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Adenosine decreases post-ischaemic cardiac TNF-α production: anti-inflammatory implications for preconditioning and transplantation

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SUMMARY

Tumour necrosis factor-α (TNF-α) is an autocrine contributor to myocardial dysfunction and cardiomyocyte death in ischaemia–reperfusion injury (I/R), sepsis, chronic heart failure and cardiac allograft rejection. Cardiac resident macrophages, infiltrating leucocytes, and cardiomyocytes themselves produce TNF-α. Although adenosine reduces macrophage TNF-α production and protects myocardium against I/R, it remains unknown whether I/R induces an increase in cardiac TNF-α in a crystalloid-perfused model (in the absence of blood), and, whether adenosine decreases cardiac TNF-α and protects function after I/R. To study this, isolated rat hearts were crystalloid-perfused using the Langendorff method and subjected to I/R, with or without adenosine pretreatment. Post-ischaemic cardiac TNF-α (enzyme-linked immunosorbent assay and bioassay) and function were determined (Langendorff). I/R increased cardiac TNF-α and impaired myocardial function. Adenosine decreased cardiac TNF-α and improved post-ischaemic functional recovery. This study demonstrates that: first, I/R induces an increase in cardiac tissue TNF-α in a crystalloid-perfused model; second, adenosine decreases cardiac TNF-α and improves post-ischaemic myocardial function; third, decreased cardiac TNF-α may represent a mechanism by which adenosine protects myocardium; and fourth, adenosine-induced suppression of cardiac TNF-α may provide an anti-inflammatory link to preconditioning and have implications for cardiac allograft preservation.

INTRODUCTION

Recent investigations have implicated cardiac tumour necrosis factor-α (TNF-α) as a mediator of cardiovascular disease. TNF-α appears to be involved in the pathogenesis of acute myocardial infarction, chronic heart failure, atherosclerosis, viral myocarditis, cardiac allograft rejection and sepsis-associated cardiac dysfunction.[1–5] Although initially described solely as a lipopolysaccharide (LPS)-induced macrophage product, evidence now indicates that, cardiac myocytes themselves produce substantial amounts of TNF-α.[6] Indeed, ischaemia-provoked cardiac TNF-α production may prove more clinically significant than sepsis-induced cardiac TNF-α production.

Hart and Fabre[7] first reported that heart interstitial connective tissue contains Ia-positive dendritic cells. Spencer and Fabre[8] subsequently characterized resident cardiac macrophages. Furthermore, the heart is a rich source of several inflammatory cytokines, including TNF-α.[3,4,6,9] In fact, the heart produces as much TNF-α per gram of tissue as either the liver or the spleen, both of which possess large macrophage populations and are major sources of TNF-α.[6] Unexpectedly, cardiac myocytes themselves produce TNF-α. Kapadia *et al.*[6] demonstrated that production of endotoxin-induced cardiac TNF-α is almost evenly distributed between cardiomyocyte and resident cardiac macrophage cell types. Thus, local TNF-α production is a potential source of TNF-α affecting myocardial function. Strategies designed to limit cardiac TNF-α production may improve post-ischaemic myocardial function.

In this regard, the heart possesses an intrinsic defence mechanism against ischaemic injury which is elicited by brief periods of ischaemia and is termed 'cardiac preconditioning'.[10] Although these findings are paradoxical, recent clinical evidence suggests that the human myocardium adapts to the stresses of repeated sublethal ischaemia. Indeed, a protective role for angina which precedes myocardial infarction by 24–48 hr has been independently observed. This phenomenon has also been confirmed in *ex vivo* human myocardial trabeculae[14,15] These findings suggest that the stress of angina induces an endogenous adaptive mechanism that protects the myocardium. Interrogation of the mechanisms of preconditioning have incriminated adenosine as a mediator of protection; however, the mechanisms responsible remain undefined.[15–22]

