Abstract

Cell death due to ischemia is observed frequently under conditions in which an organ is deprived of blood flow and is subsequently reperfused, or restored with blood supply. Myocardial ischemia occurs when there is an interruption in the blood supply to the heart muscle, most often the result of a thrombus (arterial blood clot). Subsequent reperfusion of ischemic tissue results in extension of the tissue injury beyond that due to the deprivation of blood flow; it is due to the reintroduction of oxygen to the previously ischemic heart muscle, a paradoxical event referred to as “reperfusion injury.” Estrogen, a steroid sex hormone, has been shown to protect against such cell death. It is still unknown which of two estrogen receptors (ERα/β) mediates the mechanism for the protective effects against reperfusion injury.

An in vivo rabbit model was used in which ER-specific compounds were administered, and the extent of myocardial protection was examined. The ERα agonist PPT along with 17β-estradiol produced significant reductions in myocardial infarct size when compared to vehicle-treated controls, whereas the ERβ agonist DPN did not. The results of the experiment support ERα as a mediator of estrogen’s cardioprotection against myocardial ischemia and reperfusion injury.

Introduction

One of the most common types of cellular damage in clinical medicine is a result of ischemia. Ischemia is a deficiency of blood supply to a tissue or organ. Clinical examples include arteriosclerosis, thrombotic arterial occlusions, and reduced cardiac output. As the center for blood circulation and distribution, it is evident that the heart plays a major role in proper blood supply to tissues and organs. The heart muscle (myocardium) is itself susceptible to an ischemic incident, usually due to an obstruction of one or more of the coronary arteries, the vessels which provide nutrient blood flow to the heart muscle. Myocardial ischemia leading to the progressive death of heart muscle cells represents the most common cause of heart failure. The sudden obstruction of a major coronary artery, most often due to the rapid formation of a thrombus is the immediate cause of a myocardial infarction (heart attack) and sudden cardiac death.

When myocardial tissue is subjected to a brief ischemic insult (less than 30 minutes), it is possible for the cells to recover fully. However, if ischemia is prolonged (45 minutes or more), there exists a subsequent transformation of cells from reversible damage to a state of irreversibility [1]. With restoration of blood flow, termed reperfusion, a paradox is manifested, in which oxygen-rich blood, essential to tissue survival, causes an increase in the extent of irreversible cell damage [1]. This escalation of cellular damage is referred to as reperfusion injury. Although it frequently occurs in the myocardium, reperfusion injury can also affect any other ischemic tissue or organ that is subject to restoration of blood flow [1]. Such cases include organ transplantation and some types of surgery (e.g., open-heart surgery) in which blood flow must be interrupted in order to provide a bloodless field to permit the surgical procedure to be conducted under direct visualization.

Damage to cells after myocardial ischemia/reperfusion injury arises through several mechanisms. The cells of ischemic tissue recruit neutrophils (the most abundant of leukocytes, acting as phagocytes against foreign particles) to the site of damage and in turn, become activated. Upon neutrophil activation and accumulation, release of reactive oxygen free radicals as well as cytotoxic constituents (hydrogen peroxide, hypochlorous acid, proteolytic enzymes) released from the invading neutrophils cause further damage [1,2]. Previous studies have shown that inhibitors of neutrophil activation and accumulation attenuate damage to ischemic tissue after reperfusion. Aside from neutrophil activation, tissue damage can result from activation of the complement system, which is composed of two pathways, converging to form the membrane attack complex (MAC), which forms a pore in the cell membrane. When activated, the products
of the complement cascade can damage cells indirectly through recruitment of neutrophils (anaphylatoxins C5a and C3a), or through cell lysis elicited by assembly of the MAC (C5b-9) on the cell membranes in the area of reperfusion [2].

