CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

APROTININ PRESERVES MYOCARDIAL BIOCHEMICAL FUNCTION DURING COLD STORAGE THROUGH SUPPRESSION OF TUMOR NECROSIS FACTOR

David A. Bull, MD
Rafe C. Connors, BS
Aida Albanil, BA
Bruce B. Reid, MD
Leigh A. Neumayer, MD
Ryan Nelson, BS
James C. Stringham, MD
Shreekanth V. Karwande, MD

Objective: Inflammatory cytokines, particularly tumor necrosis factor, contribute to myocardial dysfunction after ischemia-reperfusion injury. Aprotinin may improve outcomes in cardiac surgery through suppression of inflammatory mediators. We hypothesized that aprotinin may exert its beneficial effects through suppression of tumor necrosis factor α . **Methods:** Adult rat hearts were precision cut into slices with a thickness of 200 μm and stored in crystalloid cardioplegic solution alone or with one of the following additions: aprotinin or tumor necrosis factor α , aprotinin plus tumor necrosis factor α , a monoclonal antibody to tumor necrosis factor α , or a polyclonal antibody to the tumor necrosis factor α receptor. Myocardial biochemical function was assessed by adenosine triphosphate content and capacity for protein synthesis immediately after slicing (0 hours) and after 2, 4, and 6 hours of storage at 4°C. The content of tumor necrosis factor α was measured by an enzyme-linked immunosorbent assay. Six slices were assayed at each time point for each solution. The data were analyzed by analysis of variance and are expressed as the mean $\pm$ standard deviation. **Results:** When stored in cardioplegic solution containing aprotinin, the heart slices demonstrated (1) an increase in adenosine triphosphate content and protein synthesis ($P < .0001$), (2) a decrease in intramyocardial generation of tumor necrosis factor α ($P \leq .0311$), and (3) a decrease in uptake of tumor necrosis factor α into the myocardium ($P \leq .002$) compared with storage in cardioplegic solution alone. The presence of an antibody to tumor necrosis factor α or an antibody to the tumor necrosis factor α receptor in cardioplegic solution increased intramyocardial adenosine triphosphate content and protein synthesis ($P < .0001$). **Conclusions:** Aprotinin preserves myocardial biochemical function during cold storage. This preservation of biochemical function is mediated through suppression of the release, uptake, and activity of tumor necrosis factor α . (J Thorac Cardiovasc Surg 2000;119:242-50)

From the Division of Cardiothoracic Surgery, Department of Surgery, University of Utah Health Sciences Center, Salt Lake City, Utah.

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Address for reprints: David A. Bull, MD, Division of Cardiothoracic Surgery, Department of Surgery, University of Utah, 50 N Medical Dr, Salt Lake City, UT 84132 (E-mail: david.bull@hsc.utah.edu).

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Myocardial dysfunction after ischemia-reperfusion continues to be an important problem in cardiac surgery.¹ Evidence implicates inflammatory cytokines, particularly tumor necrosis factor α (TNF- α), in the myocardial dysfunction observed after ischemia-reperfusion injury.² TNF- α induces myocardial depression through multiple mechanisms including disruption of calcium homeostasis and induction of secondary depressants such as interleukin 1.¹ The alteration in calcium release and response depletes adenosine triphosphate (ATP) stores and depresses myocardial contractile efficiency, resulting in systolic and diastolic

dysfunction.¹ The mechanisms of TNF- α -induced myocardial depression can be divided into nitric oxide-dependent and nitric oxide-independent pathways.¹ The nitric oxide-dependent pathway mediates TNF- α -induced myofilament desensitization to calcium.¹

In contradistinction, aprotinin may improve outcomes in cardiac surgery through suppression of inflammatory mediators.³ Aprotinin has protective effects on the myocardium after ischemia and reperfusion: improved contractility, faster decline of ischemic contracture, and preservation of adenine nucleotide stores.⁴ Clinically, aprotinin reduces TNF- α blood levels in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass.⁵ Mechanistically, aprotinin can inhibit cytokine-induced nitric oxide synthase expression, reducing subsequent nitric oxide production by bronchial epithelial cells.⁶

Linking these two lines of investigation, we hypothesized that aprotinin mediates preservation of myocardial biochemical function through inhibition of the release, uptake, or activity of TNF- α . To test this hypothesis, we specifically investigated whether: (1) aprotinin can preserve myocardial biochemical function during ischemic cold storage; (2) the myocardium releases TNF- α during cold storage, before reperfusion; (3) the release of TNF- α is associated with a decrease in myocardial biochemical function during cold storage; (4) aprotinin preserves myocardial biochemical function during cold storage by suppression of the release, uptake, or activity of TNF- α ; and (5) aprotinin suppresses TNF- α -induced nitric oxide production within the myocardium.

