Hypoxia predisposes neonatal rat ventricular myocytes to apoptosis induced by activation of the Fas (CD95/Apo-1) receptor: Fas activation and apoptosis in hypoxic myocytes

Gal Yaniv a, Mark Shilkrut a, Rona Lotan b, Gideon Berke c, Sarit Larisch b, Ofer Binah a, *Gal Yaniv , Mark Shilkrut , Rona Lotan , Gideon Berke , Sarit Larisch , Ofer Binah a

Received 10 September 2001; accepted 17 January 2002

Abstract

Objective: Since apoptosis is an important contributor to heart diseases in which ischemia and hypoxia are key elements, we tested the hypothesis that hypoxia predisposes neonatal rat ventricular myocytes (NRVM) to Fas-mediated apoptosis, by shifting the balance between antiapoptotic and proapoptotic proteins towards the latter.

Methods: Normoxic or hypoxic (22 h, 1% O2) cultured NRVM were exposed to recombinant Fas L (rFasL) for 7 h, and apoptosis measured thereafter. Results: Whereas in normoxic NRVM, rFasL did not cause apoptosis measured by the TUNEL assay (4.8±0.5% in control versus 4.5±0.9% in rFasL), in hypoxic cultures rFasL increased the background apoptosis level by 100%. That Fas was functional in normoxic NRVM, despite its inability to mediate apoptosis, was evidenced by the finding that Fas activation increased the diastolic [Ca2+]i levels measured by Fura 2 fluorescence, and caused arrhythmias. In support of our working hypothesis, hypoxia increased Fas expression by 200% (measured by quantitative Western blot), and the expression of the proapoptotic proteins ARTS and FADD by 323 and 250%, respectively, and decreased the expression of the antiapoptotic proteins ARC and FLIP by 90 and 60%, respectively.

Conclusion: By upregulating Fas expression and key proapoptotic proteins, and by downregulating antiapoptotic proteins, hypoxia predisposes ventricular myocytes to Fas-induced apoptosis. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Calcium (cellular); Hypoxia/anoxia; Ischemia; Myocytes

1. Introduction

Fas is a ubiquitous receptor belonging to the TNF/NGF superfamily, and is activated by Fas ligand (FasL), which may cause apoptosis in Fas-bearing cells [1]. Recent studies suggest that in several important heart diseases, such as myocarditis, ischemia and heart failure, Fas activation results in apoptotic, as well as in non-apoptotic effects, both contributing to cardiac dysfunction [2,3]. The primary mediators affecting the heart by activating Fas, are cytotoxic T lymphocytes (CTL), which contribute to heart diseases such as transplant rejection, myocarditis and the resulting dilated cardiomyopathy (DCM) [3,4]. Neverthe-

less, recent studies have shown that Fas activation is involved not only in myocardial pathologies inflicted by immune effectors, but also in lymphocyte-independent diseases such as ischemia/reperfusion injuries [5–7]. In this regards, it was recently proposed that FasL can be cleaved by a metalloprotease, to form soluble FasL (sFasL), which can cause apoptosis in susceptible cells. Therefore, sFasL, which may be secreted from the failing heart and is elevated in patients with advanced congestive heart failure [8], is a potential contributor to apoptosis in this wide-spread pathology.

The consequence of Fas activation in the normoxic heart is intriguing, since its engagement by CTL or by the activating Fas mAb Jo2, causes marked diastolic [Ca2+]i,
rise, action potential alterations and arrhythmias, but not apoptosis [9–11]. Since apoptosis plays an important role in a variety of diseases in which hypoxia is a key player [12,13], we tested the hypothesis that hypoxia predisposes ventricular myocytes to Fas-mediated apoptosis by down-regulating the expression of key anti-apoptotic proteins and by up-regulating pro-apoptotic proteins.

2. Methods

The present investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.1. Cultured NRVM and Fas activation

Experiments were performed on cultured neonatal rat ventricular myocytes (NRVM) 4–6 days after plating [14]. To induce hypoxia, cultures were maintained for 22 h in a N₂–CO₂–O₂ incubator (Tutenhauer, Jerusalem, Israel) adjusted to 1% O₂. Fas was activated by recombinant FasL (rFasL) (50 ng/ml) plus the enhancing antibody (1 μg/ml) (Alexis, San Diego, CA, USA), a combination causing ~100% apoptosis of A20B cells [15]. Drugs were added to the culture 30 min before exposure to rFasL.

