Hypoxia-induced cleavage of caspase-3 and DFF45/ICAD in human failed cardiomyocytes

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Todor, Anastassia, Victor G. Sharov, Elaine J. Tanhehco, Norman Silverman, Alvise Bernabei, and Hani N. Sabbah. Hypoxia-induced cleavage of caspase-3 and DFF45/ICAD in human failed cardiomyocytes. Am J Physiol Heart Circ Physiol 283: H990–H995, 2002; 10.1152/ajpheart.01003.2001.—It has been proposed that the hemodynamic deterioration associated with heart failure (HF) may be due in part to ongoing loss of viable cardiac myocytes through apoptosis. Hypoxia has been shown to promote apoptosis in normal cardiomyocytes. Adaptation and maladaptations inherent to heart failure can modify the susceptibility of cells to different stress factors. We hypothesized that HF modifies the threshold of cardiomyocytes to hypoxia-induced apoptosis. Cardiomyocytes were isolated from 18 human hearts explanted at the time of cardiac transplantation due to either ischemic cardiomyopathy (ICM) (n = 9) or idiopathic dilated cardiomyopathy (IDC) (n = 9). Tissue from five normal donor hearts (NL) for whom no suitable recipient was available was used as control. Cardiomyocytes were cultured for 3 h under normoxic (95% air-5% CO2) or hypoxic (95% N2-5% CO2) conditions. Expression of caspase-3 and DNA fragmentation factor-45 (DFF45/ICAD) was detected by Western blot analysis. Three hours of hypoxia did not affect the expression of these proteins in NL cardiomyocytes. In contrast, hypoxia led to cleavage of caspase-3 and DFF45/ICAD both in ICM and IDC. In conclusion, failing cardiomyocytes exhibit increased susceptibility to hypoxia-induced apoptosis.

heart failure; apoptosis

The presence of reactive interstitial fibrosis, depending on its severity, has the potential to subject cardiomyocytes to chronic hypoxia (19). It is also known that cardiomyocytes can be a target for intercurrent ischemic events due to ischemic coronary disease. However, the role of hypoxia in cardiomyocyte apoptosis remains controversial. Some studies have shown that hypoxia alone is sufficient to induce apoptosis in primary cultures of neonatal (34) and adult normal cardiomyocytes (14). In contrast, Webster et al. (39) showed that activation of apoptosis in cardiomyocytes requires reoxygenation or a shift in pH. In yet another investigation, Seko et al. (26) reported that both hypoxia and hypoxia, followed by reoxygenation, stimulate apoptosis-related protein kinases in normal rat cardiomyocytes. To date, most of the studies that examined the effects of hypoxia on cardiomyocyte apoptosis have been conducted on normal cells. Information regarding the impact of hypoxia on apoptosis in failing cardiomyocytes is limited and clearly warrants further exploration. Adaptations and maladaptations that occur during HF may alter the threshold for hypoxia-induced apoptosis. Activation of caspase-3 is essential for apoptosis (10, 40). Inactive caspase-3 consists of a 32-kDa proenzyme (CPP32), whereas activation generates large (17–24 kDa) and small (10–12 kDa) fragments (40). DNA fragmentation factor-40 (DFF40)/caspase-3-activated DNase (CAD), the caspase-activated DNase (6), interacts with the inhibitory subunit DFF45/ICAD of caspase-3-activated DNase (ICAD) to form an inactive complex thought to be sequestered in the cytoplasm. Cleaved caspase-3 cleaves DFF45/ICAD into 24- and 12-kDa fragments (21), thus releasing DFF40/CAD. DFF40/CAD subsequently translocates to the nucleus and cleaves genomic DNA, a key step in apoptosis (22).

