Cardioplectic ischemia or reperfusion: Which is a main trigger for tumor necrosis factor production?

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Abstract

Background: Tumor necrosis factor alpha (TNF-α) is a key cytokine in the pathogenesis of ischemia–reperfusion injury (I/R) that also possesses negative inotropic and direct cardiotoxic effects. We investigated whether myocardial ischemia and/or reperfusion is the trigger for TNF-α synthesis and whether TNF-α release is time dependent.

Methods: Isolated rat hearts undergoing 30 min of coronary perfusion with modified Krebs–Henseleit solution followed by cardioplectic arrest for 60 min of global cardioplectic normothermic ischemia (GCI) and 30 min of reperfusion using a modified Langendorff model. Myocardial TNF-mRNA expression and TNF-α protein levels in effluent from the coronary sinus were measured at baseline and then after 15, 30, and 60 min of GCI and after 10 and 30 min of reperfusion.

Results: GCI induced myocardial TNF-α mRNA expression and elevation protein TNF-α levels in a time-dependent manner after 30 min of ischemia from 78± 17 pg/ml to 915±287 pg/ml after 60 min (p<0.0015). Reperfusion did not cause time-dependent increase of TNF-α synthesis and release but was accompanied by progressive decrease of left ventricular (LV) function. There was a correlation between TNF-α protein levels and depression of LV function immediately after GCI but not with TNF-α protein levels at 30 min of reperfusion.

Conclusion: This study demonstrated that myocardial ischemia rather than reperfusion is the main trigger for time-dependent TNF-α synthesis. Depression of LV function during reperfusion correlated significantly only with TNF-α levels at the end of GCI.

Keywords: TNF; Ischemia/reperfusion injury; Isolated heart; Cardioplegia

1. Introduction

Cardioprotection is an important goal of cardiac surgery typically achieved with cardiopletic solutions primarily designed to minimize myocardial oxygen demands to limit ischemic injury. While necessary to restore myocardial oxygen and substrate delivery, reperfusion itself may induce tissue damage by activating multiple potentially deleterious oxidative pathways [1]. It has been shown that the restoration of blood flow to the ischemic myocardium may be associated with deleterious changes of the myocardium, including arrhythmias, enzyme release, and contractile dysfunction [2].

Knowledge of the various pathologic events that occur separately in ischemia and in reperfusion is, therefore, important to the understanding of the pathogenesis of ischemic–reperfusion (I/R) injury.

Tumor necrosis factor alpha (TNF-α), a key cytokine in the pathogenesis of I/R injury, is a polypeptide hormone that has a wide range of biological activities [3–8]. TNF-α does not exist in a stored form but is rather synthesized de novo after cellular activation by a number of cells, including monocytes/macrophages, lymphocytes, Kupffer’s cells of...
the liver, cardiomyocytes and others [1,3,5,9]. TNF-α was found to have a direct cardiotoxic effect and to cause activation of other inflammatory proteins [2,4]. TNF-α was shown to be synthesized and released in the human heart at the time of coronary artery bypass grafting (CABG) [10,11].

We and others have recently reported that TNF-α produced by the heart following experimental I/R in animals and that TNF-α directly decreases animal and human myocardial contractility (3–5,10). However, it is not clear whether ischemia or reperfusion is the main trigger for TNF-α synthesis and release. The present study aimed to examine influence of global cardioplegic ischemic arrest (GCI) and reperfusion on direct changes in TNF-α protein release and determine whether TNF-α release is time dependent at time of ischemia and reperfusion.

2. Materials and methods

The present study protocol was approved by the Animal Care Committee of Tel Aviv University, Tel Aviv, Israel (in accordance with NIH recommendations for animal care). Male Wistar rats were selected for experimentation by the institutional veterinarian according to age, weight and general health status. All rats were anesthetized by intraperitoneal injection of phenobarbital sodium (30 mg/kg body weight). Their hearts were rapidly excised, immersed in cold saline solution (4 °C), and mounted on the stainless steel cannula of a modified Langendorff perfusion system.

Anterograde aortic perfusion was initiated at a perfusion pressure of 85 mm Hg with an oxygenated modified Krebs–Henseleit (KH) buffer solution of the following composition (mmol/l): NaCl 118, KCl 4.7, CaCl2, MgSO4 7, H2O 1.2, KH2PO4 1.2, glucose 11.1 and NaHCO3 25. The 95% O2 and 5% CO2 were continuously added to the perfusate to maintain pO2 between 450–550 mm Hg and pCO2 between 25–30 mm Hg.