Materials and methods

Materials. The crystalloid cardioplegic solution used for all experiments was D5 0.2%NS plus 20 mEq KCl (Baxter, Deerfield, Ill) to which 10 mEq HCO₃⁻ was added. Aprotinin was obtained from Bayer (West Haven, Conn) and used at a concentration of 200 KIU/mL with NaOH added to adjust the pH to 5.5 in all experiments. TNF- α was obtained from R&D Systems (Minneapolis, Minn) and used at a concentration of 100 pg/mL in all experiments. The monoclonal antibody to TNF- α (anti-TNF- α antibody) was obtained from R&D Systems and used at a concentration of 100 pg/mL in all experiments. TNF- α receptor blockade was accomplished with a polyclonal antibody to the TNF- α receptor (anti-TNF- α receptor antibody) obtained from Santa Cruz Biochemical (Santa Cruz, Calif) and used at a concentration of 100 pg/mL in all experiments.

Animals. All animals received humane care in compliance with the guidelines of the Institutional Animal Care and Use Committee at the University of Utah and the "Guide for the

Care and Use of Laboratory Animals" (NIH Publication 85-23, revised 1985).

Sprague-Dawley rats (Sasco, Wilmington, Mass) weighing 200-250 g were anesthetized with halothane. With the use of strict sterile technique, a median sternotomy incision and cardiectomy of the whole rat heart were rapidly performed.

Slice preparation and storage. Immediately after cardiectomy, the whole rat hearts were placed into 1 of 6 storage solutions: crystalloid cardioplegic solution alone or crystalloid cardioplegic solution with one of the following additions: aprotinin, TNF- α , aprotinin plus TNF- α , an anti-TNF- α antibody, or an anti-TNF- α receptor antibody. The whole rat hearts were then precision cut into slices with a thickness of 200 μ m in the same solution used for storage. Slicing was performed with a Vitron tissue slicer (Vitron, Tucson, Ariz). The time from cardiectomy to completion of slicing was 10 minutes. This time interval was strictly controlled and did not vary between experiments. Ten minutes represents the minimum time from cardiectomy to generation of a set of slices. Each rat heart was used to generate 4 slices on which biochemical assays were performed immediately after slicing (0 hours) or after 2, 4, and 6 hours of storage at 4°C. Each myocardial slice was stored in a cold room at 4°C for 2, 4, or 6 hours in a separate 100-mL flask containing 50 mL of solution. The flasks were gently shaken on an orbital shaker during storage. The experiments were repeated 5 times on separate occasions for each solution. Slices from 6 different rat hearts were measured at each time point for each storage solution. A total of 120 rat hearts were used to complete all of the experiments in the study.

Measurement of myocardial biochemical function. Myocardial biochemical function was assessed by ATP content and capacity for protein synthesis immediately after slicing (0 hours) and after 2, 4, and 6 hours of storage at 4°C. ATP content was measured with the use of a luciferin-luciferase bioluminescent assay.⁷ After being weighed, the tissue slice was placed in 1 mL of 10% trichloroacetic acid, homogenized, and then snap frozen in liquid nitrogen and stored at -80°C. When the collected specimens were ready for assay, the samples were thawed and centrifuged (4200 rpm $\times$ 12 minutes). Ten microliters of the supernatant was taken and diluted with 2 mL of HEPES buffer. Plastic tubes were used to minimize binding of the ATP. Two hundred microliters of each sample was pipetted into luminometer cuvettes (Turner Designs, Sunnyvale, Calif). The luciferin-luciferase solution was prepared to a volume of 15 mL with 30 μ L of luciferase (Amgen Biologicals, Thousand Oaks, Calif), 750 μ L of luciferin (Sigma Chemical Co, St Louis, Mo), 7.5 mL of stabilizing buffer, and 6.75 mL of HEPES buffer. One hundred microliters of luciferin-luciferase was added to each luminometer cuvette. As a reference, an ATP standard was run for each set of ATP assays. ATP levels were measured after addition of the luciferin-luciferase to each cuvette with a luminometer (Turner Designs, Sunnyvale, Calif), referenced to the ATP standard. So that a direct effect of aprotinin on the ATP assay could be avoided, aprotinin at 200 KIU/mL was added to the ATP standards and the stan-

dard curve was repeated. Similarly, the anti-TNF- α antibody and the anti-TNF- α receptor antibody at 100 pg/mL were separately added to the ATP standards and the standard curve repeated. Results are expressed as nanomoles ATP per milligram wet weight.