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In this regard, adenosine is known to regulate the activities of immune and inflammatory cells[23] and is released by the transiently ischaemic myocardium during preconditioning.[24] We hypothesized that first, ischaemia induces an increase in cardiac TNF-α in a crystalloid-perfused model of ischaemia–reperfusion injury which is not confounded by circulating, blood-borne TNF-α, and that second, adenosine pretreatment decreases post-ischaemic cardiac TNF-α and improves post-ischaemic myocardial function.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (weight 325–350 g, Sasco Inc., Omaha, NE) were fed a standard diet and acclimated in a quiet quarantine room for 2 weeks before the experiments. The animal protocol was reviewed and approved by the Animal Care and Research Committee of the University of Colorado Health Sciences Centre. All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication no. 85-23, revised 1985). Chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Experimental design and groups

Each ischaemia–reperfusion (I/R) experiment totalled 80 min, beginning with a mandatory equilibration period. Stability control hearts (with and without 125 μM adnosine pretreatment) underwent 80 min of oxygenated perfusion without any periods of ischaemia to ensure preparation stability. Injury control hearts were perfused for 8 min (equilibration) and then received 2 min infusion of vehicle, followed by a standard I/R insult, 20 min 37° global ischaemia and 40 min reperfusion. Adenosine-pretreated hearts were perfused for 8 min (equilibration) and then received 125 μM adenosine for 2 min followed by of 10 min of perfusion (washout) and standard I/R. The adenosine dose was based on dose–response curves generated in our laboratory which demonstrated that a higher dose of adenosine did not provide additional protection, whereas lower doses did not provide optimal protection.[24] All drug solutions were prepared in deionized water and infused through a port above the aortic root at 0·1 ml/min (not re-circulated). haemodynamic parameters were continuously recorded before, during and following infusion.

I/R of the isolated rat heart: developed pressure, end diastolic pressure, coronary flow and heart rate measurements

The isolated crystalloid-perfused rat heart model was used as we have previously described[25,26] and is shown schematically in Fig. 1. In brief, following anaesthesia and heparinization [sodium pentobarbital, 60 mg/kg intraperitoneally (i.p.) and heparin-sodium, 500 units i.p.] hearts were excised into 4° Krebs–Henseleit and perfused with oxygenated buffer within 45 seconds. Hearts were retrogradely perfused in the isolated, isovolumetric Langendorff mode (70 mmHg) with modified Krebs–Henseleit solution (in mM: 5·5 glucose, 1·2 Ca^{2+}, 4·7