In this study, we examined the effects of hypoxia on expression of caspase-3 and DFF45/ICAD in cardiomyocytes obtained from failing human hearts due to ischemic cardiomyopathy (ICM) or idiopathic dilated cardiomyopathy (IDC) versus normal adult human cardiomyocytes.
MATERIAL AND METHODS

Tissue specimens were obtained from 18 human hearts explanted at the time of cardiac transplantation. Of these, HF was caused by ICM in nine patients and IDC in another nine patients (Table 1). Tissue specimens from five normal donor hearts for whom no suitable recipients were available were used as controls.

Isolation of cardiomyocytes. Explanted hearts were immediately placed in ice-cold oxygenated cardioplegic solution (Abbott Laboratories; Chicago, IL), containing 15 meq of potassium, and were rapidly transferred from the operating room to the laboratory. Cardiomyocytes were isolated from the LV free wall, as previously described (12). Approximately 20 g of LV tissue was used to isolate myocytes. The yield of rod-shaped quiescent myocytes from failed explanted hearts that excluded trypan blue was in the range of 70–90%. This range was in the range of 80–95% for cardiomyocytes isolated from control donor hearts. Thin transmural slices ~1 mm thick were cut from tissue blocks and immediately placed in normal Tyrode solution (4 mM K+, 2 mM Ca2+) saturated with 95% O2-5% CO2 at 37°C. The tissue was then rinsed twice in HEPES solution A composed of (in mM) 115 NaCl, 5 KCl, 35 sucrose, 10 glucose, 10 HEPES, and 4 HEPES, and 4 tauirine (pH 6.95) to remove any residual blood. The slices were then placed in a 250-ml polyethylene beaker containing 100 ml of HEPES solution with 15 μM Ca2+ (solution B) and placed in a 36°C water bath. A Harvard respirator (maximal 100% displacement 100 ml) was connected to the needle end of a syringe with each pump cycle. The respirator was designed to permit the HEPES solution plus tissue to be drawn up to 7/8 of the syringe height, at a rate of 25 cycles/min, a procedure referred to as trituration. A stream of O2 was applied continuously to the beaker during the isolation procedure. The tissue was then trituated for 15 min each, once with HEPES solution A for 5 min, once with HEPES solution B, and twice with HEPES solution B containing 0.05% collagenase (type 2, Worthington), 0.025% collagenase (type 1, Worthington), and 0.13 mg/ml protease (type 14, Sigma). All subsequent triturations were performed without the use of protease. In some cases, the addition of type 7 collagenase (Sigma) improved the yield of cardiomyocytes isolated from IDC hearts. The dissociated dead cells and debris from the first four triturations were discarded, and the cardiomyocytes from the fifth through the ninth trituration were combined. The combined suspension was collected and centrifuged at 500 g for 3 min. The pellet was resuspended in 50 ml of HEPES solution B and the resulting suspension placed in 2 × 50-ml polystyrene tubes. The suspension was allowed to stand for 5 min to allow the rod-shaped cardiomyocytes to settle by gravity. This procedure was repeated multiple times until the proportion of noncardiomyocyte cell fell <3%. To make cardiomyocytes calcium tolerant, the settled cardiomyocytes were resuspended in 50-ml HEPES buffer with increasing concentrations of 50, 100, 200, 350, and 500 μM Ca2+. The cardiomyocytes were allowed 30 min to settle by gravity after each calcium buffer change. Finally, the settled cardiomyocytes were resuspended in HEPES buffer containing 500 μM Ca2+.

Incubation of isolated cardiomyocytes. Cardiomyocytes were plated in petri dishes (Corning) coated with 0.5 μg/cm² of laminin (Sigma) at a density of 2 × 10⁴ cells/cm². Before being plated, cardiomyocytes were washed free of bovine serum albumin and resuspended in medium 199 with Earle’s balanced salts (Sigma) containing 25 mM HEPES and supplemented with 1.3 mM l-glutamine, 2 mM l-carnitine, 5 mM creatine, 5 mM taurine, 0.1 μM insulin, 0.1 nM triiodothyronine, 2.5 mM pyruvate, 1.0 mM NaHCO₃, Ca2+, 100 U/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml transferrin, and 500 μM Ca2+. The serum-free medium was changed 30 min after being plated to remove myocytes that did not attach to the dish, thus improving the yield to ~100%. Cultures with attached cells were incubated for 12 h at 37°C in an atmosphere containing 5% air-5% CO2. The medium had an initial pH of 7.2.

