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Heparin interference with reverse transcriptase polymerase chain reaction of RNA extracted from lungs after ischemia-reperfusion

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Abstract The reverse transcriptase polymerase chain reaction (RT-PCR) is a rapid and sensitive method for detecting gene expression. However, when we used this technique to study gene expression of cytokines in ischemic and ex-vivo-reperfused rat lungs as a model for transplantation, significant inhibition of RT-PCR reaction was observed. To optimize RT-PCR conditions, RNA was extracted from rat lungs after flushing, preservation, and reperfusion. RNA was further purified and PCR conditions were modified with various strategies. We found that heparinase I pretreatment completely overcame the inhibitory effects of RT-PCR using RNA extracted from lung tissues after ischemia-reperfusion. With this treatment, a dramatic increase in tumor necrosis factor-α (TNF-α) mRNA was revealed from lung tissues after ischemia-reperfusion. This result suggests that residual heparin in lung tissue interferes with RT-PCR. Because heparinization is routinely used during clinical and experimental organ transplantation, we recommend the treatment of RNA samples with heparinase prior to RT-PCR.

Key words Reverse transcriptase polymerase chain reaction · Transplantation · Heparin · Ischemia-reperfusion · Gene expression · Cytokines

Abbreviations *PCR* Polymerase chain reaction · *RT* Reverse transcriptase · *TNFα* Tumor necrosis factor-α

Introduction

Lung transplantation is used to treat patients with a variety of end-stage pulmonary diseases [12]. While indications for lung transplantation continue to expand, widespread application of this procedure remains limited by the lack of suitable donor organs [13]. Therefore, many investigations have focused on the question of how to minimize lung injury induced by the obligatory ischemic and reperfusion period [7]. There is increasing evidence suggesting that proinflammatory cytokines play an important role in acute lung injury after ischemia-reperfusion. For example, in a canine lung allograft transplantation model, an early local release of interleukin-2, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) has been associated with ischemia-reperfusion lung injury [15]. Further investigation of cytokine gene expression may provide clues to the under-

lying molecular mechanisms of ischemia-reperfusion injury. The expression of cytokine genes could potentially be used as molecular markers for acute lung injury [14].

The message levels of cytokines in normal lung tissue are usually very low. During inflammatory reactions, the expression of cytokines may increase significantly. However, the collection of sufficient specimens from animal models or from human patients could be a major obstacle due to technical or ethical reasons. As a quick and sensitive technique requiring only very small amounts of tissue, the reverse transcriptase polymerase chain reaction (RT-PCR) has been widely used for detecting gene expression [6]. However, when we used RT-PCR to study gene expression of cytokines in ischemic and ex-vivo-reperfused rat lungs, significant inhibition of RT-PCR reaction was observed. In this study we describe our successful approach to overcome this effect by a pretreatment of RNA samples with heparinase I prior to RT-PCR.

Materials and methods

Lung preparation

Adult male Wistar rats (300–450 g; Charles River Inc., St. Constant, Quebec, Canada) were used. In a previous study, we described the procedure of donor lung preparation for ex vivo perfusion in detail [5]. Briefly, following intraperitoneal injection of sodium pentobarbital (50 mg/kg), a tracheotomy was performed, and animals were ventilated with a volume-cycled ventilator with room air. A midline laparotomy was followed by systemic *heparinization* (1000 IU/kg) through the inferior vena cava. A median sternotomy was performed, the thymus resected, and the great vessels exposed. A no. 16 catheter was inserted into the main pulmonary artery via the right ventricular outflow tract and allowed to fill with blood prior to being occluded. The inferior vena cava and abdominal aorta were transected and the left atrial appendage amputated. The left ventricle was partly excised to allow free flow of effluent out of the lung. Following cannulation of the main pulmonary artery, the lungs were flushed with low potassium dextran solution [10], which we routinely use for clinical and experimental lung transplantation. The heart-lung block was removed with the lungs inflated to 50% of total lung capacity [4] and stored at 4 °C for 12 h. Lung blocks used as deoxygenators were prepared the same way. For normal fresh lungs as controls, animals were sacrificed by overdose intraperitoneal injection of sodium pentobarbital without heparinization.

