Simulation of Ischemic Reperfusion in Endothelial Cell Culture Increases Apoptosis

Carl Holleyman, BS; Douglas Larson, PhD., CCP; Kyler Hunter, BS

Circulatory Sciences Graduate Perfusion Program, Sarver Heart Center, University Medical Center, University of Arizona, Tucson, Arizona

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Abstract: The endothelial layer of the myocardial vasculature serves as an important protective barrier between blood and myocardium. Ischemic reperfusion (I/R) of the endothelium has been shown to initiate a series of events that leads to ischemic reperfusion injury in the heart. At the onset of ischemic reperfusion, endothelial cells initiate apoptosis, a process whereby the cells self-destruct. Ischemic reperfusion was simulated to study its effects on the induction of apoptosis in cultured human endothelial cells (ECV 304). In addition, the cells were treated with nitric oxide (NO) to test its effect on induction of apoptosis. To mimic hypoxia, four ECV 304 cultures were placed in a medium that had been bubbled with pure nitrogen gas for 24 hours. A continuous flow of nitrogen gas was applied to the culture flasks during the course of the 2-hour ischemic period. After 2 hours, the nitrogen was removed from the hypoxic cultures to simulate reperfusion. Exposure to NO was achieved through the NO-donor (±)-S-nitroso-N-acetylpenicillamine (SNAP) at 100 μM. Cell cultures were exposed to hypoxia only, hypoxia and SNAP, and SNAP only. One positive control was established by exposure to staurosporine. A second positive control was established by exposure to a 30-min heat treatment at 43°C. Two cultures were left untreated to serve as negative controls. All cell cultures were incubated for 4 hours. Apoptosis was detected by the binding of annexin V-fluorescein isothiocyanate (annexin V-FITC). In addition, morphologic changes detected by electron microscopy were used. Apoptosis increased in all treated cultures, excluding SNAP only treated cells. It was concluded that I/R may lead to induction of apoptosis.

Keywords: apoptosis, endothelial cell, annexin V, reperfusion injury, NFκB, nitric oxide.

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It is recognized that cells die by two distinct pathways, necrosis and apoptosis. Necrosis, a passive process marked by cellular swelling and loss of membrane integrity, occurs when a cell sustains a severe injury. An inflammatory response follows because of the release of cellular contents into the extracellular space during cell lysis (1). In contrast, cells dying by apoptosis are responding to stimuli that trigger a complex series of intracellular events resulting in a gene-directed, energy-requiring process of self-destruction. Membrane integrity is preserved as apoptotic bodies are formed around cellular components. These bodies form blebs that separate from the cell and are phagocytosed by adjacent cells and macrophage. Cellular contents remain separated from the extracellular space, thus avoiding an inflammatory response (2).

Traditionally, it was thought that myocytes in the heart died through necrosis during ischemic reperfusion. However, the role apoptosis plays during reperfusion injury of the myocardium and cardiovascular endothelium is coming under increased scrutiny. The endothelium serves as a protective layer between blood and tissue, providing a defense against invading pathogens and mediating inflammatory immune responses. Initiation of reperfusion injury occurs in the ischemic cardiovascular endothelium within minutes of reperfusion resulting in inhibition of NO production by endothelial nitric oxide synthase (eNOS) (3). At the low physiologic concentrations produced by eNOS, NO regulates vasodilation, controls vascular smooth muscle growth, and protects the vasculature from adhesion of blood components (4). In addition, NO protects the myocardium from apoptosis by blocking caspase three activation, a proteolytic enzyme involved in apoptotic cell death (5).

With renewed oxygen delivery to the ischemic endothelium during reperfusion, adhesion molecules become activated, resulting in adhesion of platelets and neutrophils to the endothelium, vasoregulatory dysfunction, and edema (6, 7). The neutrophils become activated and release superoxide anion (O2−), hydrogen peroxide (H2O2), and hydroxyl radical (OH−). In addition, during these inflammatory responses, the neutrophils release cytokines, which activate inducible nitric oxide synthase (iNOS). This enzyme produces NO in high, cytotoxic concentra-
tions. NO and $O_2^-$ react to form peroxynitrite (ONOO$^-$), a reactive oxygen species known to trigger apoptosis in the myocardium and cardiovascular endothelial cells (8). The compromised endothelium allows infiltration of neutrophils and their subsequent release of reactive oxygen species and cytokines into the myocardium furthering reperfusion injury. It is imperative that the integrity of the cardiovascular endothelium remains intact to protect the myocardium.