Protein synthesis was measured by the incorporation of radiolabeled leucine into acid precipitable proteins. After cold storage, the slices were placed onto Teflon/titanium rollers (Vitron, Tucson, Ariz), loaded into glass scintillation vials, and incubated at 37°C in Waymouth solution (Gibco, Grand Island, NY) containing [3 H]leucine (Amersham, Elk Grove, Ill). The solution was prepared with 60 μ L of [3 H]leucine in 200 mL of Waymouth solution to a concentration of 0.3 μ Ci/mL. The slices were incubated for 4 hours in the Vitron dynamic organ incubator. The incubated slices were washed twice in buffer and homogenized in 1 mL of 1N KOH. Twenty-microliter aliquots of the homogenates were then pipetted off and 1 mL of 1.5N acetic acid was added. The solution was left to stand for 24 hours at 4°C and then centrifuged at 3250 rpm for 15 minutes. The resulting pellet was washed two more times in 1 mL volumes of 1N HCl and dissolved in 0.5 mL of 0.5N NaOH. The incorporation of [3 H]leucine into acid precipitable protein was determined by scintillation counting a 0.5 mL aliquot of the dissolved pellets after neutralization with 125 μ L of 2N HCl. Results are expressed as counts per minute of [3 H]leucine incorporated per milligram of protein.

TNF- α assay. TNF- α levels were measured with the use of an enzyme-linked immunosorbent assay for rat TNF- α (R&D Systems). Absorbance of samples and standards were measured with a spectrophotometer at an absorbance of 450 nm with a microplate reader (Bio-Rad Laboratories, Hercules, Calif). A standard curve was run with each set of assays. To exclude a direct effect of aprotinin on the TNF- α assay, we added aprotinin at 200 KIU/mL to the TNF- α standards and then repeated the standard curve. Results are expressed as picograms of TNF- α per milligram wet weight. If the addition of TNF- α to cardioplegic solution resulted in intramyocardial TNF- α levels higher than those measured during cold storage with cardioplegic solution alone, the difference was ascribed to uptake of extracellular TNF- α into the myocardium during the period of cold storage.

Nitric oxide assay. Nitric oxide levels were measured with the use of a nitrate/nitrite colorimetric assay (Cayman Chemical, Ann Arbor, Mich). Absorbance of samples and standards were measured with a spectrophotometer at an absorbance of 540 nm with a microplate reader (Bio-Rad Laboratories). A standard curve was run with each set of assays. To exclude a direct effect of aprotinin on the nitric oxide assay, we added aprotinin at 200 KIU/mL to the nitric oxide standards and then repeated the standard curve. Similarly, the anti-TNF- α antibody and the anti-TNF- α receptor antibody at 100 pg/mL were separately added to the nitric oxide standards and the standard curve was repeated. Results are expressed as nanomolar nitric oxide per milligrams of wet weight.

Presentation of data and statistical analysis. As the biochemical assays measured levels within an individual slice at

a given time point, the data were analyzed by analysis of variance (StatView, Abacus Concepts, Berkeley, Calif) and are expressed as mean $\pm$ standard deviation ($n = 6$ per group). Both P values and F values for the statistical analysis are presented (F values appear in the figure legends).

Results

Aprotinin and preservation of myocardial biochemical function. To determine whether aprotinin preserves myocardial biochemical function during cold storage, we measured ATP content and capacity for protein synthesis in heart slices stored in cardioplegic solution with aprotinin or in cardioplegic solution alone. The presence of aprotinin had no effect on the standard curve for ATP (data not shown), indicating that its addition did not affect the assay. As demonstrated in Fig 1, ATP content was higher in the slices stored in cardioplegic solution containing aprotinin compared with storage in cardioplegic solution alone at each time point from zero through 6 hours ($P < .0001$).

To determine whether the difference in ATP content between the two solutions at the zero-hour time point was due to an immediate protective effect of aprotinin or another variable, we prepared slices in cardioplegic solution containing aprotinin and converted them to storage in cardioplegic solution alone at the zero-hour time point. Similarly, slices prepared in cardioplegic solution were converted to storage in cardioplegic solution containing aprotinin at the zero-hour time point. As demonstrated in Fig 2, the slices prepared in cardioplegic solution with aprotinin and stored in cardioplegic solution alone demonstrated a decline in ATP content compared with those having ongoing storage in cardioplegic solution with aprotinin ($P \leq .0001$). The slices prepared in cardioplegic solution and stored in cardioplegic solution with aprotinin did not demonstrate an improvement in ATP content compared with those subjected to ongoing storage in cardioplegic solution alone. Continuous exposure of the myocardium to aprotinin immediately after cardiectomy through the generation of the slices, then, prevented the otherwise rapid decline in ATP stores seen with preparation and slicing in cardioplegic solution alone. Later addition of aprotinin to cardioplegic solution at the zero-hour time point did not significantly raise ATP content within the myocardium.

As demonstrated in Fig 3, capacity for protein synthesis, a measure of more complex biochemical function, was higher in the slices stored in cardioplegic solution with aprotinin than in those stored in cardioplegic solution alone for 2, 4, and 6 hours of cold storage ($P < .0001$).