2.2. Determining the expression of Fas and ARTS

2.2.1. Immunohistochemical staining for Fas and ARTS

Cultures were fixed for 30 min with 4% paraformaldehyde, permeabilized by Triton X-100 and washed three times with phosphate buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was neutralized by a 20-min incubation with 3% H₂O₂ in methanol, and then the cultures were washed three times in PBS. Cultures were then incubated with mouse anti-Fas at 1:100 dilution, followed by incubation with biotinylated goat anti-mouse IgG secondary antibody (Zymed, South San Francisco, CA, USA) at 1:750 dilution, and streptavidin-peroxidase conjugated. 3-Amino-9-ethylcarbazol in N,N-dimethylformamide dissolved in acetate buffer, pH 5.2, served as a substrate (Histostain-SP kit, Zymed). Non-immune mouse serum served as control. Counterstaining was performed with Mayer’s hematoxylin.

2.2.2. Immunofluorescence staining for Fas and ARTS in cultured NRVM

NRVM cultures were fixed, permeabilized and blocked in 5% BSA in PBS for 1 h, then incubated for 2 h with mouse-anti Fas or with, rabbit-anti ARTS mAb, and with mouse anti-slow muscle β-myosin mAb (Chemicon International, Temecula, CA, USA), followed by 1-h incubation with fluorescein or rhodamin anti-mouse or anti-rabbit secondary antibody (Pierce, Rockford, IL, USA). After washing, a drop of the mounting solution containing DAPI (Vector Laboratories, Burlingame, CA, USA) was added to each slide.

2.2.3. Immunohistochemical staining for Fas and ARTS in ventricular sections of neonatal rat hearts

Formalin fixed, paraffin embedded hearts were cut into 7-μm sections, and reaction performed according to a biotin–streptavidin–peroxidase technique using a Histostain Plus kit (Zymed). Anti-Fas mAb was used at 1:100 dilution in PBS, and an anti-ARTS mAb was used at 1:100 dilution in PBS. Negative controls treated with non-specific immune serum were processed simultaneously. Sections were counterstained with hematoxylin, and representative sections photographed.

2.2.4. Immunofluorescence staining for ARTS in adult rat ventricular myocytes

Adult rat ventricular myocytes were prepared as previously described for mice, with minor modifications [9]. A drop of myocytes suspension was mounted on glass slides and dried, followed by 30-min fixation with 4% paraformaldehyde. Immunofluorescence staining for rabbit anti-Fas mAb and for anti-slow muscle β-myosin mAb was performed as described earlier for cultured NRVM.

2.2.5. Western blot analysis

Lysates were prepared from normoxic and hypoxic cultures, and protein concentration was determined by the Bradford assay. A 50-μg sample of total cellular protein was loaded on 12% acrylamide gels, followed by electrophoretic transfer to nitrocellulose membranes (Micron Separation, Westboro, MA, USA), which were stained by Ponceau Concentrate (Sigma) to verify equal loading of protein. Membranes were blocked with 5% dry milk in DDW and Tween (0.05%). Subsequently, the membranes were exposed to different antibodies (as described below) in 5% dry milk in DDW (0.05% Tween). Finally, the immune complexes were detected using the ECL detection system (Pierce) with a secondary antibody coupled to horseradish peroxidase, followed by autoradiography. Antibodies were obtained from the following sources: Fas from Transduction Laboratories (Lexington, KY, USA), bcl-2 from Sigma (Rehovot, Israel), FADD from Chemicon International, FLIP from ProSci (Poway, CA, USA), and ARC from Santa Cruz (Santa Cruz, CA, USA), while ARTS was provided by Dr Sarit Larisch, a co-author of this manuscript. Peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Quantification of protein expression was performed by densitometry scanning analysis. To express the tested proteins relative to actin, membranes were rebotted with anti-actin antibody (Oncogene, Boston, MA, USA), and relative intensities of protein and actin calculated.
2.3. Determination of apoptosis in cultured NRVM

In most experiments apoptosis was identified by the DAPI assay, which is a reliable and specific tool for detecting apoptosis in cultured myocytes [16,17]. In some experiments (Fig. 2), apoptosis was also detected by the TUNEL assay. For each experimental protocol, apoptosis was determined separately in two different wells (500 myocytes measured in each well), which were prepared from three different litters, for a total of 3000 myocytes.

2.3.1. TUNEL assay

In situ labeling of fragmented DNA was performed with TdT UTP nick end-labeling (TUNEL) [18] using a commercial kit (Boehringer-Mannheim, Indianapolis, IN, USA). Myocytes counterstained with mouse anti-slow muscle β-myosin heavy chain were identified as apoptotic if they showed unequivocal positive TUNEL staining in the nucleus.

2.3.2. DAPI assay

After β-myosin heavy chain staining, cultures were counterstained with DAPI to visualize nuclear morphology. Myocytes were scored as apoptotic if they exhibited unequivocal nuclear chromatin condensation and fragmentation.