The heart temperature was monitored by a thermistor implanted in the right ventricular wall and carefully maintained at 37 °C or 31 °C (at the time of ischemia) by a water-jacket. The right atrium was incised, and the hearts were paced to 300 beats/min at 4 V with the use of an external pacemaker (Devices Limited, Implants Division, type E4162 Garden City), ensuring an identical heart rate for all hearts. A water-filled balloon was placed in the left ventricular cavity through a small incision in the left atrium after which it was connected to a Mennen Medical PI 32284 pressure transducer (Hamburg, Germany). The balloon was tied and inflated to a volume that produced 5 mm Hg diastolic pressure.

Left ventricular (LV) peak systolic pressure was measured and the first derivate of the rise in LV pressure (dP/dt max) and the area under the LV-developed pressure curve (pressure–time integral) were calculated. These variables were continuously recorded, and measurements were taken at 10-minute intervals. The control measurements were recorded at the end of a 15-minute period of stabilization (baseline).

Cardioplegia with KH containing 20 mEq/L of KCl was then administered for two min. After 60 min of GCI anterograde aortic perfusion was restarted.

3. Protocol

The animals were divided into the following groups each containing 10 animals.

3.1. Determination of TNF-α mRNA expression at time of Global Cardioplegic Ischemia

Group A : hearts were immediately excised after the stabilization period to assess the baseline TNF-α mRNA myocardial expression.

Group B : hearts were excised after 15 min of GCI to assess LV TNF-α mRNA expression. Nine additional hearts were reperfused after 15 min of GCI to determine the TNF-α protein level in 1st ml of effluent from coronary sinus.

Group C : hearts were excised after 30 min of GCI to assess TNF-α mRNA expression. Nine additional hearts were reperfused after 30 min of GCI to determine the TNF-α protein level (1st ml).

Group D : hearts were excised after 60 min of GCI to assess TNF-α mRNA expression.

3.2. Determination of TNF-α mRNA expression and TNF-α protein levels at time of reperfusion

Group E : hearts underwent GCI for 1 h followed by reperfusion with KH solution for 15 min and were excised to assess TNF-α mRNA expression. The TNF-α protein level for this group was determined in effluent collected during first 1 min (TNF-α protein level at end of GCI).

Group F : hearts underwent GCI for 1 h followed by reperfusion with KH solution for 30 min and were excised to assess TNF-α mRNA expression after effluent collection during 1 min for the determination of TNF-α protein for 15 and 60 min of reperfusion.

3.3. TNF-α mRNA determination

Total RNA was extracted from LV myocardial samples using the guanidium thiocyanate method [12]. Pellets of RNA were kept at –20 °C with 75% ethanol until assay. Dried sediments were dissolved in sterile RNase-free water and quantitated spectrophotometrically at a wave length of 260 nm.

Two μg of total RNA was subjected to reverse transcription reaction in 20 μl using a reverse transcription system (Promega). After completion of the reaction, 5 μl of this reaction mixture was used for TNF-α complementary DNA (cDNA) polymerase chain reaction (PCR).
amplification, and 5 µl of 1:10 diluted reaction mixture was used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification. Our PCR negative control contained water instead of cDNA, and the cDNA negative control contained water instead of RNA. For cDNA amplification the primers sense-CACGCTCTTCTGTC-TACTGA and antisense-GGACTCCGTGATGTCTAAGT were used, producing a 546-base pair fragment. An annealing temperature of 57 °C was chosen for this reaction. For GAPDH cDNA amplification, the primers AATG-CATCCGCACCACCAA and antisense-GTAGCCATATT-CATTGTCATA were used, producing a 515-base pair fragment [13]. The annealing temperature was 60 °C. The optimal cycle number for TNF-α is 30, and a minicycler TM (MJ Research Inc. Watertown, Massachusetts) was used for PCR amplification and reverse transcription reaction.

The PCR products (10 µl) were separated in 1.8% agarose gel, stained with ethidium-bromide, visualized by ultraviolet irradiation and photographed with Polaroid film. The images were taken to evaluate band densities, using the Fujiifilm Thermal Imaging System FTJ-500, computer-based Image Capture Software (Pharmacia Biotech, Jerusalem, Israel) and the TINA program package (Ryest Isotope Messgerate, GmbH, Staubenhardt, Germany). The intensities were normalized by their respective GAPDH values. Each PCR reaction was performed at least twice [14].

4. Determination of TNF-α protein concentration in the effluent

Coronary sinus effluent samples for measuring TNF-α protein levels were drawn at the following points: baseline, 15, 30 and 60 min of ischemia and following 15 and 30 min of reperfusion.