Fig 1. Myocardial ATP content in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing aprotinin (—◇—) or cardioplegic solution alone (—■—). ATP content is higher in the heart slices stored in cardioplegic solution containing aprotinin than in cardioplegic solution alone for 0 ($F = 149.33$, $P < .0001$), 2 ($F = 660.21$, $P < .0001$), 4 ($F = 356.12$, $P < .0001$), and 6 hours of cold storage ($F = 124.43$, $P < .0001$). $N = 6$ slices at each time point for each solution.

Fig 2. Myocardial ATP content in the heart slices: (1) prepared and stored in cardioplegic solution containing aprotinin (—◇—); (2) prepared in cardioplegic solution containing aprotinin and converted to storage in cardioplegic solution alone at the zero-hour time point (—Δ—); (3) prepared in cardioplegic solution and converted to storage in cardioplegic solution containing aprotinin at the zero-hour time point (—●—); (4) prepared and stored in cardioplegic solution alone (—■—). The slices prepared in cardioplegic solution with aprotinin and stored in cardioplegic solution alone demonstrate a decline in ATP content compared with ongoing storage in cardioplegic solution with aprotinin for 2 ($F = 360.29$, $P < .0001$), 4 ($F = 139.24$, $P < .0001$), and 6 ($F = 36.52$, $P < .0001$) hours of cold storage. The slices prepared in cardioplegic solution and stored in cardioplegic solution with aprotinin do not demonstrate an improvement in ATP content compared with ongoing storage in cardioplegic solution alone. $N = 6$ slices at each time point for each solution.

Fig 3. Capacity for protein synthesis in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing aprotinin (—◇—) or cardioplegic solution alone (—■—). Protein synthesis is higher in the heart slices stored in cardioplegic solution containing aprotinin compared with cardioplegic solution alone for 2 ($F = 189.62$, $P < .0001$), 4 ($F = 495.56$, $P < .0001$), and 6 ($F = 53.64$, $P < .0001$) hours of cold storage. $N = 6$ slices at each time point for each solution.

Fig 4. Intramyocardial generation of TNF- α in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing aprotinin (—Δ—) or cardioplegic solution alone (—●—). With storage in cardioplegic solution containing aprotinin, the heart slices demonstrate a decrease in intramyocardial generation of TNF- α for 2 ($F = 34.43$, $P = .0042$), 4 ($F = 108.27$, $P = .0019$), and 6 ($F = 10.63$, $P = .0311$) hours of cold storage compared with storage in cardioplegic solution alone. $N = 6$ slices at each time point for each solution.

Aprotinin and intramyocardial generation of TNF- α . To determine whether aprotinin inhibits the generation of TNF- α within the myocardium during cold storage, we measured TNF- α levels in the heart

Fig 5. Intramyocardial levels of TNF- α in the heart slices stored in cardioplegic solution containing aprotinin ($-\Delta-$), cardioplegic solution containing TNF- α plus aprotinin ($-\diamond-$), cardioplegic solution containing TNF- α ($-\blacksquare-$), or cardioplegic solution alone ($-\bullet-$) immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C. The addition of TNF- α to cardioplegic solution results in intramyocardial TNF- α levels higher than those measured during cold storage with cardioplegic solution alone, indicating that extracellular TNF- α can be taken up into the myocardium during cold storage. Shaded areas illustrate intramyocardial TNF- α levels attributed to uptake of TNF- α . With aprotinin present in cardioplegic solution containing TNF- α , there is a decrease in the uptake of TNF- α into the myocardium for 0 ($F = 48.22$, $P < .0001$), 2 ($F = 17.82$, $P = .002$), 4 ($F = 73.08$, $P < .0001$), and 6 ($F = 208.90$, $P < .0001$) hours of cold storage, compared with storage in cardioplegic solution containing TNF- α (Fig 5).

slices stored in cardioplegic solution with aprotinin or in cardioplegic solution alone. The presence of aprotinin had no effect on the standard curve for TNF- α (data not shown), indicating that its addition did not affect the assay. With aprotinin present in cardioplegic solution, the heart slices demonstrated a decrease in intramyocardial generation of TNF- α for 2, 4, and 6 hours of cold storage compared with storage in cardioplegic solution alone ($P \leq .0311$, Fig 4).

Aprotinin and uptake of TNF- α into the myocardium. To determine whether aprotinin inhibits the uptake of TNF- α into the myocardium, we measured TNF- α levels in the heart slices stored in cardioplegic solution containing TNF- α plus aprotinin or cardioplegic solution containing TNF- α . The addition of TNF- α to cardioplegic solution resulted in intramyocardial TNF- α levels higher than those measured during cold storage with cardioplegic solution alone, indicating that extracellular TNF- α can be taken up into the myocardium during cold storage (Fig 5). With aprotinin present in cardioplegic solution containing TNF- α , there was a decrease in the uptake of TNF- α into the myocardium for zero through 6 hours of cold storage, compared with storage in cardioplegic solution containing TNF- α ($P \leq .002$, Fig 5).

