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

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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 incubated 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)/inhibitor of caspase-3-activated DNase (ICAD) were 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.

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/inhibitor 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.

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PROGRESSIVE WORSENING of left ventricular (LV) function constitutes one of the primary characteristics of heart failure (HF). The exact cause of this hemodynamic deterioration is not known but has been attributed in part to the ongoing loss of viable cardiac myocytes possibly through apoptosis. Studies (15, 16, 27) performed in animal models of experimentally induced HF and in end-stage failed explanted human hearts have suggested that apoptosis may contribute to cardiomyocyte loss in HF. Many studies (5, 7, 9, 17, 18, 23–25, 30, 36) have also detected cardiomyocyte apoptotic activity in human HF of varying etiologies.

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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 taurine (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 displacement 100 ml) was connected to the needle end of a 20-ml plastic syringe (without plunger) with the wide end. A stream of O2 was blown into the syringe with each pump cycle. The respirator was adjusted 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 (100%) was applied continuously to the beaker during the isolation procedure. The tissue was then triturated 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), 0.02% 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 95% 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 studied. 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 rod-shaped 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 deoxycholate) 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 polyacryl-
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 × mm². 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 α = 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 ± 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. 1, B 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. 1, B and C).

![Fig. 1.](http://alpheatheart.physiology.org)
DFF45/ICAD. DFF45/ICAD was detected in cardiomyocytes isolated from normal donor hearts as a single band in its intact uncleaved form (44 kDa molecular mass). Hypoxia did not cause cleavage of DFF45/ICAD in normal cells (Fig. 2A). DFF45/ICAD was significantly increased in both ICM and IDC compared with controls (Fig. 2, B and C). There were no cleaved fragments of DFF45/ICAD detected in ICM and IDC under normoxic conditions. However, hypoxia led to the appearance of p24 and p12 fragments of DFF45/ICAD in both ICM and IDC myocytes (Fig. 2, B and C).

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-

Fig. 2. A–C, left: Western blots depicting the expression of DNA fragmentation factor-45 (DFF45) and its cleavage products in cardiomyocytes obtained from NL and cardiomyocytes obtained from explanted failed hearts due to ICM and IDC under NX and HX conditions. The 24-kDa marker indicates the molecular mass of the p24 fragment, and the 12-kDa marker indicates that of p12. A–C, right: bar graphs depicting the densitometric analysis of the expression of DFF45 in cardiomyocytes isolated from NL (n = 5), IDC (n = 9), and ICM (n = 9) hearts, in units of optical density × mm². A: expression of DFF45, p24, and p12 in normal cardiomyocytes. B: expression of DFF45, p24, and p12 in IDC cardiomyocytes. C: expression of DFF45, p24, and p12 in IDC cardiomyocytes. *P < 0.05 vs. NL + NX.
ern blotting in the present study. The cause of increased susceptibility of failing cardiomyocytes to hypoxia remains unclear. It may be that cleavage of caspase-3 in failing cardiomyocytes occurs more easily compared with normal cells. It is now clearly established that cytochrome c release from the mitochondria to the cytosol is a critical event for caspase-3 cleavage (4, 31). This process depends on the integrity of the outer mitochondrial membrane. In normal cells, additional physiological stress, such as anoxia, leads to voltage-dependent anionic channel closure and isolates mitochondria from the cytosol (2). If the outer membrane is partially destroyed, which could be the case in HF (20), it can more easily lose its barrier function thus facilitating cytochrome c release and caspase-3 cleavage. Fas/Fas-L interaction is a well-known pathway that leads to cytochrome c release with subsequent cleavage of caspase 3 through activation of proapoptotic Bcl-2 family proteins (1, 11, 13, 37). The mechanism of increased susceptibility of failing cardiomyocyte to hypoxia may be complex and possibly etiology dependent. It was shown that Fas is downregulated in IDC and is unchanged in IDC. Fas-L, on the other hand, is downregulated in IDC but upregulated in ICM (35). The downregulation of Fas-L in the face of unchanged Fas in IDC makes a Fas-mediated mechanism of increased susceptibility to cell death through apoptosis not likely in this etiology. The marked upregulation of Fas-L in ICM makes the possibility of cell death through apoptosis more likely. c-Jun, a downstream of stress-activated protein kinase cascade that can lead to apoptosis (29), is upregulated in IDC and downregulated in ICM, whereas p38 mitogen-activated protein kinase also can trigger hypoxia-stimulated apoptosis (38) is downregulated in IDC and upregulated in ICM (35). With regard to possible mechanisms of susceptibility of failing cardiomyocytes to hypoxia, the stress-activated protein kinase pathway appears to play a more central role in cells isolated from the heart with IDC, whereas the p38 mitogen-activated protein kinase cascade is appears to prevail ICM.

There are several limitations to the study that warrant consideration. Myocytes isolated from donor hearts that served as controls were from younger individuals than myocytes from failed hearts. Therefore, one could argue that the aging process itself may be conducive to increased susceptibility to the hypoxic stimulus. Another limitation of the study is absence of a direct evaluation or quantification of apoptosis. Knowledge of that would have been helpful in the interpretation of the study results as a whole. Finally, whereas the possible mechanisms by which hypoxia mediates the cleavage of caspase-3 and GFF45-ICAD are addressed, a direct attempt at elucidating these mechanisms was not part of the present study but nonetheless must be addressed in future work.

In conclusion, the study results demonstrate that normal cardiomyocytes are resistant to changes in the level of expression of caspase-3 and DFF45/ICAD induced by short-term severe hypoxia. In contrast, failing cardiomyocytes taken from ICM or IDC hearts exhibit increased 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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