2.4. Measurement of intracellular Ca\(^{2+}\) transients

Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(\_i\)) transients were measured by means of methods routinely used in our laboratory [9]. Briefly, NRVM were loaded for 25 min with Fura 2-AM (Molecular Probes, Eugene, OR, USA) and the fluorescence ratio \(R = F_{340}/F_{380}\) measured from cultures maintained at 37°C and stimulated at 1 Hz [9].

2.5. Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma. Other agents purchased were: BHQ and xestospongin C (Calbiochem, La Jolla, CA, USA), ryanodine (Alomone Labs, Jerusalem, Israel), U73122 and U73343 (Biomole, Plymouth Meeting, PA, USA).

2.6. Statistical analysis

The results were expressed as the mean±S.E.M. The means of two populations were compared using Student’s t-test for unpaired observations.

3. Results

3.1. Fas expression in NRVM, and the effects of rFasL in normoxic cultures

At the onset of this study, we ascertained by means of three different techniques that Fas is constitutively expressed in ventricular myocytes. (1) RT-PCR analysis (Fig. 1A) demonstrates that Fas mRNA is expressed in cultured NRVM. (2) Immunohistochemical staining was used to confirm Fas expression in NRVM. As depicted in Fig. 1B, myocytes are extensively stained with brown-red, indicative of Fas expression. Without the primary antibody, staining was absent (data not shown). (3) By demonstrating that Fas is constitutively expressed in left ventricular sections of neonatal rat hearts (Fig. 1C), we confirmed that Fas expression in cultured NRVM was not caused by the dissociation procedure of the heart into isolated myocytes and/or by the culture conditions.

3.1.1. Fas-mediated apoptosis in normoxic myocytes

The background apoptosis level in normoxic NRVM was determined by means of the two widely used assays: DAPI and TUNEL [7,16,19]. As depicted in Fig. 2A, whereas healthy myocytes display a large, sphere-like nucleus (stained in blue), the apoptotic nucleus appears either fragmented (using DAPI), or is characterized by orange staining, resulting from the positive TUNEL reaction with the fragmented DNA strands. Altogether, the background apoptosis levels in normoxic NRVM were 2.5–5% (depending on the method used), in complete agreement with previous reports [16,19]. To determine the capability of Fas activation to cause apoptosis in normoxic myocytes, NRVM were exposed to rFasL for 7 h, and apoptosis measured immediately thereafter by the TUNEL and DAPI assays. As depicted in Fig. 2B, rFasL did not cause apoptosis above the background level. In additional experiments (data not shown), exposure of NRVM to rFasL for 24 h did not increase significantly the apoptosis level, indicating that normoxic myocytes are indeed resistant to Fas-mediated apoptosis. To ascertain that the apoptotic machinery is functional in normoxic NRVM, we measured (500 myocytes counted in two different cultures) the apoptosis level induced by 24-h exposure to 1 μM staurosporine, which was >90%.

3.2. Apoptosis induced by Fas activation in hypoxic cultures

Next, we determined whether hypoxia predisposes NRVM to Fas-mediated apoptosis by means of the following protocol. Cultures were initially incubated for 22 h under hypoxic conditions (1% O\(_2\)). Subsequently, the cultures were returned to normoxic conditions for 7 h, in the presence (rFasL) or absence (Control) of rFasL, and apoptosis measured thereafter. In agreement with previous studies [20], we found that neither hypoxia alone nor hypoxia followed by normoxia were harmful to cultured NRVM, which displayed spontaneous contractions and intact morphology. The major finding in these experiments was that in hypoxic cultures, rFasL increased the background apoptosis level by ~100%, compared to control.
Fig. 1. Fas expression in cultured NRVM and in left ventricular sections. (A) RT-PCR analysis in cultured NRVM: 34 cycles of 1-min denaturation at 94 °C, 1-min annealing at 55 °C, 1-min elongation at 72 °C, and a final elongation of 5 min at 72 °C. Isolated RNA were converted to cDNA, amplified by PCR technique and resolved on a 1% agarose gel. The size of the RT-PCR product of Fas was 344 bp. Two samples were analyzed and shown. M, marker; –, reverse transcriptase negative; 1 and 2, two separate samples from different animals. (B,C) Fas expression demonstrated by immunohistochemical analysis performed in cultured NRVM and in ventricular sections of newborn rats, respectively. Fas expression is indicated by brown staining. In both panels, staining was not observed in the absence of the primary antibody (data not shown).

(rFasL) cultures (Fig. 2C). In agreement with the pivotal role of caspases activation in apoptosis, the all-caspase inhibitor Boc-D(OMe)-FMK (20 μmol/l) completely blocked rFasL-induced apoptosis in NRVM (Fig. 2C).