Fig 6. Myocardial ATP content in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing an anti-TNF- α receptor antibody (*anti-TNF- α -R1 Ab*, $-\diamond-$), an anti-TNF- α antibody (*anti-TNF- α Ab*, $-\triangle-$), or cardioplegic solution alone ($-\blacksquare-$). The presence of an anti-TNF- α antibody or an anti-TNF- α receptor antibody in cardioplegic solution increases myocardial ATP content for 0 ($F = 339.60$, $P < .0001$), 2 ($F = 146.20$, $P < .0001$), 4 ($F = 160.29$, $P < .0001$), and 6 ($F = 101.06$, $P < .0001$) hours of cold storage compared with storage in cardioplegic solution alone. $N = 6$ slices at each time point for each solution.

tin present in cardioplegic solution containing TNF- α , there was a decrease in the uptake of TNF- α into the myocardium for zero through 6 hours of cold storage, compared with storage in cardioplegic solution containing TNF- α ($P \leq .002$, Fig 5).

Anti-TNF- α antibodies and preservation of myocardial biochemical function. To determine whether TNF- α mediates the decline in myocardial biochemical function during cold storage, we measured ATP content and capacity for protein synthesis in heart slices stored in cardioplegic solution containing an anti-TNF- α antibody, an anti-TNF- α receptor antibody, or in cardioplegic solution alone. The presence of an anti-TNF- α antibody or an anti-TNF- α receptor antibody had no effect on the standard curve for ATP (data not shown), indicating that their addition did not affect the assay. The presence of an anti-TNF- α antibody or an anti-TNF- α receptor antibody in cardioplegic solution increased intramyocardial ATP content for zero through 6 hours of cold storage compared with storage in cardioplegic solution alone ($P < .0001$, Fig 6). Similarly, the presence of an anti-TNF- α antibody or an anti-TNF- α receptor antibody in cardioplegic solution increased intramyocardial protein synthesis for 2, 4, and 6 hours of cold storage compared with storage in cardioplegic solution alone ($P < .0001$, Fig 7).

Aprotinin and nitric oxide production. To deter-

Fig 7. Capacity for protein synthesis in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing an anti-TNF-α receptor antibody (*anti-TNF-α-R1 Ab*, —◇—), an anti-TNF-α antibody (*anti-TNF-α Ab*, —▲—), or cardioplegic solution alone (—■—). The presence of an anti-TNF-α receptor antibody in cardioplegic solution increases myocardial protein synthesis for 2 ($F = 391.10$, $P < .0001$), 4 ($F = 428.75$, $P < .0001$), and 6 ($F = 185.49$, $P < .0001$) hours of cold storage compared with storage in cardioplegic solution alone. The presence of an anti-TNF-α antibody in cardioplegic solution increases myocardial protein synthesis for 2 ($F = 77.34$, $P < .0001$), 4 ($F = 151.12$, $P < .0001$), and 6 ($F = 174.11$, $P < .0001$) hours of cold storage compared with storage in cardioplegic solution alone. $N = 6$ slices at each time point for each solution.

mine whether the effects of aprotinin might be mediated through a TNF-α-induced nitric oxide-dependent pathway, we measured nitric oxide levels in the heart slices stored in cardioplegic solution containing aprotinin, an anti-TNF-α antibody, an anti-TNF-α receptor antibody, or cardioplegic solution alone. The presence of aprotinin, an anti-TNF-α antibody, or an anti-TNF-α receptor antibody had no effect on the standard curve for nitric oxide (data not shown), indicating that their addition did not affect the assay. The addition of aprotinin, an anti-TNF-α antibody, or an anti-TNF-α receptor antibody to cardioplegic solution decreased nitric oxide levels within the myocardium for 2, 4, and 6 hours of cold storage compared with storage in cardioplegic solution alone ($P < .0001$, Fig 8).

Discussion

In these experiments, aprotinin improves preservation of myocardial ATP content and capacity for protein synthesis during cold storage. This improvement in myocardial biochemical function correlates with a suppression in the intramyocardial generation of TNF-α when aprotinin is present. The addition of TNF-α to

Fig 8. Myocardial generation of nitric oxide in the heart slices immediately after slicing (0 hours) and at 2, 4, and 6 hours of storage at 4°C in cardioplegic solution containing aprotinin (—◇—), an anti-TNF-α antibody (*anti-TNF-α Ab*, —▲—), an anti-TNF-α receptor antibody (*anti-TNF-α-R1 Ab*, —●—), or cardioplegic solution alone (—■—). The addition of aprotinin, an anti-TNF-α antibody or an anti-TNF-α receptor antibody to cardioplegic solution decreases nitric oxide levels within the myocardium for 2 ($F = 40.65$, $P < .0001$), 4 ($F = 102.58$, $P < .0001$), and 6 ($F = 206.45$, $P < .0001$) hours of cold storage compared with storage in cardioplegic solution alone. $N = 6$ slices at each time point for each solution.