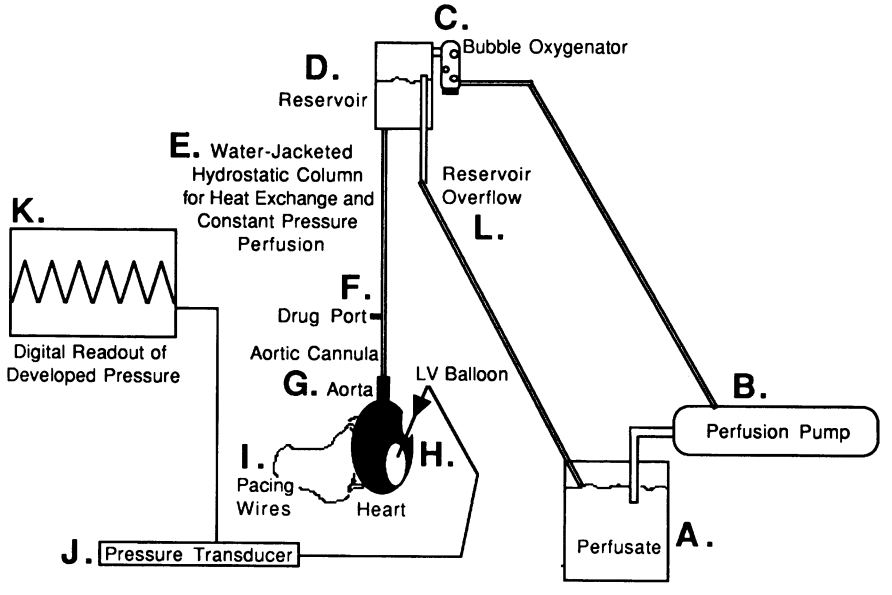

Figure 1. Schematic representation of crystalloid perfusion of the isolated rat heart using the Langendorff method. After the hearts are harvested they are perfused by a hydrostatic column (fixed pressure) of oxygenated, normothermic, glucose-enriched crystalloid solution (see the Materials and Methods for details). The Langendorff aparatus consists of the following features: (A) a major perfusate reservoir from which perfusate is pumped (B) through an oxygenator (C) and into a fixed volume minor reservoir (D); from the minor reservoir, perfusate enters a water-jacketed hydrostatic column which provides set heat exchange for normothermic perfusion and a constant perfusion pressure (E); attached to the hydrostatic column is a side port (F) which can be used for agent (adenosine) infusion immediately prior to perfusate use by the myocardium; the perfusate enters the heart (coronary arteries) from the hydrostatic column via the ascending aortic arch which is attached to an aortic cannula fixed to the column (G); the heart (H) contains a water-filled balloon in its left ventricle (LV) which is paced (I) at a constant rate (NB the native heart rate on this apparatus is approximately 300 beats per minute, similar to the rat endogenous rate; hearts are paced in order to prevent varying rate from affecting developed pressure); LV balloon pressures are transmitted to a pressure transducer (J) which provides information for real-time computerized display of myocardial function (K).

KCl, 25·0 $NaHCO_3$) and saturated with 92·5% O_2/7·5% CO_2, to achieve a pO_2 of 440–460 mmHg, pCO_2 of 39–41 mmHg, and pH of 7·39–7·41 (ABL-4 blood gas analyser, Radiometer, Copenhagen, Denmark). A pulmonary arteriotomy and left atrial resection were performed prior to insertion of a water-filled latex balloon through the left atrium into the left ventricle. This balloon was then adjusted to a left ventricular end-diastolic pressure (EDP) of 6 mmHg during the initial equilibration. This preload volume was held constant during the entire experiment to allow continuous recording of the ventricular pressure during I/R. Pacing wires were fixed to the right atrium and pulmonary outflow tract and hearts were paced at approximately 6 Hz (355 beats per minute) in order to compare functional measurements using a standardized heart rate.

Measured indices of myocardial function were left ventricular developed pressure (DP), EDP and coronary flow (CF). Data were continuously recorded using a computerized MacLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and an Apple Quadra 800 computer (Apple Computer Inc., Cupertino, CA). Paced hearts that did not produce 105±25 mmHg DP at 6 mmHg EDP were discarded. A three-way stopcock above the aortic root was used to create global ischaemia during which time the heart was placed in a 37° degassed organ bath. Coronary flow was measured by collecting pulmonary artery effluent. Following reperfusion, left ventricular myocardium was excised added to 10 volumes of cold isotonic homogenization buffer (in mM: 50 imidiazole acetate, 10 magnesium acetate, 4 KH_2PO_4, 2 ethylenediaminetetraacetic acid; and in μM 50 *N*-acetylcysteine as antioxidant and 12·5 sulphur in 0·8% ethanol to inhibit adenylate cyclase; pH 7·6). Samples were homogenized in a vertishear tissue homogenizer (parallel blades 0·5 cm apart) at half maximal speed for 20 seconds (10 equally spaced bursts) followed by centrifugation at 2000 ***g*** for 15 min. Supernatant total protein concentration was quantified using the Lowry assay and then stored at −70° until used in the TNF-α assays.