The incidence of coronary vascular disease increases two-fold among women after menopause, which is associated with a decrease in the synthesis and release of estrogen. However, it has been demonstrated that the administration of estrogen may have adverse effects due to the lack of specificity for heart muscle. Thus, hormone replacement therapy, once believed to be beneficial for the prevention of cardiovascular events in the post-menopausal patient, has limitations to its usage [3]. Several controlled, double-blind clinical trials have generated unfavorable outcomes, and the recommendation is for short-term usage of hormone replacement therapy for the relief of vasomotor symptoms (e.g., hot flashes).

However, recent experimental studies have shown estrogen (17β-estradiol) to be cardioprotective in terms of reducing the extent of reperfusion injury. Two estrogen receptors (ER) have been identified, mainly ERα and ERβ. The exact mechanism by which estrogen protects against reperfusion injury is still unknown [3]. The focus of the study was to learn more about the mechanism behind this cardioprotection, and specifically, whether it is mediated through ERα or ERβ. Experimental data show 17β-estradiol, the naturally occurring form of the hormone estrogen, to be protective, but finding a more selective compound would provide the benefits without the adverse effects exerted upon the uterus, breast and blood clotting system. To do this, estrogen receptor-specific compounds were used and examined for the extent to which they offered cardioprotection against myocardial ischemia and reperfusion injury.

Materials and Methods

Surgical Preparation

Female New Zealand White rabbits were anesthetized with ketamine and xylazine, administered intramuscularly. Sodium pentobarbital was administered intravenously as needed throughout the duration of the experiment to maintain a state of surgical anesthesia. An endotracheal tube was inserted that allowed for controlled respiration with the use of a positive pressure respirator. The left jugular vein was isolated and a catheter was inserted for drug administration and blood sampling. A Millar™ micro pressure transducer was inserted into the left carotid artery to record the aortic blood pressure, and an electrocardiogram was monitored throughout the duration of the protocol. The chest was opened through the left side to expose the heart, and the pericardium was opened to expose the surface of the heart and in order to visualize the coronary arteries, thereby concluding the preliminary surgical preparation [Figure 1].

Administration

The animals were allowed to equilibrate for 15 minutes after the surgery. After the stabilization period, one of four drug treatments was administered: vehicle (n = 8), composed of 20% dimethyl sulfoxide (DMSO) and 80% polyethylene glycol (PEG); 17β-estradiol (10 µg; n = 8), non-selective, binding to both ERα and ERβ; 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)triphenol, or PPT (3 mg/kg; n = 8), a selective ERα agonist; or 2,3-bis(4-Hydroxyphenyl)-propionitrile, or DPN (3 mg/kg; n = 8), a selective ERβ agonist. After 30 minutes, the left anterior descending coronary artery was occluded by passing a suture underneath the artery and tying it around a segment of plastic tubing thereby occluding the vessel [Figure 2]. Regional myocardial ischemia was maintained for a period of 30 minutes and was confirmed by a distinct
The heart was then removed from the apparatus and three cross sections of the left ventricular tissue were obtained and examined [Figure 3]. Heart sections were traced and scanned. Adobe Photoshop was used to calculate the number of pixels for the areas of total left ventricle, area at risk, and infarct size. Along with examining the myocardial tissue, blood samples were also analyzed. Samples were taken at different time points in the experiment for all treatment groups and were assessed for troponin I levels. Troponin is a protein complex, an isoform that exists in the cardiac muscle (cTnI), and its levels correlate positively with tissue damage. Antibodies

**Figure 2:** The Heart: A picture of an animated heart. The left anterior descending (LAD) coronary artery is identified. The location of the ligature is also marked, and some tissue below the occlusion was subject to ischemia. Finally, three cross-sectional slices of left ventricular tissue were examined for infarct size.