Exposure of cardiomyocytes to hypoxia. In 12 h, cardiomyocytes prepared as previously described were incubated for 3 h under normoxic or hypoxic conditions. The 3 h of hypoxia was used based on evidence showing that this duration of hypoxia did not affect the viability (yield of rod-shaped myocytes) of cardiomyocytes. Preliminary data in our laboratory showed that exposure of cardiomyocytes to 6 h of hypoxia leads to necrosis of ~10–30% of cardiomyocytes as evidenced by failure of these cardiomyocytes to exclude trypan blue and/or loss of their characteristic rod shape and appearance of cross striation. Accordingly, only cell preparations subjected to 3 h of hypoxia that had ≤5% change in cell viability were used. For studies (28) involving exposure of cells to a hypoxic stimulus, small volumes of culture medium at a height of ~0.64 mm were used to minimize the oxygen diffusion gradient. Hypoxic conditions were produced by placement of the dishes containing the isolated cardiomyocytes in an airtight incubator (Lab-Line) at 37°C for 3 h, where normal air was replaced by 95% N₂-5% CO₂. At the end of incubation, the PO₂ in the media reached the level of 10–15 mmHg. Identical preparations of cells were also placed simultaneously in a CO₂ incubator at 37°C where normal air was replaced by 95% air-5% CO₂. Once exposure to any of the above stimuli was completed, cell viability based on the percentage of cell types with attached cells, the presence of cross striation, and trypan blue exclusion was determined using an inverted microscope. The pH of the medium was assessed to ensure that no significant changes in pH took place during hypoxic exposure. Only preparations showing ≤5% change in cell viability and ≤0.2 shift in extracellular pH after incubations were used for further investigations. The latter allowed us to separate the effect of pure hypoxia (low PO₂) from a potential influence of ischemia, which is followed by lactate dehydrogenase (LDH) contamination and decreased pH. Aliquots of cells were placed in cryostat microtubes, flash frozen in liquid nitrogen, and stored at −70°C until ready to use.

Western blotting. Western blots were performed as previously described (3, 32, 33) with some modifications. Briefly, ~100 mg of frozen cardiomyocytes were homogenized in a lysis buffer (10 mM Tris base pH 7.5; 10 mM EDTA, 0.4% deoxycholate; 1% NP-40; 0.1% sodium dodecyl sulfate) containing protease inhibitor (phenylmethylsulfonyl fluoride 1 μl/100 μl of lysis buffer). The homogenate was centrifuged and the supernatant was saved. Approximately 10–15 μl of the supernatant containing 55 μg of protein was subjected to electrophoresis with the use of 4–20% Tris-glycine polyacrylic-
amide gels (Bio-Rad). The separated proteins were transferred to a membrane (Immuno-Lite Assay Kit, Bio-Rad). The membrane was then incubated with a primary antibody and then with a secondary antibody for 2 h. The antibody bound antigen was identified by chemiluminescence followed by autoradiography. The density of the bands corresponding to the protein of interest was measured using a desktop high-performance imaging densitometer (GS-670, Bio-Rad). The unit of densitometric measurement was optical density \( \times \) mm\(^2\). The molecular mass of the proteins is 32 kDa for caspase-3 and 44 kDa for DFF45. In addition, activation fragments of caspase-3 (p17: 17 kDa) and DFF45 (p24: 24 kDa and p12: 12 kDa) were detected. Polyclonal anti-active caspase-3 and anti-DFF45 antibodies were obtained from Calbiochem (San Diego, CA).