Cardiac TNF-α

Cardiac homogenate TNF-α content was determined by enzyme-linked immunosorbent assay (ELISA) and bioactivity was determined by WEHI-164 clone cytotoxicity assay. ELISA was performed by adding 100 ml of each sample (equal protein and tested in duplicate) to wells in a 96-well plate of a commercially available ELISA kit (R&D Systems, Inc., Minneapolis, MN). The antibodies used in this ELISA are not influenced by either the type 1 or type 2 TNF-α receptors (TNFR1 or TNFR2, respectively). According to the manufacturer, the detection limit of this assay was determined to be 15 pg/ml after statistical analysis of the ELISA results. Furthermore, the manufacturer has determined that the ELISA is highly specific for TNF-α. Concentrations as high as 10^6 pg/ml of interleukin-1α (IL-1α), IL-3, IL-4, IL-6, IL-7, TNF-α, interferon-γ (IFN-γ), and murine granulocyte–macrophage colony-stimulating factor (mGM-CSF) did not yield detectable cross-reactivity. Similarly, concentrations as high as 10^4 pg/ml IL-1β or 10^5 pg/ml IL-2 did not yield detectable cross-reactivity. TNF-α ELISA was performed according to the manufacturer's instructions. Final results were expressed as pg of TNF-α per gram of protein. TNF-α bioactivity in the cardiac samples was determined by utilizing the WEHI-164 cell clone cytotoxicity assay as previously described.[27–31] Final results were expressed as units of TNF-α activity per gram of protein.

Presentation of data and statistical analysis

All reported values are mean±SEM (n=4–6 animals in each of the control and experimental groups). Differences at the 95% confidence level were considered significant. Data were compared at the corresponding time-points between groups using one-way analysis of variance (ANOVA) with *post hoc* Bonferroni/Dunn test (StatView 4·0, Abacus Concepts, Berkeley, CA).

RESULTS

Cardiac TNF-α

Cardiac TNF-α protein content as determined by ELISA is shown in Fig. 2. Ischaemia–reperfusion resulted in a profound increase (347±43 to 841±108 pg/g) in cardiac TNF-α which was decreased with adenosine pretreatment (373±85 pg/g). Cardiac TNF-α bioactivity as determined by cytotoxicity assay is shown in Fig. 3. In parallel with the cardiac TNF-α protein content as determined by ELISA, I/R myocardium resulted in an increase of WEHI-164 cell clone cytotoxicity (14·75±7·8

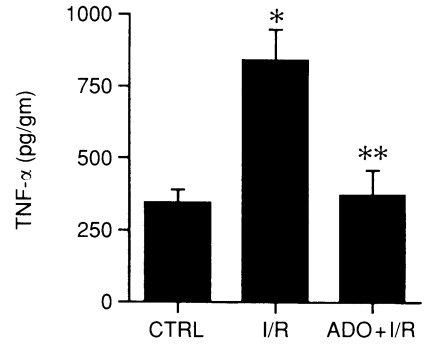

Figure 2. Effect of adenosine pretreatment of post-ischaemic cardiac TNF-α levels. I/R induced an increase in cardiac tissue TNF-α levels which was decreased by adenosine (ADO) pretreatment. *P<0·05 versus control (CTRL); **P<0·05 versus I/R.

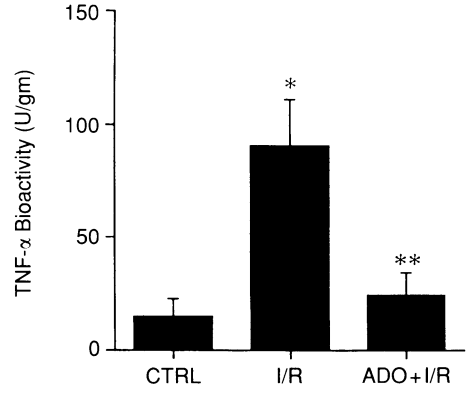

Figure 3. Effect of adenosine pretreatment of post-ischaemic cardiac TNF-α bioactivity. I/R induced an increase in cardiac tissue TNF-α bioactivity which was decreased by adenosine (ADO) pretreatment. *P<0·05 versus control (CTRL); **P<0·05 versus I/R.

to 90·75±20·6 U/g) which also decreased following adenosine pretreatment (24±9·9 U/g).