crystalloid cardioplegic solution results in intramyocardial TNF-α levels higher than those measured during cold storage with cardioplegic solution alone, indicating that extracellular TNF-α can be taken up into the myocardium during cold storage. This uptake of TNF-α into the myocardium is suppressed in the presence of aprotinin. Myocardial ATP content and capacity for protein synthesis during cold storage improved with an anti-TNF-α antibody or an anti-TNF-α receptor antibody present. This implicates TNF-α as a primary mediator of the decline in myocardial biochemical function during cold storage in cardioplegic solution alone. Linking these experimental results, we can conclude that aprotinin mediates improvement in the preservation of myocardial biochemical function during cold storage through the suppression of the release, uptake, and activity of TNF-α. Finally, aprotinin suppresses TNF-α-induced nitric oxide production within the myocardium during cold storage. The depression of myocardial biochemical function by TNF-α may, therefore, be mediated through a nitric oxide-dependent pathway.

Inflammatory cytokines, in particular TNF-α, are principal mediators of myocardial dysfunction after ischemia-reperfusion injury.¹ Both myocardial macro-

phages and cardiac myocytes synthesize TNF- α .¹ Cardiac myocytes produce substantial amounts of TNF- α in response to ischemia.¹ In fact, the myocardium can produce as much TNF- α per gram of tissue as either the liver or the spleen.¹ TNF- α produced within the myocardium is likely the primary contributor to myocardial dysfunction after ischemia-reperfusion.¹ Locally produced TNF- α contributes to postischemic myocardial dysfunction via direct depression of contractility and induction of myocyte apoptosis.¹ Mechanistically, TNF- α appears to depress systolic function by disrupting calcium release by the sarcoplasmic reticulum.¹

Temporally, the effects of TNF- α can be divided into early and late phases.¹ The early phase occurs within minutes of the release of TNF- α .¹ This pathway is independent of nitric oxide and is mediated by sphingosine disruption of calcium transients.¹ The late phase occurs hours after release of TNF- α and is correlated with induction of nitric oxide synthase, subsequently resulting in release of nitric oxide, myofilament desensitization to calcium, and sustained contractile dysfunction.¹ Suppression of TNF- α release within the myocardium, therefore, might modify the myocardial dysfunction after ischemia-reperfusion injury and improve patient outcomes.

The use of crystalloid cardioplegic solution in our experiments eliminates the confounding variable of macrophage production of TNF- α with blood storage. Gurevitch and associates⁸ have demonstrated TNF- α production within the myocardium during reperfusion after arrest with crystalloid cardioplegic solution. They concluded that TNF- α production occurs during the reperfusion phase.⁸ Our results demonstrate that TNF- α production also occurs during ischemic cold storage before reperfusion.

Aprotinin was used at a concentration of 200 KIU/mL in all our experiments because this corresponds to the concentration observed to produce anti-inflammatory effects *in vivo*.⁹ In our studies, aprotinin helps to maintain ATP levels during the period of ischemic cold storage before reperfusion. This extends the findings of Sunamori and colleagues,⁴ who previously demonstrated improved preservation of adenine nucleotides with aprotinin after ischemia and reperfusion. Further, the protective effects of aprotinin extend to preservation of capacity for protein synthesis during cold storage, indicating general preservation of cell function beyond conservation of energy stores. Exposure of the slices to aprotinin, an anti-TNF- α antibody, or an anti-TNF- α receptor antibody during the 10 minutes from cardiectomy to slice generation resulted in an elevation in ATP levels compared with

preparation and slicing in the cardioplegic solution alone group, as measured at the zero-hour time point. Our crossover studies with the slices at the zero-hour time point demonstrate that the improved ATP content is due to an immediate protective effect conferred by aprotinin. The further elevation in ATP levels in the presence of an anti-TNF- α antibody or an anti-TNF- α receptor antibody suggests more effective blockade of TNF- α -mediated depletion of ATP stores. Mechanistically, the rapid depletion of ATP stores in the absence of aprotinin and the conservation of ATP stores over hours of cold storage when aprotinin is present suggests that depletion of ATP stores may proceed through both nitric oxide-dependent and nitric oxide-independent pathways. Depletion of ATP stores occurring through both nitric oxide-dependent and nitric oxide-independent pathways would account for the difference in ATP levels seen at the zero-hour time point when TNF- α levels have not yet diverged.