Animals received humane care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research, the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science and published by the National Institutes of Health (NIH Publication No. 86–23 revised, 1985), and the *Guide to the Care and Use of Experimental Animals* formulated by the Canadian Council of Animal Care.

The lung reperfusion circuit

The perfusion circuit was primed with 20 ml of fresh venous blood obtained from two *heparinized* (1000 IU/rat) rats [5]. To control organ function of the single left study lung, blood samples of 0.3 cc were taken during reperfusion. The effluent of the study lung was continuously deoxygenated by a second double-lung block (deoxy lung), which was ventilated with a hypoxic gas mixture ($F_iO_2 = 0.04$, $F_iCO_2 = 0.08$, $V_t = 3.0$ ml, at a rate of 70 bpm, with 2 cm H_2O PEEP. This inspired gas composition and the ventilatory settings yielded blood gases similar to in vivo mixed venous gases. Both lung blocks were housed in a warmed (36–38 °C) and humidified plexiglas chamber. A double-head roller pump was used to maintain continuous blood flow. The deoxy lung effluent was continuously collected in a reservoir and pumped into the main pulmonary artery of the study lung. Similarly, the study lung effluent was collected in another reservoir and pumped into the main pulmonary artery of the deoxy lung block.

Tissue collection and total RNA extraction

Lung biopsies were collected from normal rat lungs ($n = 3$) as controls, from lungs after ischemic preservation ($n = 3$), and from lungs 2 h after the initiation of reperfusion ($n = 3$). Lung tissues were snap-frozen in liquid nitrogen and stored at −70 °C. For total RNA extraction, the guanidinium isothiocyanate method was used [3]. Briefly, frozen tissue samples (about 0.1 g) were crushed in a liquid-nitrogen-cooled mortar. The crushed tissue powder was mixed with 5 ml of 4 M guanidinium isothiocyanate buffer (Gibco/BRL, Gaithersberg, Md.) containing 0.7% of freshly added β-mercaptoethanol and was homogenized with a Polytron tissue homogenizer (Brinkman, Mississauga, Ontario, Canada) for 1 min at medium speed. The lysate was mixed with 0.1 ml 2 M sodium acetate, pH 4.2, followed by the addition of 5 ml phenol, pH 4.5, and 1 ml chloroform:isoamyl alcohol (49:1). The aqueous phase was collected after centrifugation at 8000 rpm for 40 min at 4 °C with a high-speed centrifuge (J2–21 centrifuge, JA-17 rotor, Beckman, Fullerton, Calif.). The supernatant was transferred to a new tube, and RNA was precipitated by mixing with equal volume of isopropanol. RNA concentration was measured with a spectrophotometer (Beckman DU 640B). To evaluate the RNA quality, 10 µg of total RNA was visualized by running a formaldehyde-agarose gel.

Semiquantitative RT-PCR

Total RNA (5 µg) was used for the RT reaction, using a Superscript II kit (Gibco/BRL), according to the manufacturer's instructions. The RT reaction from 0.5 µg of total RNA was used for PCR. When samples were subjected to heparinase treatment, 5 µg of total RNA was incubated with heparinase I (Sigma, St. Louis, Mo.) in 5 mM Tris, pH 7.5, 1 mM $CaCl_2$, and 40 U of RNase (Promega, Madison, Wis.) for 2 h at 25 °C prior to RT reaction [9].

PCR primers specifically for rat TNFα [11] and β-actin [1] were synthesized by ACGT Corporation (Toronto, Ontario, Canada). The PCR reaction mixture was set up in a total volume of 30 µl containing 3 µl 10X PCR buffer (200 mM Tris-Cl, pH 8.4, 500 mM KCl), 1 µl 50 mM $MgCl_2$, 0.7 µl 10 mM dNTP mix, 0.5 µl of each PCR primer (10 µM), and 0.3 µl Taq polymerase (Gibco/BRL). PCR was performed with a programmable thermal cycler (PTC-100, MJ Research, Watertown, Mass.) for 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C for 25 (β-actin) or 35 cycles (TNFα). PCR product (10 µl) was loaded on 1% agarose gel with ethidium bromide staining for visualization. After electrophoresis, gels were photographed and quantified with a gel documentation system (Gel Doc1000, Bio-Rad, Mississauga, Ontario, Canada).