Myocardial function

Twenty minutes of ischaemia and 40 min of reperfusion resulted in a dramatic decrease in myocardial function as demonstrated by: a DP decrease from 99±7 to 46±4 mmHg (Fig. 4); a CF decrease from 21±2 to 12±1·7 ml/min (Fig. 5); and an EDP increase (decreased compliance) from 6·1±1·7 to 61±7 mmHg (Fig. 6). In concert with decreased post-ischaemic cardiac TNF-α, adenosine improved post-ischaemic functional recovery by increasing DP (46±4 to 77±8 mmHg) and CF (12±1·7 to 17±1·5 ml/min), and decreasing EDP (61±7 to 26±4 mmHg).

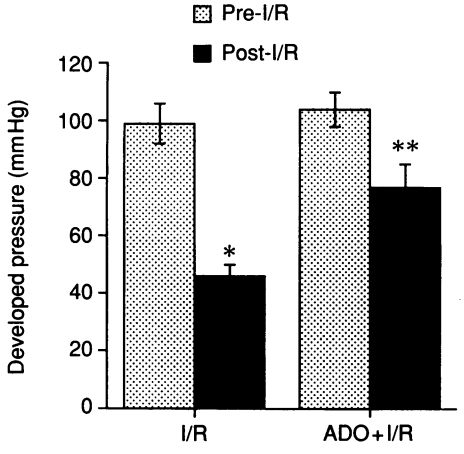

Figure 4. Effect of adenosine pretreatment on post-ischaemic recovered left ventricular developed pressure (DP). I/R impaired post-ischaemic myocardial DP recovery which was improved with adenosine (ADO) pretreatment. $*P<0.05$ versus Pre-I/R; $**P<0.05$ versus Post-I/R-value for I/R alone group.

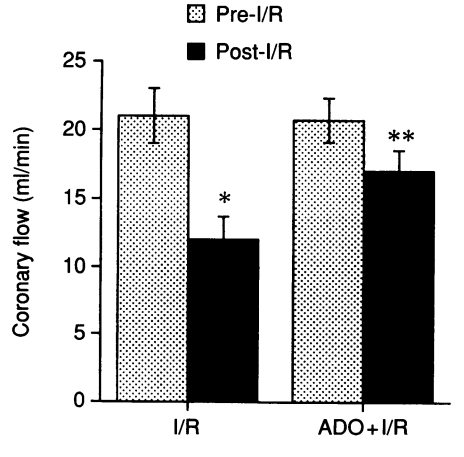

Figure 5. Effect of adenosine pretreatment on post-ischaemic coronary flow (CF). I/R impaired post-ischaemic myocardial CF which was improved with adenosine (ADO) pretreatment. $*P<0.05$ versus Pre-I/R; $**P<0.05$ versus Post-I/R-value for I/R alone group.

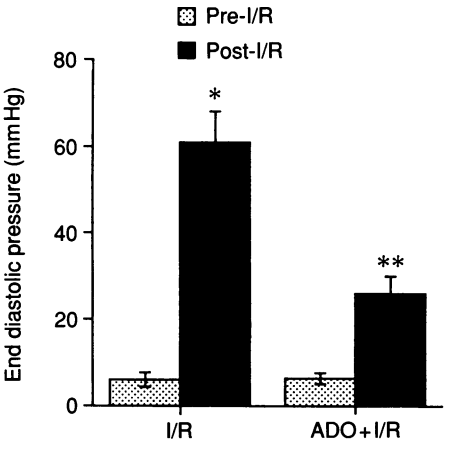

Figure 6. Effect of adenosine pretreatment on post-ischaemic end diastolic pressure/compliance (EDP). I/R impaired post-ischaemic left ventricular compliance which was improved with adenosine (ADO) pretreatment. $*P<0.05$ versus Pre-I/R; $**P<0.05$ versus Post-I/R-value for I/R alone group.