3.2.1. Are 1,4,5-inositol trisphosphate (1,4,5-IP<sub>3</sub>) and [Ca<sup>2+</sup>] rise involved in Fas-mediated apoptosis?

We have previously shown that in adult murine ventricular myocytes, the electrophysiological perturbations and arrhythmias, as well as the marked rise in diastolic [Ca<sup>2+</sup>], caused by a short (3 h) exposure to the anti-Fas mAb Jo2 (an activating antibody), were exclusively dependent on an intact 1,4,5-IP<sub>3</sub> cascade and on Ca<sup>2+</sup> release from the sarcoplasmic reticulum (SR) [9,10]. Therefore, in the present study we tested whether the phospholipase C (PLC)→1,4,5-IP<sub>3</sub>→SR [Ca<sup>2+</sup>] release pathway is involved in Fas-mediated apoptosis. As shown in Fig. 2D, the following pharmacological interventions which interfere with this pathway, did not prevent Fas-mediated apoptosis; (1) blocking PLC (which generates 1,4,5-IP<sub>3</sub> from PIP<sub>2</sub>) with U73122; (2) depleting SR Ca<sup>2+</sup> release with the Ca<sup>2+</sup>-ATPase inhibitor BHQ; and (3) blocking SR Ca<sup>2+</sup> release with ryanodine. These results indicate that the 1,4,5-IP<sub>3</sub>-induced SR [Ca<sup>2+</sup>] release is not involved in Fas-mediated apoptosis in hypoxic cultures. This observation is important since it demonstrates that despite the well-known contribution of [Ca<sup>2+</sup>] rise to a variety of myocardial pathologies, it does not participate in the apoptotic pathway triggered by Fas activation.

3.3. Is Fas receptor functional in normoxic myocytes?

The incapacity of Fas activation to induce apoptosis in normoxic NRVM, raised the question whether under these conditions, Fas is functional, despite its unequivocal expression. This critical issue was addressed by testing in
Fig. 2. Apoptosis induced by rFasL in normoxic and hypoxic cultured NRVM. (A) Detection of apoptosis in normoxic cultures using the DAPI and TUNEL assays (see text for details). (B) Resistance of normoxic NRVM to rFasL-induced apoptosis, measured by DAPI and TUNEL. (C) Hypoxia predisposes NRVM to rFasL-induced apoptosis (measured by DAPI). Blockade of rFasL-induced apoptosis in hypoxic cultures by the caspase inhibitor Boc-D(OMe)-FMK (20 μmol/l). *P, 0.002 compared to Control. (D) Involvement of sarcoplasmic reticulum [Ca\textsuperscript{2+}] release and 1,4,5-inositol trisphosphate (1,4,5-IP\textsubscript{3}) in apoptosis (measured by DAPI) induced by rFasL in hypoxic NRVM. Percentage of apoptosis is shown in Control, in rFasL-treated hypoxic cultures (rFasL, second column from left), and in rFasL-treated hypoxic cultures plus BHQ (100 μmol/l), ryanodine (50 nmol/l) or U73122 (2 μmol/l). In all three drug-treated cultures, [Ca\textsuperscript{2+}] levels were not significantly different from rFasL alone. Each protocol was performed in cultures prepared from three different litters, for which apoptosis was measured in two wells, in which 1000 myocytes were counted.

normoxic NRVM the effect of rFasL on [Ca\textsuperscript{2+}], transients, represented by Fura 2 fluorescence signals (fluorescence counts at 340 and 380 nm yielding the ratio \(R = F_{340}/F_{380}\)). The rationale for conducting these experiments emanated from our recent study showing that in adult murine ventricular myocytes, Fas activation (3 h exposure to Jo2), caused pronounced diastolic [Ca\textsuperscript{2+}], rise and arrhythmogenic activity [9]. Panel A in Fig. 3 depicts representative traces of the fluorescence ratio recorded from a control culture, a culture exposed to rFasL, rFasL+U73122 or rFasL+U73343. Cultures were paced at 1 Hz, and the stimulated beats are marked by asterisks. As shown by the representative [Ca\textsuperscript{2+}] transients and by the summary of these experiments (Fig. 3B), Fas activation in normoxic NRVM caused two related effects: (1) diastolic [Ca\textsuperscript{2+}], rise represented by the increase in the fluorescence ratio from ~0.6 to ~0.8; and (2) arrhythmogenic activity, indicated by the non-stimulated [Ca\textsuperscript{2+}] transients, which probably resulted from early- and/or delayed afterdepolarizations. In agreement with our previous study [9], both diastolic [Ca\textsuperscript{2+}], rise and the arrhythmias were prevented by BHQ, ryanodine, U73122 (but not by the inactive analogue U73343), and xestospongin C, which is a specific blocker of the 1,4,5-IP\textsubscript{3}-operated, SR Ca\textsuperscript{2+} channels [21] (Fig. 3B). Hence, these findings clearly show that in normoxic myocytes Fas is responsive, and its activation causes 1,4,5-IP\textsubscript{3}-mediated functional disturbances.