**Analysis**

The degree of protection afforded by each treatment was assessed by determining the extent of myocardial tissue undergoing irreversible tissue injury (myocardial infarction). After the 4 hours of reperfusion, the hearts were removed and placed on a Langendorff perfusion apparatus that circulated buffer through the heart, clearing the coronary vascular bed of blood cellular elements and plasma. A solution of 1% triphenyltetrazolium chloride (TTC) in phosphate buffer was passed through the heart, staining the viable, non-infarct tissue that was subject to ischemia a bright red color. The cells with irreversible damage do not stain, and remain a pale yellow color. The coronary artery was then ligated just above the original point of occlusion that induced myocardial ischemia to insure the same region of myocardium was now tied off (area at risk). After stopping the perfusion pump, a solution of Evans blue dye was infused through the heart, marking all tissue that was not subjected to ischemia. The heart was then removed from the apparatus and three cross sections of the left ventricular tissue were obtained and examined [Figure 3]. Heart sections were traced and scanned. Adobe Photoshop was used to calculate the number of pixels for the areas of total left ventricle, area at risk, and infarct size. Along with examining the myocardial tissue, blood samples were also analyzed. Samples were taken at different time points in the experiment for all treatment groups and were assessed for troponin I levels. Troponin is a protein complex, an isoform that exists in the cardiac muscle (cTnI), and its levels correlate positively with tissue damage. Antibodies
specific to cardiac troponin I were used to detect levels of the complex at different stages during the experiment.

Results

Administration

The area at risk as a percent of the total left ventricle was calculated to ensure consistency with the amount of tissue subject to ischemia from animal to animal. In determining the area at risk (AR) as a percent of the total left ventricular tissue (LV), the treatment group averages were as follows: 59.2 ± 3.0 with vehicle, 55.2 ± 2.8 with 17β-estradiol, 59.4 ± 4.2 with PPT, and 58.7 ± 2.2 with DPN [Figure 4].

Analysis

The infarct size as a percent of the area at risk (tissue subject to ischemia) was calculated to determine the extent of damage in each treatment group. When calculating the infarct zone (IZ) as a percent of the area at risk (AR), there were significant differences among the treatment groups. The average infarct size decreased among the 17β-estradiol (ERα/β agonist) group (17.7 ± 2.9) when compared to the vehicle group (45.3 ± 2.4; p < 0.001). The group pretreated with PPT (ERα) also showed a similar decrease in infarct size (18.1 ± 2.3) when compared to vehicle (p < 0.001). The ERβ agonist DPN, however, showed no significant reduction of infarct size (44.5 ± 4.1) as compared with vehicle [Figure 5].

Troponin levels seemed to increase from the baseline to the 2-hour reperfusion mark, and even more at the 4-hour reperfusion mark. However, the rabbits treated with 17β-estradiol and PPT showed a lesser increase in troponin levels than in vehicle or DPN treated animals [Figure 6].

Discussion

When analyzing the results, the data showed that a consistent amount of tissue was subject to ischemia during the surgical procedure across all treatment groups. Therefore, any differences in infarct size were due to the drug treatment interventions. The calculations indicate that both 17β-estradiol and PPT result in a smaller region...
of infarct tissue, suppressing the damage caused by reperfusion injury. The DPN group did not show this decrease in tissue damage, exhibiting similar results to the control group. The reduced troponin levels in the 17β-estradiol- and PPT-treated groups further support the evidence for cardioprotection elicited by these drugs. The data compiled from this study, consequently, do not support ERβ as having a major role in mediating estrogen’s protective activity. However, results from this experiment do indeed show evidence for ERα as a primary component in estrogen’s mechanism for cardioprotection against myocardial ischemia and reperfusion injury. With these results and future studies, more will be known of the mechanism behind estrogen’s cardioprotection. The adverse effects of current hormone replacement therapy will be minimized without compromising the benefits through novel therapeutic interventions.

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**References**

Figure 6: **Troponin Levels:** Troponin is a protein complex released in damaged muscle cells. The more damaged a cell becomes, the higher its concentration of troponin release. Plasma samples were obtained for all treatment groups at time points, and troponin levels were examined using antibodies. All treatment groups showed an increase in troponin level from baseline to 2-hour reperfusion, and from 2- to 4-hour reperfusion. However, the vehicle and DPN (ERβ agonist) groups showed a much larger increase in troponin levels than did either 17β-estradiol (ERα/β agonist) or PPT (ERα agonist).