During cardiopulmonary bypass (CPB) surgery, the perfusionist presides over the reperfusion of the ischemic heart. It is well known that CPB can trigger the release of cytokines through increased serum levels of lipopolysaccharide and through blood contact with the bypass circuit (9). Specifically, the increased serum levels of the cytokine tumor necrosis alpha have been shown to activate iNOS in the myocardium (10). Taken together, the events described above become clinically relevant to the perfusionist. Interest in developing pharmacological interventions to prevent apoptosis in the heart is driving research at an
accelerating pace. Some of these interventions may soon find their way into the hands of the perfusionist to administer during CPB. Knowledge of apoptosis may prove to be useful to the perfusionist in the future.

Cardiomyocyte cell culture models have been used extensively in past studies of apoptosis. Arstall et al. found cytokines induced apoptosis in cultures of rat ventricular myocytes mediated through increased iNOS expression and exposure to peroxynitrite (8). Inssete et al. employed nitrogen gas and hydrogen peroxide to simulate oxidative stress and ischemia in their cell culture model of rat cardiomyocytes resulting in an increase of apoptotic cells (11). Pinsky et al. found that nitric oxide produced by the donor (±)-S-nitroso-N-acetylpenicilliamine induced apoptosis in cultures of rat ventricular myocytes. In addition, scavenging of nitric oxide attenuated apoptosis (12). The following experiment was designed to study the induction of apoptosis caused by oxidative stress from nitric oxide exposure and simulation of ischemic reperfusion in an endothelial cell culture model.

MATERIALS AND METHODS

ECV-304 human endothelial cells (American Type Culture Collection, Rockville, MD) were incubated (37°C, 5% CO₂) in 10 25 cm² culture flasks (Corning, 0.2-µm vented cap) with Dulbecco’s Modified Eagles Medium F-12 HAM (Sigma, Saint Louis, MO) until confluent. Two cultures were untreated and incubated for 4 h as negative controls. To establish positive controls for apoptosis, one culture flask was immersed in a 43°C water bath for 30 min followed by 4 hours of incubation. A second positive control was established by adding staurosporine to culture as described in Annexin V-FITC Apoptosis Detection Kit A2214 (Sigma) followed by incubation for 2 hours. 25 mL of ischemic medium was prepared by bubbling pure nitrogen gas through it for 24 hours. Ischemia was simulated by removing existing culture medium from four culture flasks and replacing it with 3 mL of ischemic medium in each flask. Pure nitrogen gas was blown over the medium in each flask for a 2-hours incubation period. To simulate ischemic reperfusion, the nitrogen was turned off. At this time, the NO donor (±)-S-nitroso-N-acetylpenicilliamine (SNAP, Sigma) was added to two ischemic reperfusion cultures and two untreated cultures. Incubation continued for 2 hours.

At the end of the 4-hours incubation period, cells from each group were prepared for electron microscopy as follows. The cells were centrifuged in cell medium at 5000 rpm for 10 min. The medium was removed; the cells were resuspended in 0.1M cacodylate buffer and centrifuged at 5000 rpm for 10 min. The buffer was removed, and the cells were resuspended and fixed in 3% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.2 for 1 hours at room temperature. At the end of the 1-hours period, the fixative was removed, and the cells were resuspended in buffer and refrigerated at 4°C.