3.4. How does hypoxia predispose NRVM to apoptosis induced by Fas activation?

To decipher the molecular mechanisms whereby hypo-
Fig. 3. The functionality of Fas in normoxic myocytes: effects of rFasL on \([\text{Ca}^{2+}]_i\) transients and the role of 1,4,5-IP_3 and SR \([\text{Ca}^{2+}]_i\) stores. (A) Representative \([\text{Ca}^{2+}]_i\) transients (measured by Fura 2 fluorescence ratio, \(R = F_{360}/F_{380}\)) from a control culture, and from three different cultures treated for 3 h with rFasL, rFasL + U73122, or rFasL + U73343. Paced beats (at 1 Hz) are marked by asterisks. The horizontal solid bars mark arrhythmogenic activity, and the dashed line, diastolic \([\text{Ca}^{2+}]_i\), level in the control culture. (B) Summary of diastolic \([\text{Ca}^{2+}]_i\), levels in control cultures (\(n = 90\) myocytes, seven cultures), and in cultures treated with: rFasL (\(n = 41\) myocytes, three cultures), rFasL + U73122 (2 \(\mu\)mol/l, \(n = 20\) myocytes, two cultures), rFasL + U73343 (2 \(\mu\)mol/l, 20 myocytes, two cultures), rFasL + xestospongin C (10 \(\mu\)mol/l, \(n = 40\) myocytes, two cultures), rFasL + BHQ (100 \(\mu\)mol/l, 20 myocytes, two cultures) and rFasL + ryanodine (50 \(\mu\)mol/l, 20 myocytes, two cultures). *\(P < 0.01\) versus Control. In the presence of rFasL + U73122, rFasL + xestospongin C, rFasL + BHQ and rFasL + ryanodine, diastolic \([\text{Ca}^{2+}]_i\), levels did not differ significantly from Control.

xia predisposes NRVM to Fas-mediated apoptosis, we investigated how hypoxia affects the expression of Fas, the proapoptotic proteins ARTS and FADD and the antiapoptotic proteins FLIP, ARC and bcl-2.

### 3.4.1. The effects of hypoxia on Fas expression

As shown by the representative experiments (Fig. 4A), Fas expression detected by the immunofluorescence staining (represented by the bright green spots), was markedly increased in the hypoxic culture (panel A2), as compared to the normoxic culture (panel A1). These findings were repeated in normoxic and hypoxic cultures from three different litters. The increase in Fas expression by hypoxia, as witnessed by the immunofluorescence detection, was confirmed by Western blot analysis. As shown by the representative immunoblots (Fig. 4B) and by the quantitative Western blot analysis (Fig. 4C), hypoxia markedly augmented (>200%) Fas expression, a finding suggesting that Fas upregulation is contributing to the sensitization of hypoxic ventricular myocytes to apoptosis induced by Fas activation.

Recently, Stephanou and co-workers have suggested that in NRVM, phosphorylation of p38 MAPK (SB203580-inhibitable) occurs as an acute and rapid response to simulated ischemia/reperfusion, and is responsible for the consequential increase in Fas expression [22]. To test whether a similar mechanism is operative in our experimental setting, we tested if hypoxia-induced increased Fas expression is affected by inhibiting p38 MAPK with SB203580. In these experiments, NRVM were incubated with SB203580 (Sigma) for 22 h under normoxic or hypoxic conditions, and Fas expression determined by Western blot (Fig. 5). As seen by the representative blots (Fig. 5A), in the presence of p38 MAPK blockade, hypoxia...
Fig. 4. The effect of hypoxia on Fas expression in cultured NRVM. (A) Immunofluorescence staining of Fas expression in NRVM in a normoxic (panel 1) and hypoxic culture (panel 2). Fas expression, indicated by bright green spots, was markedly increased by hypoxia. (B) Representative immunoblots from two normoxic cultures (N1 and N2) and one hypoxic culture (H). (C) Quantitative Western blot analysis of Fas expression in normoxic (n=4 litters) and hypoxic cultures (n=4 litters). *P<0.05. Equal protein loading is indicated by the size of the actin message.

