubation, cells were harvested and assayed for caspase activity. Results represent means ± S.E.M. ($N = 3$). B: H9c2 cells were incubated for 24 h in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia), in absence or in presence of 160 nM PMA. Cell extracts were analyzed for caspase 3 processing by Western blotting. C: H9c2 cells were incubated in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia), in absence or in presence of 160 nM PMA. At the indicated times, cell death was assessed by trypan blue exclusion. Results represent means ± S.E.M. ($N = 3$).

apoptosis. Serum withdrawal induced a time-dependent increase in caspase activity, that was first evident after 8 h of incubation. Oxygen deprivation of cells cultured in serum-free medium increased caspase activity significantly. Hypoxia alone did not induce caspase activity with respect to the control cells during the time examined, in agreement with previous reports [21,22] (Fig. 1A). The caspase activation induced by serum deprivation and hypoxia was accompanied by a reduction in cell viability (Fig. 1B). Low oxygen concentrations alone did not reduce cell viability of H9c2 cells, consistent with the low caspase activity measured in hypoxic conditions in the presence of serum. To rule out the possibility that the loss of viability observed in hypoxic serum-starved H9c2 cells was due to necrotic process, a TUNEL quantification of apoptotic cells was performed. Fig. 1C shows that after 8 h of incubation no DNA strand break fragmentation occurred in treated cells, whereas at the same time caspase activity was already detectable. The percentage of apoptotic cells increased in time-dependent manner in serum-starved and in hypoxic serum-starved cells, with a precise correspondence with the caspase activation and the loss of cellular viability.

These data suggest that serum deprivation and hypoxia, components of ischemia in vivo, induced apoptosis in H9c2 cells. In the light of these results on apoptosis induction, we adopted in the following experiments a model of simulated ischemia consisting of H9c2 cells cultured in hypoxic serum-starved conditions.

3.2. *Caspase activation in H9c2 cells cultured in hypoxic serum-starved conditions is accompanied by mitochondrial dysfunction*

Activation of downstream caspase cascade may take place either within death receptor complexes of the cytoplasmic membrane involving the activation of the initiator caspase 8 or by a mitochondrion-dependent mechanism in the cytosol involving the activation of the initiator caspase 9 [4]. Therefore, we measured the activity of caspase 8 and caspase 9 by the cleavage of the specific fluorogenic peptide substrates in H9c2 cells grown in hypoxic serum-deprived medium for 24 h. Fig. 2A shows that caspase 9 activity is largely increased in ischemic cells, whereas the degree of activation of caspase 8 is much lower. Caspase 8 activity was not affected at all after 8 h of incubation (not shown).

Since the peptide sequence DEVD, used as a substrate in the caspase 3 activity assay, is cleaved by caspase 3 and also by caspases 6 and 7, effector members of the caspase family, we next examined if these caspases were activated. Under control conditions, only the uncleaved proforms of endogenous caspases were detected by immunoblotting (Fig. 2B). Exposure of H9c2 cells to serum withdrawal and to hypoxia induced proteolytic cleavage of caspases, as revealed by the appearance of the characteristic fragment at p20 kDa of caspase 3 and by the disappearance of the uncleaved precursors of caspases 6 and 7. This proteolytic activation was nearly complete after 24-hours incubation.

The apoptotic responses involve mitochondrial dysfunction, so we evaluated an impairment in mitochondrial functions by the MTT assay. The conversion of the tetrazolium dye MTT to its reduced form, a reaction mediated by mitochondrial reductases, was used as an index of mitochondrial viability. The simulated ischemia induced a time-dependent decrease of