With regard to our experimental model, the use of tissue slices has advantages over both *in vivo* whole organ models and *in vitro* cellular models to study organ function. Advantages include a reduction in the number of animals used, a decrease in experimental variation, more rapid production of experimental results, and elimination of humoral and neuronal systemic influences.¹⁰ Preservation and homogenization of tissue in a measured, standardized fashion is much simpler with myocardial slices rather than with whole organs, facilitating the measurement of quantifiable biochemical end points. Compared with *in vitro* cell culture or cell suspension models, tissue slices maintain the multicellular composition of intact tissue, preserving the intracellular connections used in maintaining contact inhibition, signal transmission, and hormonal and ion transport.¹⁰ These intracellular connections are typically lost with the protease digestion necessary to isolate single cell types used with *in vitro* models.¹⁰ With optimization of the slice thickness, efficient gas and nutrient exchange can be maintained with diffusion into the tissue of entering nutrients and oxygen and egress of cellular by-products.¹⁰ Tissue slices, therefore, can serve as a proxy for whole organ models.^{11,12}

In our experience, limitations of the application of tissue slicing to myocardial tissue include the need to precisely maintain a slice thickness of 200 μ m, as greater slice thicknesses do not allow satisfactory diffusion into the slice of oxygen and nutrients, resulting in cell death. Similarly, myocardial slices at 37°C are quite sensitive to changes in oxygen tension, and significant hypoxia must be avoided to prevent cell death.

Assessment of biochemical function is a requirement for determining effectiveness of cellular preservation

with cold storage. Preservation of myocardial biochemical function can be assessed by measurement of ATP levels and capacity for protein synthesis. ATP stores in the heart are critical for energy production during and after reperfusion, as well as for prevention of structural injury from ischemic contracture during storage. Capacity for protein synthesis is a general indicator of biochemical function because it requires the integration of several complex enzymatic pathways. Maintenance of the capacity for protein synthesis is therefore a more sophisticated measure of the ability of the myocardium to carry out biochemical functions integral to cell viability after cold storage.

In conclusion, TNF- α depresses myocardial biochemical function during cold storage through depletion of ATP stores and reduction in capacity for protein synthesis. In contradistinction, aprotinin improves myocardial ATP levels and protein synthesis during cold storage. Aprotinin mediates this improvement in preservation of biochemical function through suppression of the release, uptake, and activity of TNF- α .

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Discussion

Dr Robert Robbins (*Stanford, Calif*). The authors have tested the hypothesis that aprotinin mediates preservation of myocardial biochemical function through inhibition of the release, uptake, or activity of TNF- α . They specifically investigated the following points: whether aprotinin can preserve myocardial biochemical function during cold storage; whether the myocardium releases TNF- α during cold storage before reperfusion; whether the release of TNF- α is associated with a decrease in myocardial biochemical function during cold storage; whether aprotinin preserves myocardial biochemical function during cold storage by suppression of the release, uptake, or activity of TNF- α ; and, finally, whether aprotinin suppresses TNF- α -induced nitrous oxide production within the myocardium. They used tissue slices of rat myocardium for the assessment of ATP content, protein synthesis, TNF- α levels, and nitric oxide levels for up to 6 hours at 4°C. Myocardial tissue slices stored in a solution of aprotinin demonstrated higher ATP levels and increased capacity for protein synthesis, a decreased level of TNF- α production and uptake, and a decreased level of nitric oxide over time compared with control groups not treated with aprotinin.

I have a few comments and questions concerning these data.

You have demonstrated that aprotinin preserves ATP stores and likewise that anti-TNF- α monoclonal antibodies to the receptor and to TNF- α preserve ATP stores; however, these data do not directly prove that aprotinin preserves ATP stores through the reduction of TNF- α . Since aprotinin is a widespread nonspecific inhibitor of enzymatic activity, could this lead to some preservation of ATP stores that is independent of TNF- α ?

Dr Bull. In the manuscript we discuss in detail that ATP is likely preserved through both nitric oxide-dependent and nitric oxide-independent pathways.

Dr Robbins. You have highlighted some of the advantages of using these tissue slices for this study; however, there are obviously some important limitations to this technique, most important, what happens during reperfusion. In an in vivo study by Suzuki, which was referenced in your manuscript, left ventricular function was certainly worse in isolated dog hearts that were treated with aprotinin compared with control hearts not treated with aprotinin. Do you have any additional data on reperfusion data in your laboratory?

Dr Bull. No. We specifically did not look at reperfusion in the study. I want to emphasize that our study examines changes in the myocardium during cold storage before reperfusion. As you know, TNF- α is made by the myocardium primarily and also by macrophages within the myocardium and in the periphery. To control these factors, we conducted the experiments under conditions of cold storage. To extrapolate our data to the setting of reperfusion would require significant additional experiments that involve a whole set of other variables that have to be introduced and tested for, especially macrophage production of TNF- α .

Dr Robbins. Have you tried pretreatment of donor rats with systemic aprotinin before cardiectomy instead of just taking the heart out and putting it into a solution of aprotinin?