3.4.2. Effects of hypoxia on the proapoptotic proteins ARTS and FADD

It was recently discovered that the proapoptotic protein ARTS (for apoptotic-related protein in the TGF-β signaling pathway), which is expressed in many cell types, enhances cell death induced by TGF-β, and to a lesser extent by other apoptotic stimuli. ARTS is a mitochondrial septin-like protein derived from alternative splicing of the H5/PNUTL2/hCDCrel2a/2b gene [23]. As illustrated by the immunofluorescence staining of cultured NRVM and by the ventricular sections from neonatal rat hearts, ARTS is expressed in neonatal ventricular myocytes (Figs. 6A and B). Further, as shown by the representative immunofluorescence staining (Fig. 6C), ARTS is also expressed in adult rat ventricular myocytes. These findings indicate that ARTS is constitutively expressed in the neonatal as well as in the adult rat heart. Next, we determined whether ARTS is modulated by hypoxia. As depicted by the representative immunoblots (Fig. 7A) and by the quantitative Western blot analysis (Fig. 7C), in hypoxic myocytes, ARTS expression was increased three-fold compared to normoxic myocytes. The second important proapoptotic protein studied is FADD (Fas-associated death domain), a death domain required to transmit the Fas-related apoptotic signal intracellularly [24]. As depicted in Figs. 7B and D, hypoxia pronouncedly elevated FADD expression. Collectively, these findings suggest that the increase in ARTS and FADD expression by hypoxia, contributes to the potentiation of Fas-mediated apoptosis in hypoxic cultures.

3.4.3. The effects of hypoxia on the antiapoptotic proteins FLIP, ARC and bcl-2

In these experiments we determined how hypoxia affects the expression of the antiapoptotic proteins FLIP, ARC and bcl-2. FLIP (FLICE-inhibitory proteins) is a caspase-8-like protein, which by preventing caspase-8 recruitment to FADD following Fas activation, inhibits Fas-mediated apoptosis [24]. As shown by the representative immunoblots (Fig. 8A), FLIP is highly expressed in normoxic...
myocytes. In agreement with our working hypothesis, hypoxia caused a 60% reduction in FLIP expression (Fig. 8D), an effect that may increase the sensitivity of hypoxic myocytes to Fas-induced apoptosis. Next, we determined the effect of hypoxia on ARC (an apoptosis repressor with a CARD) expression, a protein primarily expressed in cardiac and skeletal muscle [25]. As presented in Figs. 8B and E, hypoxia reduced ARC expression by >90%, rendering this antiapoptotic protein an excellent candidate that can contribute to the potentiation of Fas-mediated apoptosis by hypoxia. Finally, we determined the effect of hypoxia on the antiapoptotic protein bcl-2, which can suppress apoptosis by several mechanisms such as direct antioxidant effect, inhibition of the release of proapoptotic mitochondrial proteins and inhibition of the proapoptotic regulators bax and bak [2]. As shown in Figs. 8C and F, bcl-2 expression was unaffected by hypoxia.

4. Discussion

Our main aim was to test the hypothesis that hypoxia, a primary contributor to ischemic heart disease, predisposes ventricular myocytes to apoptosis caused by Fas activation, by shifting the balance between antiapoptotic and proapoptotic proteins towards the latter. In support of this hypothesis we found that: (1) Fas activation caused apoptosis in hypoxic, but not in normoxic myocytes; and (2) hypoxia markedly augmented the expression of Fas and that of the proapoptotic proteins FADD and ARTS, and diminished the expression of the antiapoptotic proteins FLIP and ARC.

4.1. Hypoxia predisposes NRVM to Fas-mediated apoptosis

In agreement with previous reports [10,11], we found that although Fas is expressed in cultured NRVM, neonatal ventricles and adult ventricular myocytes, its activation in normoxic myocytes did not cause apoptosis. That the Fas receptor is not only expressed in normoxic NRVM, but is also functional, was clearly demonstrated by showing that Fas activation resulted in diastolic $[Ca^{2+}]_i$ rise and arrhythmias. In accord with our previous findings in adult murine ventricular myocytes [10], these effects were caspase-independent, and were attenuated by blocking the PLC→1,4,5-IP$_3$→SR $Ca^{2+}$ release pathway. In contrast, Fas-mediated apoptosis in hypoxic cultures, was caspase-dependent, but 1,4,5-IP$_3$ and SR $[Ca^{2+}]_i$-release-independent. Hence, we have demonstrated for the first time that hypoxia alone (not ischemia) potentiates apoptosis induced by Fas activation, a finding baring considerable relevance to mechanisms underlying ischemic heart disease. In a related study Jeremias and coworkers [7] found that 6 h of ischemia simulated by glucose- and serum-free medium, O$_2$ tension <10%, increased cell death (myocytes death was measured rather than apoptosis) caused by the anti-Fas mAb Jo2. Our own study and that of Jeremias are dissimilar in three principle aspects: (1) the hypoxia used in our study (1% O$_2$) is different from the ischemic protocol used by Jeremias’ group; (2) in their study, at the end of the ischemia, 33% of the myocytes were already dead, whereas in our study, hypoxia per se did not cause apoptosis. In other words, it is likely that Jeremias’ group tested the effect of Jo2 on progressively deteriorating cultures, rather than apoptosis conditioned by hypoxia, as was done in the present work; and (3) we have measured apoptosis only in β-myosin-stained cells (i.e. ventricular myocytes), whereas Jeremias’ group measured non-specific cell death.