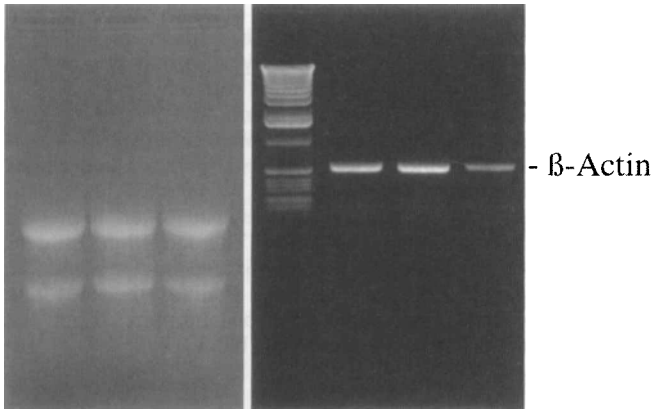

Fig. 1 Inhibition of reverse transcriptase polymerase chain reaction (*RT-PCR*) of RNA extracted from lungs after ischemia-reperfusion. *Left panel* To examine the integrity of RNA, total RNA (5 μg) from lung tissues was separated by 1 % agarose-formaldehyde gel. The quality of RNA from lung samples after preservation, ischemia-reperfusion was equal. Both 28S and 18S ribosomal RNAs were well preserved. *Right panel* Total RNA extracted from rat lung tissues was subjected to RT-PCR with primers for β-actin mRNA. PCR products were resolved on 1 % agarose gel containing ethidium bromide and visualized by a gel documentation system. RT-PCR was inhibited with RNA extracted from lung tissue after ischemia-reperfusion (*M* Molecular markers, *C* control, normal lung without heparin, *P* preserved lung, *R* reperfused lung)

Results

Inhibition of RT-PCR reaction in RNA extracted from lung samples after ischemia-reperfusion

To test the integrity of RNA, total RNA (5 μg) isolated from lung tissue was loaded on 1 % agarose gel containing 3.7 % formaldehyde. After gel electrophoresis, the quality of total RNA was visualized with a gel documentation system. All of the RNA samples were of good quality. The bands representing 28 S and 18 S ribosomal RNA were well preserved. No significant RNA degradation was observed (Fig. 1, left panel). We have noted that the mRNA levels of β-actin remain consistent in rat lungs after preservation or ischemia-reperfusion, as examined by Northern blotting analysis (unpublished observation). Thus, this highly abundant gene was used as an internal control to monitor the RNA concentration and the efficiency of the RT reaction. Surprisingly, in all three samples collected after ischemia-reperfusion, the levels of β-actin mRNA were reduced to different degrees (Fig. 1, right panel). This indicates that RT-PCR reaction was partially inhibited.

In an effort to overcome the inhibitory effect, we tried to purify RNA further by repeated ethanol precipitation, changing Mg^{2+} concentrations, varying annealing temperatures, and increasing PCR cycles. None of these interventions were able to overcome the observed inhibitory effect. In some samples, even after 40 cycles of PCR, a weak band was only occasionally visualized with the gel documentation system by increasing the integration time significantly. Furthermore, when RNA extracted from normal lung tissue was mixed with RNA from lung tissue after ischemia-reperfusion, the RT-PCR was also inhibited (data not shown).

Inhibition of RT-PCR is due to heparin

In the current clinical practice of organ transplantation, heparinization is routinely used to prevent thrombosis and to optimize the microcirculation in donor and recipient organs. Lungs and other organs are commonly flushed with a preservation solution, which removes blood from the vasculature. After implantation, transplanted organs are reperfused with heparinized blood. This is also true for experimental in vivo and ex vivo models in transplantation research. It has been reported that heparin binds to DNA [2] or RNA [8] isolated from whole blood and interferes with PCR or RT-PCR. Thus, it is possible that heparin in the lung tissue after reperfusion also interferes with RT-PCR, which was seen in our initial experiments.