Apoptosis Assay by Flow Cytometry

The Annexin V-FITC Apoptosis Detection Kit (Sigma) was used to quantify apoptotic cell death by flow cytometry. During the early stages of apoptosis, the membrane lipid phosphatidylserine flips from the intracellular membrane to the extracellular membrane (13). The Annexin V protein binds to phosphatidylserine on the outer membrane of apoptotic cells with high affinity in the presence of Ca²⁺ while emitting a green fluorescence (13). Propidium iodide binds to DNA and is detected by a red fluorescence inside the cell, indicating membrane disruption and necrosis. Cells from each group were washed twice with PBS, buffered with a calcium binding buffer, stained with propidium iodide and Annexin V-FITC, and incubated in the dark at room temperature for 10 min. Fluorescence of the cells was then determined by flow cytometry.

RESULTS

The data indicate that ischemic reperfusion alone, and ischemic reperfusion with NO increases apoptosis; whereas, NO alone decreases apoptosis. Figure 2 shows the change in percentage of apoptotic cells between the control group (4.7%) and treated groups as determined by flow cytometry detection of Annexin V expression. The two positive controls, heat treatment and staurosporine treatment, resulted in 45.9% and 82.6% increases of apoptosis, respectively. Ischemic reperfusion increased apoptosis 18.5%; whereas, ischemic reperfusion and SNAP showed a 22.7% increase. SNAP treatment alone resulted in a 34.8% decrease in the number of apoptotic cells.

DISCUSSION

Ischemic reperfusion refers to the resumption of adequate blood flow through the ischemic tissue. Although essential for functional recovery, reperfusion of ischemic tissue can cause further damage and possibly cause cell death by either apoptosis or necrosis.

The purpose of this study was to simulate ischemic reperfusion and determine its effect with nitric oxide on the apoptotic process in endothelial cells. The data indicate that ischemic reperfusion of human endothelial cells induces apoptosis; whereas, nitric oxide attenuates apoptosis. In addition, nitric oxide in the presence of ischemic reperfusion seems to cause a greater increase in apoptosis than ischemic reperfusion alone (22.7% vs. 18.5%). These contradictory roles for NO have been shown to be enzyme- and concentration-dependent. Endothelial nitric
oxide synthase produces low physiologic concentrations of nitric oxide that protect the vasculature; whereas, inducible nitric oxide synthase triggers release of high, damaging levels resulting in oxidative stress, cytokine expression, and inflammation (4, 8). In this experiment, the increased NO provided by SNAP seems to be responsible for increased apoptotic cell numbers in culture. Further tests must be done to identify the apoptotic pathway.

Induction of apoptosis occurs through a series of steps that can be activated by reactive oxygen species and cytokines produced during ischemic reperfusion of the myocardial vasculature. These molecules trigger intracellular signaling pathways that might be interrupted with pharmacological compounds before the cell is committed to apoptosis. If apoptosis can be prevented in the endothelium where ischemic reperfusion injury begins, greater protection of the ischemic myocardium might be achieved. Areas of research into the inhibition of apoptosis include antioxidant therapy, inhibition of pro-apoptotic proteases, and prevention of cytokine damage.

Significant ischemic reperfusion injury results from the adhesion of activated neutrophils to activated endothelium and decreased NO expression by eNOS. In a recent study, Lindemann et al. have shown a correlation between decreased levels of NO and up regulation of intracellular adhesion molecule-1 resulting in increased neutrophil adhesion to the endothelium (14). Activated neutrophils release reactive oxygen species, which can further damage the tissue because of disruption of membranes, DNA, and endothelium (7, 15). Use of, and research into antioxidants as pharmacological agents to block these processes is ongoing. Carvedilol, used in the treatment of hypertension, protects the myocardium and cardiovascular endothelium during ischemic reperfusion by scavenging reactive oxygen species that activate intracellular adhesion molecule one, thus attenuating activation of neutrophils (16, 17). Although mannitol is known to scavenge the extremely toxic hydroxyl radical (18), there are conflicting reports on its apoptotic effects. Korenkov et al. have shown that mannitol significantly reduces apoptotic cell numbers in infarct areas of ischemic reperfused mouse cerebellum (19). Surprisingly, mannitol has also been reported to induce apoptosis at clinical concentrations in bovine aortic endothelial cells (20).