4.2. Hypoxia alters the balance between antiapoptotic and proapoptotic proteins

To explore the mechanisms underlying the principle finding that normoxic myocytes are resistant to Fas-mediated apoptosis, but hypoxic myocytes are not, we tested the hypothesis that hypoxia shifts the balance between
Fig. 6. Expression of the proapoptotic protein ARTS in neonatal and adult rat ventricular myocytes. (A) ARTS expression is demonstrated by immunohistochemical staining in cultured NRVM. (B) ARTS expression in a ventricular section from a neonatal rat heart, indicated by the brown staining. (C) ARTS expression in freshly dissociated adult rat ventricular myocyte. Myocytes were stained for myosin (green staining), ARTS (red staining), DAPI, and then photographed. Magnification ×100.

Antiapoptotic protein and proapoptotic towards the latter. As apoptosis is a complex process, chiefly regulated by the fine balance between antiapoptotic and proapoptotic factors, it is conceivable that hypoxia will alter this equilibrium, and thus predispose the myocardium to Fas-mediated apoptosis.

4.2.1. Hypoxia upregulates Fas expression

By means of quantitative Western blot analysis, we demonstrated that hypoxia increased Fas expression in NRVM, an effect which increases the responsiveness of hypoxic myocytes to Fas-mediated apoptosis. This finding complements the report by Tanaka’s group, who found a twofold increase in Fas mRNA in hypoxic NRVM [26], and is in agreement with reports that Fas is upregulated in myocytes during hypoxia and ischemia [13,27], and in volume-overload heart [11], which is frequently associated with heart failure, and possibly with apoptosis. Based on a recent study demonstrating that the increase in Fas expression in cardiac myocytes following ischemia/reperfusion is p38 MAPK-dependent [22], we determined whether a similar mechanism is functioning in hypoxic cultured NRVM. In agreement with the study of Stephanou and co-workers, we found that p38 MAPK blockade with SB203580 prevented hypoxia-mediated increased Fas expression. This finding suggests that the effect of hypoxia on Fas expression is mediated by p38 MAP, which belongs to a large family of MAP kinases, extensively studied for their role in apoptosis [28]. In this regard, Yin and co-workers have shown that in the rabbit Langendorff heart preparation, ischemia and reperfusion caused a 3.5- and a 6.3-fold increase in p38 MAPK over the basal level, respectively [29]. Accordingly, SB203580 administration before the ischemia caused a dose-dependent inhibition of p38 MAPK and a >50% reduction in apoptosis. Hence, the present study as well as others call for a detailed investigation of the involvement of p38 MAPK in hypoxia-induced increased expression of Fas and other proapoptotic proteins in ventricular myocytes.
4.2.2. Hypoxia upregulates the expression of the proapoptotic proteins ARTS and FADD

The increased apoptotic response of hypoxic NRVM to Fas activation can be accomplished not only by increasing Fas expression, but also by augmenting the expression of important proapoptotic proteins. In accordance with this notion, ARTS and FADD expression was increased by hypoxia. It was recently reported that ARTS, which is expressed in many cell types, is the first member of the septin family shown to localize to the mitochondria, or to play a role in apoptosis. The mitochondrial localization of ARTS and its mitochondrial-to-nuclear translocation during apoptosis suggest that ARTS represents yet another key factor in the regulation of caspase activation and cell death. Importantly, we demonstrate here for the first time that in ventricular myocytes, ARTS resides in the Fas apoptotic pathway, and therefore its upregulation by hypoxia may contribute to myocyte apoptosis in ischemic heart disease. Hence, this finding calls for future studies in which the role of ARTS in ischemic heart disease as well as in other myocardial pathologies will be investigated.

The second proapoptotic protein tested, FADD, was also augmented by hypoxia. This protein actually links Fas activation and activation of the caspase family, by recruiting and activating procaspase-8. Therefore, enhancement of FADD expression by hypoxia is likely to contribute to the increased susceptibility of hypoxic myocytes to Fas-induced apoptosis.