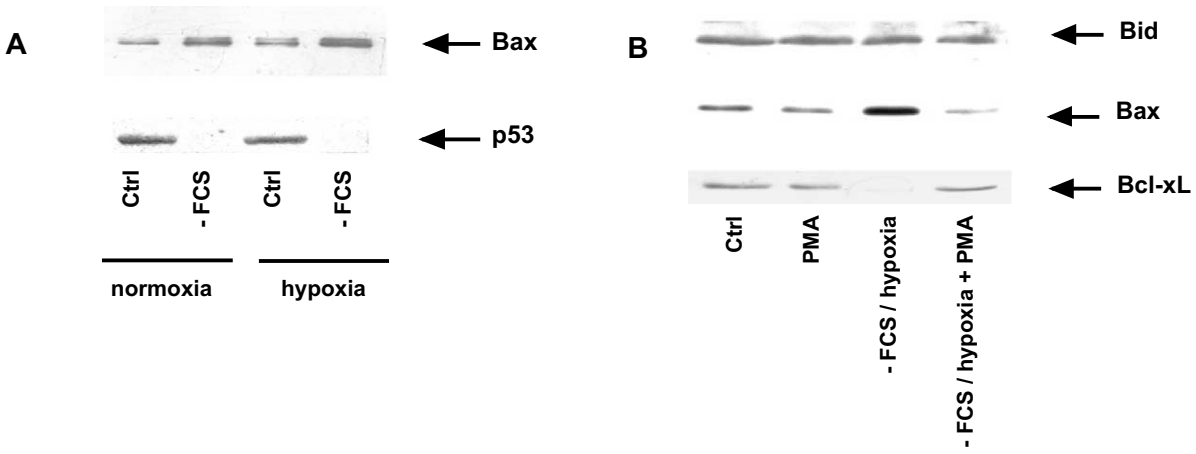

Fig. 4. Effect of simulated ischemia and PMA on the level of some Bcl-2 family proteins. A: H9c2 cells were incubated in standard DMEM (Ctrl) or in serum-deprived DMEM (-FCS), either in normoxic or in hypoxic conditions for 24 h. At the end of the incubation, cell extracts were prepared and analyzed by Western blotting for Bax and for p53 proteins. B: H9c2 cells were incubated for 24 h in standard DMEM (Ctrl) or in ischemic culture conditions (-FCS/hypoxia), in absence or in presence of 160 nM PMA. At the end of the incubation, cell extracts were prepared and analyzed by Western blotting for Bid, Bax and Bcl-xL proteins. Results are representative of three independent experiments.

MTT reductase activity, that was first evident after 24 h of initiation of the apoptotic stimulus (Fig. 2C). In the light of these changes in mitochondrial function [5,6], we assessed whether there was any decrease in $\Delta\Psi_m$ using the potential-sensitive dye JC-1. We observed a loss of $\Delta\Psi_m$ after 24-h incubation in ischemic conditions (Fig. 2D). Loss of membrane potential was not detectable before 24 h. After 8 h of incubation we were not able to measure any significant decrease in MTT reductase activity as well as in $\Delta\Psi_m$ suggesting that mitochondrial dysfunction follows caspase activation during apoptosis of H9c2 cells induced by serum deprivation plus hypoxia.

3.3. *PMA inhibits apoptosis induced by simulated ischemia in H9c2 cells*

The phorbol ester PMA is known to modulate apoptosis induced by various stimuli [23–25]. In order to assess whether PMA could prevent apoptosis induced by simulated ischemia, we determined the effect of PMA on caspase activation, that represents an early event in this model of apoptosis. H9c2 cells were incubated for 24 h in hypoxic serum-deprived DMEM in the presence of 160 nM PMA, given at the beginning of the incubation. As shown in Fig. 3A, PMA significantly reduced the induction of caspase activity elicited by simulated ischemia. The effect of PMA was similar to that of 100 μM z-DEVD-cmk, an inhibitor of effector caspases. Exposure of control cells to PMA and z-DEVD-cmk did not modify basal caspase activity. PMA also inhibited the processing of procaspase 3 (Fig. 3B) and partially restored cell viability (Fig. 3C) in ischemic H9c2 cells. PMA blocked caspase activation also in serum-deprived cells, incubated in normoxic culture conditions (not shown).