All samples were then immediately stored at −70 °C until assay. TNF-α levels were determined using the commercially available ELISA kit (Cytoscreen tm rat TNF-α, Camarillo, CA). The limit of detection is 4 pg/ml.

4.1. Data analysis

The results are presented as the mean±standard deviation. Measurements of LV function were subjected to two-way analysis of variance (ANOVA) with repeated measurements. Statistical significance was established at p<0.05. One-way ANOVA was used to assess differences between relative optical densities of the mRNA bands and between TNF-α levels in the effluent in each group and between the groups. Pearson’s correlation coefficient regression analysis was calculated between TNF-α levels in the effluent and the percentage of reduction LV function measured at the end of the reperfusion.

All the statistical analyses were performed with the SPSS computer program (SPSS Inc., Chicago, Illinois) in the Statistics Department at our Medical Center.

Fig. 1. Effect of global cardioplegic ischemia and reperfusion on myocardial performance of isolated rat heart. After 1 h of GCI (during reperfusion), there was a significant decrease in left ventricular peak systolic pressure (A, p<0.02), in dP/dt max = first derivative of the rise of left ventricular pressure (B, p=0.005) and in the time–pressure integral (C, p=0.001) compared to pre-ischemic measurements.
5. Results

5.1. Hemodynamics

There were no differences in LV hemodynamic variables among the experimental groups (groups B to F) during 30 min perfusion before cardioplegic ischemia (\(p=\text{NS}\), Fig. 1). Significant decrease in LV peak systolic pressure (\(p=0.02\)), in \(dP/dt\) max (\(p=0.005\)) and in the time–pressure integral (\(p=0.001\)) at time of reperfusion compared to pre-ischemic measurements were obtained (Fig. 1).

5.2. TNF-\(\alpha\) mRNA expression and TNF protein release

5.2.1. Ischemia

Baseline TNF-\(\alpha\) mRNA expression was detected in myocardial specimens from the LV at baseline after the period of stabilization (Fig. 2A,B). We found no significant changes in the intensities of TNF-\(\alpha\) mRNA bands after 15 min of GCI (Fig. 2A,B). More prolonged ischemia (30 min) caused an increase of TNF-\(\alpha\) mRNA expression compared to baseline and compared with 15 min of GCI (\(p=0.03\), Fig. 2A,B). One hour of ischemia increased the intensities of TNF-\(\alpha\) mRNA bands 2.7-fold compared with baseline (\(p=0.02\)) and 1.7 fold compared with 30 min of GCI (Fig. 2A; \(p=0.04\)).

The amount of TNF-\(\alpha\) protein in isolated heart effluent was non-detectable at baseline and after 15 min of GCI. More prolonged ischemia caused a significant time-depen-

dent increase of this cytokine to 78±17 pg/ml after 30 min and to 915±287 pg/ml after 60 min of ischemia (\(p<0.002\)).

5.2.2. Reperfusion

There was no significant difference between the TNF-\(\alpha\) mRNA band intensities after 60 min of myocardial ischemia nor 15 min and 30 min after reperfusion (Fig. 3A,B).

TNF-\(\alpha\) protein concentrations in the effluent at 15 and 30 min of reperfusion were 150 ±73.4 pg/min and 130 ±54.7 pg/min, respectively. There was no expected statistically significant difference in concentrations at 15 and 30 min of reperfusion.

5.2.3. Correlation analysis

Depression of LV function parameters — peak systolic pressure, \(dP/dt\) max and the time–pressure integral on 30 min of reperfusion significantly correlated with TNF-\(\alpha\) effluent levels immediately after 60 min of global cardioplegic ischemia in the hemodynamic (\(r=0.8\), \(p=0.01\); \(r=0.7\), \(p=0.04\) and \(r=0.8\), \(p=0.01\)). There was no significant correlation of these parameters with the TNF-\(\alpha\) levels after 30 min of reperfusion.

6. Discussion

We investigated the influence of cardioplegic ischemia and reperfusion on TNF production by the isolated heart. A blood-free model was intentionally used in this study to exclude the possibility of involvement of systemic blood factors in endogenous TNF-\(\alpha\) formation and in exogenous TNF-\(\alpha\) action on the heart. A model of cardioplegic ischemia
was chosen because of the extensive use of cardioplegic solution at the time of cardiac surgery with cardiopulmonary bypass.