DISCUSSION

The results of this study demonstrate for the first time that: firstly, I/R increases cardiac TNF-α levels and TNF-α bioactivity in an isolated, crystalloid-perfused model of myocardial I/R injury; second, adenosine, which has been demonstrated to decrease LPS-stimulated macrophage TNF-α production,[23] also decreases ischaemia-induced cardiac TNF-α production; third, increased cardiac TNF-α is associated with post-ischaemic myocardial dysfunction; and, fourth, when post-ischaemic cardiac TNF-α was decreased by adenosine pretreatment, post-ischaemic myocardial function also improved. The results of this suggest that cardiac myocytes and cardiac resident macrophages are an important source of TNF-α which affects myocardial function, since ischaemia and reperfusion provoked cardiac TNF-α production even in the absence of blood circulation. Furthermore, these results confirm earlier data which indicated that adenosine down-regulates macrophage TNF-α production *in vitro*, as well as extend these observations to indicate that adenosine down-regulates TNF-α production *in situ*. Indeed, this may have important clinical implications for preservation of myocardium during and following ischaemia. Heart transplant surgery and cardiac bypass surgery, as well as coronary angioplasty, are three clinical scenarios which obligate myocardial I/R. TNF-α has recently been appreciated as an important mediator of myocardial ischaemic damage[3,32] The present model allows the study of these mechanisms in the laboratory, as well as the opportunity to test potential therapeutic stategies. The observation that adenosine exerts anti-inflammatory effects on cardiac TNF-α production may be particularly germane to transplant surgery, where TNF-α may contribute to cardiac allograft rejection, as well as I/R injury.

This study should be interpreted with several important caveats. First, the *ex vivo* myocardial perfusion model employed here does not precisely replicate the *in vivo* situation. While we have successfully used this model to answer questions concerning myocardial physiology and pathophysiology in the past, in many ways it does not reflect the *in vivo* situation.

Since this heart model is perfused with crystalloid, not blood, aspects of blood which are either protective (anti-oxidants) or injurious (neutrophils/blood-derived cytokines) do not contribute. Indeed, this model was chosen in order to determine more accurately cardiac TNF-α production (versus accumulation from blood). Second, other cytokines such as IL-1β, IL-2, IL-6 and IFN-γ may also play a role in ischaemia-induced myocardial dysfunction.[9] Third, we have not definitively linked cardiac TNF-α production with I/R injury. Specific TNF-α blockade would more effectively answer that quesion. However, TNF-α binding proteins may not work in this model since TNF-α likely works in a paracrine fashion; i.e. since perfusate was not recirculated, TNF-α entering the the coronary circulation never re-enters the heart. Thus, TNF-α-binding proteins, which would presumably remain intravascular, may not influence interstitial or membrane-bound TNF-α. Furthermore, although important, this study was not designed to determine the portion of injury mediated by TNF-α, but rather to determine: whether ischaemia provokes cardiac TNF-α production in the absence of blood, and whether adenosine decreases both cardiac TNF-α and myocardial I/R injury, which is purported to be partly due to TNF-α.

Although considerable information exists concerning the mechanisms by which LPS induces TNF-α production, little is known about the mechanisms of ischaemia-induced cardiac TNF-α production. Reperfusion of ischaemic myocardium imposes an oxidant burden in which the reduction product of molecular oxygen, hydrogen peroxide, contributes to myocardial injury.[26] Hydrogen peroxide activates P38 mitogen-activated protein kinase (MAPK) which may contribute to ischaemia-induced TNF-α production[33,34] Oxidant stress also activates nuclear factor κ B(NFkB) which may play a role in the sequence of ischaemia-induced TNF-α production in the heart. This, however, remains to be determined.