Dr Bull. No, although it is an intriguing point. The ATP content at the zero-hour time point, which is the 10-minute interval from the time the cardiectomy is done through the preparation and generation of slicing, is significantly lower. The presence of aprotinin while the hearts were being prepared and sliced seems to significantly increase ATP content. When we performed a cross-over experiment, as described in the text, we found that when aprotinin was removed at the zero-hour time point and instead the slices were placed in cardioplegic solution alone, ATP content was rapidly lost. Similarly, adding aprotinin to the slices prepared in cardioplegic solution at the zero-hour time point did not restore ATP levels. Clearly, aprotinin seems to be conferring an immediate protective effect on ATP stores in the 10-minute interval from cardiectomy through completion of the slices. This does not hold true for protein synthesis. Protein synthesis may be a more accurate assessment of true biochemical function because it does require the integration of several important enzymatic pathways. ATP as a measured biochemical function is more ephemeral and more subject to immediate episodes of ischemia. I personally think that, of the two, capacity for protein synthesis is the more rigorous test.

Dr Robbins. How did you do this? Are you saying that you sectioned the heart in a solution of aprotinin?

Dr Bull. Right.

Dr Robbins. Am I correct that at time point zero you immediately put the heart in aprotinin?

Dr Bull. No. At the time the cardiectomy is done, the hearts immediately go into cardioplegic solution containing aprotinin or into one of the other study solutions. There is a 10-minute time interval from cardiectomy to the time we harvest the slices.

Dr Robbins. One thing you might consider is the use of cardioplegic solution immediately, before you start the experiment, so that you stop the heart and get it cold immediately.

The use of genetically modified TNF "knock out" animals for these studies would be instructive since they would permit the investigation of effects of aprotinin in the absence of TNF- α . Do you have any plans to use TNF "knock outs"? I think that would help clean up this experiment.

Dr Bull. I do not, but if you have access to them I would love to have them.

Dr Robbins. Alternatively, you could combine aprotinin and the anti-TNF- α antibody. Have you combined either the receptor antibody and the antibody to TNF- α , and do you have any data on what happens to the levels in the experiments you did? I did not see any report of what happens to the TNF- α levels with these monoclonal antibodies.

Dr Bull. We did not test specifically for that. Other groups, especially the group at Colorado, have used monoclonal antibodies to TNF- α and demonstrated salutary effects from that, but we did not specifically test that.

Dr Robbins. Finally, on the basis of these data, have you

used aprotinin more liberally despite its cost in your clinical cardiac practice, particularly in the high-risk cases?

Dr Bull. We have gradually increased our use of aprotinin in our practice. We first began using it for patients requiring complex redo operations or longer reconstructions in whom we thought bleeding was a primary issue. As we have gained experience in the use of aprotinin, we have focused on the parallel pathway of suppression of inflammatory mediators. We believe aprotinin may decrease the incidence of complications such as adult respiratory distress syndrome and pancreatitis through suppression of inflammatory mediators.

Dr Edward Verrier (*Seattle, Wash.*). I very much liked the design of the experiment, although I do have a couple of questions.

First, cutting slices is considerably different from having an ischemic reperfusion model. There is no ischemia per se because there is no lack of blood supply. You just cut the tissue and put it into storage. My biggest difficulty with this process is that protein synthesis takes time. If we get a signal on the surface of a cell (and here we are dependent on a diffusion gradient at 4°C), then we have to get a signal to the cell. It has to go in through a signal transduction pathway, hit a promoter, whatever the promoter is for TNF (it is probably NF- κ -B), and turn on the gene. The gene then has to express a messenger RNA, translate it, develop a protein, go to the surface, and have the protein expressed. That is what I do not understand about this model. How do you explain the fact that you are accounting for protein synthesis over a 4-hour period except that it happens immediately?

Dr Bull. The protein synthesis assay is done at a particular time point after 2, 4, or 6 hours of cold storage. The slices are removed and then incubated at 37°C to allow enzymatic activity. They are bathed in tritiated leucine to allow incorporation of the tritiated leucine occurring at 37°C. It is not occurring at 4°C.

Dr Verrier. But once again, to get protein synthesis at 4°C, there has to be a time frame. Maybe it is my own understanding that is different here, but I think that there may be something unique to the model, and I think you are going to need to take the next step to find out. When you take the next step, you go into a monstrous number of variables that are going to affect whether you really modify the response or induce actual protein synthesis by such a broad agent as a TNF inhibitor.

Dr Bull. An alternative means of understanding what is being measured is the maintenance of cell viability. In other words, cell viability is being maintained during that period of cold storage. Cells are lost through apoptosis or ischemia to varying degrees in different preservation solutions. The degree to which overall cell viability is maintained can be assessed by measuring the degree to which capacity for protein synthesis is maintained.

Dr Verrier. That is a very intriguing concept in that a lot of TNF is mediated through NF- κ -B. NF- κ -B goes into a cytodestructive and a cytoprotective side. The cytoprotective side is that you can inhibit apoptosis that is due to TNF, and that would be very interesting to study as an extension of this.