4.2.3. Hypoxia downregulates the expression of the antiapoptotic proteins FLIP and ARC

In agreement with our working hypothesis, we have found that hypoxia attenuated the expression of two key antiapoptotic proteins, FLIP and ARC, both regulating the activity of caspase-8. In addition to this inhibitory mechanism, ARC suppresses apoptosis via a caspase-independent mechanism, by blocking cytochrome c release from the mitochondria. In support of the present work, Ekhterae and co-workers have recently shown that ARC protein levels in H9c2 myocardial cell line were decreased by hypoxia [25], and that overexpression of transfected ARC inhibited hypoxia-induced apoptosis in this cell line. The antiapop-
The concept that Fas expression alone does not necessarily commit the cell to apoptosis (as shown in this work) was demonstrated in a variety of experimental systems. For example, Chan and co-workers studied Fas-mediated apoptosis in human medial vascular smooth muscle cells from health coronary arteries [32], and concluded that Fas sensitivity is determined not only by surface Fas expression, but also by the differential expression of Fas signaling proteins below the receptor level. Specifically, Fas-resistant cells showed reduced expression of FADD, Fas ligand, and caspase-3 and 7, and increased expression of FLIP and c-IAP-1.

### 4.2.4. Apoptosis-inducing interventions that alter the balance between antiapoptotic and proapoptotic proteins

Several studies in related experimental models provide evidence for the notion that apoptotic-inducing interventions alter the balance between antiapoptotic and proapoptotic proteins. For example, (1) Matsushita and co-workers have shown that NFkB activation by hypoxia caused aortic endothelial cell death and apoptosis through the suppression of bcl-2 [33], and (2) exposure of adult rat myocyte to $\text{H}_{2}\text{O}_{2}$, which simulates oxidative stress, stimulated the rapid translocation of the proapoptotic protein Bad to the mitochondria, which was followed by the subsequent degradation of Bad and bcl-2. $\text{H}_{2}\text{O}_{2}$, which is an apoptosis-inducing agent, caused the translocation of cyto-

---

**Fig. 8.** The effect of hypoxia on the expression of the antiapoptotic proteins FLIP, ARC and bcl-2 in cultured NRVM, determined by Western blot analysis. Representative immunoblots of FLIP (A), ARC (B) and bcl-2 (C), from normoxic (N) and hypoxic (H) cultures. Quantitative Western blot analysis of FLIP (D), ARC (E) and bcl-2 (F) in normoxic and hypoxic cultures. *P<0.05. ARC: three litters; bcl-2: five litters; FLIP: four litters.
chrome c from the mitochondria to the cytosol, which was indicative of mitochondrial dysfunction. As shown by the following examples, in vivo studies also support the notion that hypoxia/ischemia (as well as other disease conditions) shift the balance toward the proapoptotic state: (1) soon after acute coronary occlusion, bcl-2 is upregulated, especially in the salvageable myocardium [34,35], but is decreased after left ventricular adaptation to chronic pressure overload in the rat [36]; (2) overexpression of bcl-2 in the heart effectively reduces myocardial reperfusion injury by decreasing the extent of apoptotic myocyte death [37]; and (3) the ratio of bcl-2/Bax is reversed (toward the antia apoptotic state) in the heart after the placement of a left ventricular assist device [38].

4.3. Fas activation in normoxic and hypoxic myocytes leads to different consequences

Based on the present work and on our previous studies [9,10] as well as on other reports, we propose the following model for the dependency of Fas-mediated actions on the ambient oxygen level. In normoxic myocytes, whereas Fas activation does not cause apoptosis, it leads to caspase-independent, PLC activation, 1,4,5-IP₃ generation and increased [Ca²⁺]ᵢ, which is responsible for a variety of functional perturbations, including arrhythmias. On the other hand, under hypoxic conditions (still in the absence of Fas activation), while hypoxia alone (as simulated in the present work) is not harmful to myocytes, it increases the expression of Fas and the proapoptotic proteins ARTS and FADD, and decreases the expression of the antia apoptotic proteins FLIP and ARC, thus predisposing myocytes to the apoptotic effect of Fas activation. Therefore, our main conclusion is that hypoxia predisposes ventricular myocytes to Fas-mediated apoptosis, rendering this phenomenon an important pathological pathway in the ischemic heart.

Acknowledgements

This work was supported by the Israel Academy of Sciences, Minerva Foundation through the Bernard Katz Center for Cell Biophysics, the Rappaport Institute, and the Technion V.P. R Fund–Archie Basen Heart Research Fund.

References

[13] Rubin Y, Kessler-Icekson G, Navon G. The effect of furosemide on myocardial reperfusion injury by decreasing the extent of apoptotic myocyte death [37]; and (3) the ratio of bcl-2/Bax is reversed (toward the antia apoptotic state) in the heart after the placement of a left ventricular assist device [38].
protein, ARTS, mediates apoptosis dependent on its P-loop motif.