To determine whether the inhibition of RT-PCR is due to heparin, and if so, to evaluate the optimal dose of heparinase I to remove the effect of heparin, RNA (5 μg) from reperfused lung was incubated with various amounts of heparinase I prior to RT reaction. Heparinase treatment overcame the inhibitory effect very effectively at concentrations as low as 0.1 U per 5 μg of total RNA. After heparinase treatment, both β-actin and TNFα mRNAs were revealed clearly from samples collected after ischemia-reperfusion (Fig. 2). Interestingly, with 10 U of heparinase treatment (2 U/μg of total RNA), the mRNA level of β-actin was reduced (Fig. 2).

Heparinase I pretreatment permits RT-PCR detection of TNFα mRNA expression

Based on the information mentioned above, total RNA (5 μg) isolated from normal lung, ischemic preserved lung, and lung tissues after ischemia-reperfusion was pretreated with 1 U of heparinase I, and mRNA levels of β-actin and TNFα were analyzed by RT-PCR. Consistent with our Northern blot analysis, the mRNA levels of β-actin were not affected by preservation or ischemia-reperfusion (Fig. 3). In contrast, mRNA levels of TNFα significantly increased after ischemia-reperfusion (Fig. 3).

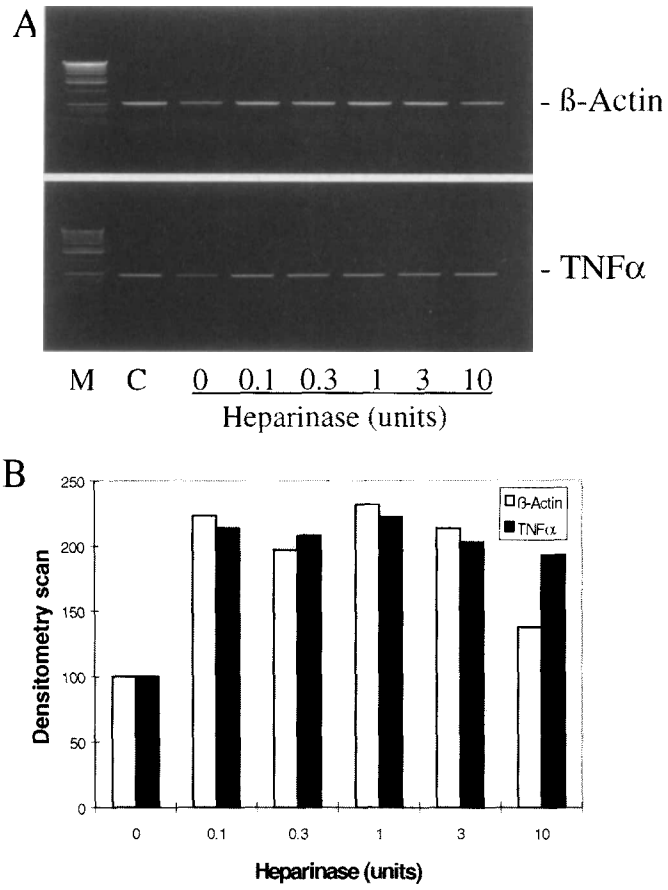

Fig. 2 Heparinase I pretreatment abolished the inhibition on reverse transcriptase polymerase chain reaction (*RT-PCR*). Total RNA (5 μg) extracted from lung tissue after ischemia-reperfusion was incubated with various concentrations of heparinase I at 25 °C for 2 h prior to RT reaction. Heparinase I treatment enhanced mRNA levels for both β-actin and tumor necrosis factor-α (*TNF-α*) with similar efficiency. **A** Original blots (*M* Molecular markers, *C* control, normal lung without heparin), **B** Density of each band was analyzed with a gel documentation system, and expressed as a percentage of that from samples pretreated in the absence of heparinase I