3.4. *Bcl-2 family proteins in apoptosis induced by simulated ischemia in H9c2 cells*

Bcl-2 family proteins play a critical role in the decision of the cell to die or survive by acting at multiple levels with a prompt impact on caspase activation [5,6]. Therefore we investigated the effect of serum withdrawal and hypoxia on the expression of the proapoptotic protein Bax in H9c2 cells. Six hours of serum deprivation induced an accumulation of the Bax protein that was further increased by hypoxia to about 1.4 times the control level, as determined by densitometric scanning of immunoblots. Bax levels continued to increase until 24 h of incubation (not shown). Fig. 4A depicts the separated effect of serum deprivation and hypoxia, components of simulated ischemia model that we used, on the Bax level after 24 h of incubation. In cells incubated at low oxygen concentration alone for the same time period, the level of Bax protein was similar to the control cells, whereas it was increased about 2.6 times in ischemic cells. Since hypoxia in some experimental models may increase levels of the proapoptotic protein p53 [21,26], and p53 itself can induce Bax [27], we next examined whether hypoxia and/or serum deprivation could influence p53 expression. Fig. 4A shows that hypoxia itself failed to cause p53 accumulation, while serum withdrawal, independently by hypoxia, induced a down-regulation of p53. This down-regulation is almost complete after 15 min of incubation (data not shown). These data suggest that in the present model of simulated ischemia, the induction of H9c2 cells apoptosis is associated to a signaling pathway involving Bax and independent of p53.

We next examined the effect of PMA on the Bcl-2 family proteins. Fig. 4B shows that PMA treatment of ischemic H9c2 cells for 24 h blocked Bax accumulation, whose level was lower than in control cells. An opposite trend was found when the expression of anti-apoptotic protein Bcl-xL was examined: after 24 h of simulated ischemia we detected a strong down-regulation of Bcl-xL that was inhibited by PMA treatment. Exposure of control cells to PMA alone did not change basal levels of Bax and Bcl-xL proteins. We could not notice significant change in the protein levels of the proapoptotic protein Bid (time points from 6 to 24 h were examinated), according to the weak activation of caspase 8 (Fig. 4B).

Inhibitor of apoptosis protein (IAP)-related proteins have been demonstrated to modulate activation of apoptosis by directly inhibiting activated effector caspases, such as caspases

3, 6 and 7 [28]. Recently, PMA has been reported to inhibit apoptosis by sustaining expression levels of the IAP-proteins [29]. However, in our experimental model expression levels of cIAP-1, cIAP-2 and X-IAP proteins was not altered by ischemic conditions (not shown).

4. Discussion

Many recent studies have indicated that either ischemia alone or in combination with reoxygenation can trigger cardiomyocyte apoptosis in a way similar to the other cellular stresses, such as growth factor withdrawal, UV radiation and chemotherapeutic agents [21,22,30,31]. The mechanisms by which the ischemic insult induces apoptosis are not well understood. Moreover, little is known about pharmacological agents that target the ischemia-induced apoptotic pathways in the heart and can exert a cardioprotective effect. In the present study we show that ischemia induces apoptosis in H9c2 myogenic cells that is accompanied by Bax up-regulation and Bc-xL down-regulation, mitochondrial dysfunction and activation of all effector caspases 3, 6 and 7.

The ischemic model of cultured cardiomyoblasts used in this study combines the following two properties of ischemia: survival-growth factor withdrawal and hypoxia. Between these stimuli, serum deprivation is the stronger one in H9c2 cells, indicating that, as in other cell types, cardiac myocytes undergo apoptosis in the absence of defined growth factors. In this regard, recently various trophic factors have captured the attention of researchers due to their possible role in cardiomyocyte survival. Factor such as interleukin-6, cardiotrophin 1 and insulin-like growth factor 1 have been shown to be cardioprotective and to inhibit cardiac myocyte apoptosis [32–34].

In H9c2 cells hypoxia alone is a very weak apoptotic stimulus. However, hypoxia largely increases the proapoptotic action of serum withdrawal, and can be seen as a modulator of the apoptotic process. Previous reports have documented the need for long term hypoxia (24–72 h) for the appearance of significant apoptosis in neonatal rat cardiac myocytes [22]. This prolonged viability in the face of severe hypoxia suggests the presence of intrinsic mechanisms that protect cardiac myocytes from hypoxia-induced apoptosis. Some of this relative resistance to hypoxia may result from the high levels of endogenous apoptosis inhibitors in neonatal and embryonal cardiac cells, such as the cellular FLICE-inhibitory proteins and ARC (apoptosis repressor with caspase recruitment domain). In this regard, ARC expression in H9c2 cells was shown to decrease in association with activation of ischemia-induced apoptosis [35].