In human coronary arteries, Li et al. have shown that the oxidative stress accompanying ischemic reperfusion causes endothelial dysfunction and enhanced apoptosis. Low-density lipid proteins in the vascular endothelial cells are oxidized during reperfusion increasing lipid peroxidation and decreasing the protective effect of superoxide dismutase, an enzyme involved in cellular antioxidant defense. Lipid peroxidation of coronary artery endothelium results in cellular membrane disruption, edema in the interstitium, and destruction of the cells (21). In addition, hypoxia and reoxygenation increases Fas expression, a cell surface signaling molecule that induces apoptosis through activation of intracellular signaling molecules (22). This coincides with decreased expression of bcl-2, an anti-apoptotic gene (21). Activation of Fas activates protein tyrosine kinases resulting in DNA fragmentation and apoptosis (23). Li et al. (21) were able to reduce endothelial apoptosis with genistein, an inhibitor of protein tyrosine kinases, suggesting a therapeutic approach to protecting coronary artery endothelium during ischemic reperfusion.

**Figure 2.** Analysis of Annexin V-labeled human vascular endothelial cells.
In an experiment conducted on immature porcine hearts by Ihnken et al., adding the antioxidants (N-(2-mercaptopyrrolionyl)-glycine and catalase) to the prime decreased lipid peroxidation during CPB. Greater cardiac function and high antioxidant reserves were achieved in treated animals as compared to controls. Ihnken et al. found that reperfusion of ischemic hearts without the antioxidant treatment negated the protective effects of blood cardioplegia, resulting in decreased contractility of the myocardium and oxidative damage (24). Interestingly, off-pump CABG (OPCABG) has resulted in short-term left ventricular dysfunction related to the short-term periods of ischemia produced by coronary artery occlusion during the procedure (25). It has been suggested that impaired endothelial function in coronary arteries may result from OPCABG (26).

The caspases are cysteine proteases responsible for cleaving proteins that maintain the viability of cells. During ischemic reperfusion, caspases are activated in both the heart and endothelium. Poly(ADP-ribose) polymerase, a protein responsible for DNA repair, is cleaved by activated caspases, rendering it unable to repair DNA damage resulting in apoptosis (27). Inhibition of caspase 3 activation prevents apoptosis (28), providing researchers with a target at which to aim new therapeutics. For example, in myocardium, the caspase inhibitors acetyl-Tyr-Val-Ala-Asp chloromethylketone and Z-Val-Ala-Asp(Ome)-CH₂F attenuated apoptosis and decreased the infarct zone (27).

Attenuation of the damaging effects of tumor necrosis factor alpha is drawing the attention of researchers in the fight against apoptosis. During CABG, activation of apoptotic pathways related to this cytokine have been observed (29). Two drugs that can block the function of tumor necrosis factor alpha are being studied currently. Treating rat myocardium with adenosine before ischemia decreased tumor necrosis factor alpha levels (30). In addition, etanercept is able to bind to tumor necrosis factor alpha, blocking its ability to bind to myocardial cells, resulting in improvement in the conditions of patients with congestive heart failure (Class III and IV) (31).

Increased expression of the gene transcription factor NFκB occurs through oxidative stress upon reperfusion of the myocardium during open-heart surgery. NFκB is capable of activating genes responsible for synthesis of several inflammatory mediators, including intracellular adhesion molecule one and tumor necrosis factor (32). Although NFκB is usually bound to inhibitory proteins, IkB, upon ischemic reperfusion oxidative stress, triggers a series of events leading to the detachment of IkB from NFκB. NFκB can translocate to the nucleus and transcribes tumor necrosis factor (10). Inhibition of translocation or activation of NFκB during ischemic reperfusion would be a likely target in the prevention of apoptosis.

CONCLUSION

Apoptosis is a complex process and presents many points of intervention. Development of cardioplegia and new pharmacological agents to add to the prime could put perfusionists on the front line in the effort to prevent apoptosis triggered by reperfusion injury. Addressing apoptosis in the cardiovascular endothelium and myocardium might result in better outcomes for patients undergoing cardiopulmonary bypass surgery. If ways can be found to attenuate ischemic reperfusion injury to the endothelium, it will be headed off in the myocardium at the same time.

REFERENCES

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