Similarly to Shames BD et al. [15] we have found that GCI alone induces TNF-α mRNA expression in a time-dependent manner. Induction of TNF-α mRNA expression and increases of TNF-α protein levels in our study were obtained at 30 and 60 min of GCI but not at 15 min of GCI. However, we did not find that the reperfusion cause a further increase in TNF-α mRNA gene expression and TNF-α levels were unchanged during 30 min of reperfusion. Correlation analysis showed that LV deterioration at 30 min of reperfusion significantly correlated only with the TNF-α effluent level immediately after ischemia but not with the TNF-α protein level measured at 30 min of reperfusion. TNF-α protein seems to be accumulated in the myocardium in high concentrations during 30 and 60 min of GCI. The decreased TNF-α protein level at the time of reperfusion (compared to the end of ischemia) could be explained by washout of TNF-α protein from the myocardium under conditions of steady-state perfusion. On the other hand, we had expected that reperfusion would cause an elevation of TNF protein (i.e., the level at 15 min compared to the level at 30 min), but the results showed no differences in TNF levels at these time points. We assume that the highest TNF-α level at the end of ischemia is the result of accumulation of TNF-α at the time of ischemia and that this accumulation is mainly responsible for the harm resulting from the deterioration of LV function.

Some recent studies reported a time-dependent increased TNF-α protein after 10–15 min of non-cardioplegic ischemia [15]. In our model, we used cardioplegic ischemia and found an induction of TNF-α mRNA gene expression with further TNF-α protein release only after 30 min of ischemia. These data could provide additional evidence for a cardioprotective role of cardioplegic solutions in I/R injury.

6.1. Role of TNF-α in I/R injury of the heart

TNF-α is a key cytokine in the pathogenesis of I/R injury. Our group previously reported that administration of monoclonal antibodies to TNF-α eliminated this cytokine in effluent and attenuated the postischemic myocardial injury [4]. Some other cytokines and free radicals have been found to play an important role in the I/R injury of the heart. These cytokines and free radicals may be produced in I/R conditions and may have cardiodepressive/cardiotoxic effects by themselves or cause increase production of TNF.

TNF-α binds cell surface receptors TNF-α-R1 and TNF-α-R2, and, on activation, mediates most of the physiological TNF-α-dependent effects, including a negative inotropic effect and apoptosis in cardiomyocytes [19]. As a myocardial depressant substance, TNF-α causes a rapid concentration-dependent decrease in the peak levels of intracellular calcium [20]. TNF-α was also reported to interfere with cardiac metabolism by enhancing lipoprotein lipase activity in animal hearts, which would lead to an increase representing a deleterious event in cases of myocardial ischemia [21]. Moreover, other factors (TNF-α-related and -unrelated) probably participated in the evolution of reperfusion injury. Recent studies have shown that ischemia caused TNF-α production following nuclear factor-kb, iCAM-1 and interleukin-1 and -6 activation at the time of reperfusion [6,22,23]. In our previous study, we reported that TNF-α during GCI led to angiotensin II synthesis and eNOS mRNA down-regulation which, in turn, influenced hemodynamic parameters of the isolated heart [13,24]. TNF-α was shown to be involved in the release of free radicals from the myocardium [25]. We had shown that the use of anti-TNF antibodies preserves LV function [4]. This means that TNF has direct negative inotropic effect on the myocardium. Experiments with mice lacking TNF-α have demonstrated a protective element against myocardial I/R injury [26]. It was also demonstrated that reperfusion caused the production of free oxygen radicals followed by up-regulation of chemokine expression in the venular endothelium of the reperfused myocardium in a TNF-α-independent manner [17].

Our study has several limitations including the in vitro study design using non-blood perfusion that excluded macrophages and other cells that might influence cytokine levels in the venous effluent. Our model of isolated perfused heart makes it possible to investigate only one of the mechanisms of TNF-α production, i.e., by the myocardium. We used different methods of effluent sampling for TNF-α protein determination at the time of ischemia and reperfusion. In addition, our non-blood perfused isolated rat heart model provided high partial oxygen pressure that can induce coronary vasoconstriction and low oxygen transport [27].

This study has shown that ischemia is the main trigger for time-dependent TNF-α synthesis. We showed that reperfusion does not change TNF-α mRNA expression and does not cause time-dependent increase TNF-α production. Deterioration of LV function at the time of reperfusion significantly correlated with TNF-α protein levels measured at the end of ischemia but not during reperfusion. The present study is an additional step in understanding the mechanism of I/R injury and sheds new light on TNF-α-related mechanisms in the evolution of I/R injury. We believe that the therapeutic strategies targeting TNF-α production before or during ischemia may be more successful than those used during reperfusion.

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References