**Data analysis.** For each protein examined, one-way analysis of variance was used to compare normal cardiomyocytes with failing cardiomyocytes exposed to normoxic condition and failed cardiomyocytes exposed to hypoxic conditions. For this test, significance was set at \( \alpha = 0.05 \). If significance was attained, then pairwise comparisons were made using the Student-Newman-Keuls test with significance set at \( P < 0.05 \). All results are presented as means \( \pm \) SE.

**RESULTS**

**Caspase-3.** Caspase-3 can be detected in its inactive form, CPP32 (32 kDa), and active form, p17 (17 kDa). In normal cardiomyocytes, caspase-3 was detected as a single band of CPP32. Hypoxia did not elicit the appearance of active caspase-3 (p17) in normal cells (Fig. 1A). Cardiomyocytes isolated from explanted hearts with either ICM or IDC showed significantly increased expression of CPP32 compared with normal control cardiomyocytes (Fig. 1B and C). P17 was not detected in either ICM or IDC cardiomyocytes under normoxic conditions. However, exposure to hypoxia stimulated the activation of caspase-3 as seen by the appearance of p17 in both ICM and IDC myocytes (Fig. 1B and C).

![Fig. 1. A–C, left: Western blots depicting the expression of caspase-3 in cardiomyocytes obtained from normal donor hearts (NL) and cardiomyocyte obtained from explanted failing hearts due to ischemic cardiomyopathy (ICM) and idiopathic dilated cardiomyopathy (IDC) under normoxic (NX) and hypoxic (HX) conditions. The 17-kDa marker indicates the molecular mass of the active form of caspase-3 (p17) and the 32-kDa marker indicates that of the inactive form (CPP32). A–C, right: bar graphs depicting the densitometric analysis of the expression of caspase-3 in cardiomyocytes isolated from NL \((n = 5)\), IDC \((n = 9)\), and ICM \((n = 9)\) hearts, in units of optical density \( \times \) mm\(^2\). A: expression of caspase-3 (CPP32 and p17) in normal donor cardiomyocytes. B: expression of caspase-3 (CPP32 and p17) in ICM cardiomyocytes. C: expression of caspase-3 (CPP32 and p17) in IDC cardiomyocytes. *\( P < 0.05 \) vs. NL+NX.](http://ajpheart.physiology.org/)
**DISCUSSION**

Compared with normal cardiomyocytes, cells isolated from ICM and IDC hearts exhibited elevated levels of inactive caspase-3 and no evidence of the active p17 fragment under normoxic conditions. Similar results were obtained when examining levels of DFF45 expression, with ICM and IDC cardiomyocytes showing increased levels of intact DFF45. Upregulation of intact DFF45/ICAD in both ICM and IDC cardiomyocytes may be an adaptive mechanism because this protein inhibits the activation of DFF40/CAD DNase (6, 22).

Hypoxia induced cleavage of caspase 3 and DFF45/ICAD in ICM and IDC cells, whereas both proteins remained intact in normal cardiomyocytes. Because cleavage of caspase 3 and DFF45/ICAD are inevitable steps for cell death through apoptosis (6, 10, 21, 22, 40), these observations suggest that failing cardiomyocytes are more susceptible to hypoxia compared with normal cardiomyocytes.

Although an increase in the incidence of apoptosis is associated with HF (15, 16, 27), cleaved fragments of caspase-3 and DFF45/ICAD were not detected in failing cardiomyocytes under normoxic conditions. This is most likely due to the very low number of cardiomyocytes undergoing apoptosis at any given time in the beating failing heart (8). Thus the levels of p17, p24, and p12 may have been too low for detection by West-
short-term severe hypoxia. In contrast, failing cardiomyocytes are resistant to changes in the level of susceptibility for cleavage of caspase-3 and DFF45/ICAD in response to hypoxia. Our results suggest that increased sensitivity of failing cardiomyocytes to hypoxia-induced caspase 3 and DFF45/ICAD cleavage may be one pathway that contributes to the ongoing loss of cardiomyocytes in HF.

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REFERENCES


CARDIOMYOCYTE APOPTOSIS IN HEART FAILURE


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