The haemodynamic effects of TNF-α are characterized by decreased myocardial contractile efficiency and reduced ejection fraction, hypotension, decreased systemic vascular resistance, and biventricular dilatation.[35] TNF-α is a myocardial depressant which has been known to mediate LPS-induced myocardial depression; however, ischaemia-provoked cardiac TNF-α production has only recently been appreciated. Indeed, ischaemia-induced cardiac TNF-α production may prove to be more clinically important than sepsis-induced cardiac TNF-α production.

The mechanisms by which TNF-α causes myocardial dysfunction include calcium dyshomeostasis, direct cytotoxicity, oxidant stress, disruption of excitation–contraction coupling, myocyte apoptosis, as well as the induction of other cardiac depressants. The biphasic (early and delayed) nature of TNF-α-induced myocardial depression suggests that TNF-α induces negative inotropic effects by at least two different mechanisms.[1] The early phase of TNF-induced functional depression occurs within minutes, whereas the delayed phase appears to require hours of TNF-α exposure.[1] Although TNF-α-induced nitric oxide (NO) production has been suggested to mediate this depression, TNF-α may not induce high levels of NO rapidly enough to account for the early phase of myocardial depression.[1] In this regard, sphingolipid metabolites are stress-induced second messengers which participate in intracellular signal transduction following TNF-α binding to the TNF receptor type 1 (TNFR1; 55 000 MW). Two important characteristics of sphingolipid metabolites led to the hypothesis[1] that sphingosine mediates TNF-α-induced myocardial contractile dysfunction: first, it is rapidly produced by cardiac myocytes (via sphingomyelin degeneration) following TNF-α triggering of TNFR1;[36] and second, sphingosine decreases calcium transients by blocking the ryanodine receptor, which mediates calcium-induced calcium-release from the sarcoplasmic reticulum.

Evidence strongly suggests that TNF-α participates in myocardial I/R injury and cardiac allograft rejection.[1–5] Thus, strategies designed to decrease cardiac TNF-α production may be of therapeutic value. In this regard, preconditioning is known to protect myocardium through an adenosine-mediated mechanism. Further, adenosine has been demonstrated to decrease macrophage TNF-α production. Given the recent evidence suggesting that myocardium produces substantial amounts of TNF-α which may participate in I/R injury, we attempted to determine whether adenosine's (preconditioning's) beneficial effects may in part be attributed to down-regulation of cardiac TNF-α (as may be suggested by the results of Parmely *et al.*[23]) Indeed, the results presented here indicate that adenosine down-regulates TNF-α production in the ischaemic heart. Parmely *et al.* concluded that adenosine decreases macrophage TNF-α production and serum TNF-α at the post-transcriptional level, since TNF-α mRNA levels were not decreased by adenosine.

Although adenosine decreases myocardial TNF-α production, adenosine has been reported to exert cardioprotective effects through other mechanisms as well.[16] Several beneficial effects of adenosine on the ischaemic myocardium have been proposed. Protective effects of adenosine during hypoperfusion include coronary artery vasodilatation, which increases oxygen supply, and negative inotropy, which decreases myocardial oxygen demand.[16] Adenosine-induced acute myocardial protection evolved from studies examining the mechanisms by which transient ischaemia results in acute myocardial adaptation against a proximal I/R insult (ischaemic preconditioning). During ischaemia, myocardial utilization of ATP leads to accumulation of the nucleotide adenosine. The mechanism of adenosine-induced protection has been shown to include activation of the ATP-sensitive potassium channel (K_{ATP} channel) in several species, including humans[15,17,38] Adenosine-induced activation of the K_{ATP} channel probably protects myocardium by limiting potassium-induced calcium loading of cardiac myocytes[39] which occurs during I/R injury[18,19] It is possible therefore that the observations presented here are an epiphenomenon of the myocardial protection which may be afforded by adenosine via other mechanisms. This remains to be determined.

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