In the model of simulated ischemia-induced apoptosis described in this paper, a definite sequence of events take place. At earlier time points (6–8 h) Bax begins to accumulate and caspases are activated. After 24 h mitochondria damage is well evident and cells can be detected with signs of nuclear apoptosis. At later time points (48–72 h) an increasing number of cells die.

It appears unlikely that the death receptor pathway is involved in ischemia-induced apoptosis of H9c2 cells since we could not detect cellular events characteristic of this pathway. Actually, caspase 8 activation is very weak and the Bid proapoptotic protein is not processed [30]. The activation of effector caspases 3, 6, 7 in this model it is likely to occur via the mitochondrial death pathway, as suggested by activation of caspase 9 and Bax accumulation.

Bcl-2 family proteins play important roles in the regulation of apoptosis and are important modulators of cardiac myocyte apoptosis [16,36]. The main site of action of Bcl-2 family proteins appears to be the mitochondrion. Homodimers of Bcl-2 or Bcl-xL associate with the mitochondrial membrane and influence membrane permeability [37]. The protective effect of Bcl-2 or Bcl-xL on mitochondria permeability is lost when Bcl-2 or Bcl-xL homodimers are sequestered by the formation of Bcl-2/Bax heterodimers [34]. In H9c2 cells the level of Bcl-2 protein is extremely low [37], so we examined the expression of another anti-apoptotic protein, i.e. Bcl-xL and we show here for the first time that Bcl-xL is expressed in H9c2 cells. The induction of apoptosis by simulated ischemia is associated with Bcl-xL down-regulation, and with an increased abundance of Bax. It is possible that induction of apoptosis involves a concerted action of Bax and Bcl-xL on caspase activation. However, it should be stressed that at the present, only speculations on the role of these proteins in apoptosis of ischemic H9c2 cells can be drawn. In this experimental model, apoptosis and Bax accumulation were completely independent of p53 protein [27]. Actually, there was no change in p53 protein during exposure of myoblasts to hypoxia alone; moreover, serum starvation itself induced a very rapid down-regulation of p53, before any change in Bax protein level. The role of p53 in apoptosis is highly dependent on cell type and cell context [21,26], and several pathways of apoptosis are independent of p53, even in cardiac myocytes [38].

Since apoptosis represents an active, gene-directed mechanism, it should be possible to control this process for therapeutic purposes. In this regard, we tested the hypothetical protective effect of PMA. PMA, a phorbol ester and activator of classical and novel protein kinase C (PKC) isoforms [39], has been reported to induce proliferation or differentiation in different cell lines [40] and to be associated with cardiac hypertrophy [41]. In addition PMA is able to modulate apoptosis in different models [23–25]. In several cases, evidence has been provided that the anti-apoptotic effect of PMA is mediated through PKC [42]. We show here that PMA attenuates ischemia-induced apoptosis in H9c2 cells, inhibiting caspase 3 activation and reducing cytotoxicity. This result suggests that the cell survival signal imparted by PMA could interfere with the apoptotic signal pathway activated by ischemia in myoblastic cells. PMA also abrogated the Bax accumulation and the down-regulation of Bcl-xL that follows exposure to ischemia. This effect on Bcl-2 family proteins level may be an important component of the anti-apoptotic action of PMA. Previous reports have suggested that PMA may exert its anti-apoptotic effect by inducing phosphorylation and inactivation of the proapoptotic Bcl-2 family member Bad [43]. It has also been reported that PMA induces Bcl-2 phosphorylation [44] and that PMA elicits a rapid decline of the steady state level of Bax in murine macrophages [25]. Moreover the anti-apoptotic effect of PMA observed in our experimental model may involve an active PKC protein. In fact, the treatment of ischemic cells with a generic PKC inhibitor Gö6850 [45], blocked the inhibitory effect of PMA on caspase activity (unpublished data). Studies are being in progress to explore the role of PKC proteins in this experimental model. Elucidation of the details by which PMA can inhibit apoptosis in cardiac

myocytes may suggest novel strategies for the treatment of ischemic heart disease.

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