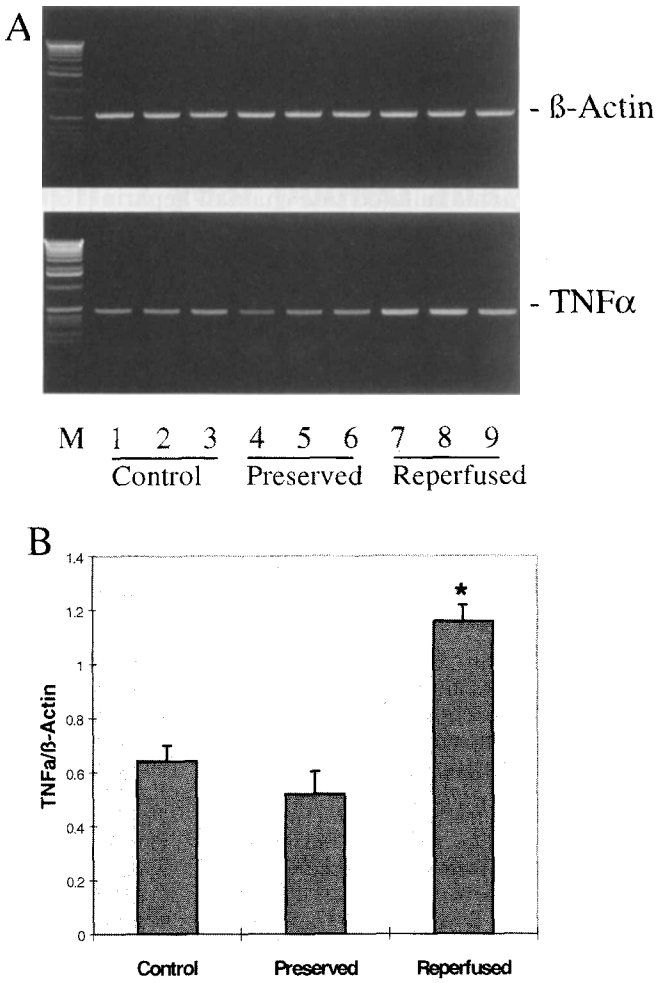

Fig. 3 Tumor necrosis factor-α (TNFα) gene expression increases in lungs after ischemia-reperfusion. RNAs were pretreated with heparinase I (1 U/5 μg of total RNA) for 2 h prior to reverse transcriptase reaction. Reverse transcriptase polymerase chain reaction (*RT-PCR*) was performed with primers for tumor necrosis factor-α (TNFα) and β-actin. The expression of β-actin was not altered during lung preservation and ischemia-reperfusion, and thus served as an internal control. The expression of TNFα was normalized with the mRNA level of β-actin from the same RT reaction for each sample. TNFα expression was significantly increased in lung tissues after ischemia-reperfusion. **A** Original blots, **B** The ratio between TNFα and β-actin, analyzed with a gel documentation system. All data are mean ± SE from three samples. *: $P < 0.05$ compared with other groups as determined by using one-way ANOVA

Discussion

During organ transplantation, heparin as a necessary anticoagulant is given to both the donor (before flushing the organs) and the recipient (before or right after organ reperfusion) to prevent thrombosis and to optimize the microcirculation. Although it has been reported that heparin inhibits RT-PCR or PCR with RNA or DNA extracted from heparinized whole blood, until now it was unknown whether the amount of heparin present in lung tissue in the transplantation setting was sufficient to interfere with the RT-PCR. In this study, we indeed found that the inhibitory effect of heparin in lung tissue after reperfusion was significant. Furthermore, heparin could not be removed from RNA samples just by repeated ethanol precipitation or by optimizing PCR conditions.

Izraeli et al. found that 1–3 U of heparinase I were required per μg RNA derived from frozen heparinized whole blood [9]. RNA extracted from lung tissue after ischemia-reperfusion needed much less heparinase I. Based on our study, 0.1 U of heparinase I is sufficient

to overcome the inhibition from heparin. This may be due to the fact that the amount of heparin in the lung tissue is much less than that in whole blood. The mechanism by which heparin inhibits RT-PCR is not well understood. It is possible that there is an interaction between critical components of the reaction mixture and the highly anionic sulfated side chain of heparin [16].

RT-PCR is the most sensitive method to study gene expression. It is widely used in medicine and other scientific fields. With the development of semiquantitative and quantitative methods, it has been applied more commonly to the study of gene expression of cytokines, chemokines, and other inflammatory mediators. In the present study, we found clear induction of TNFα gene expression as early as 2 h after the initiation of reperfusion. Because cytokines play an important role in mediating acute inflammatory reactions and allogenic responses, the examination of cytokine gene expression during and immediately after organ transplantation has been considered for both clinical diagnostic applications and research. Since patients or animals are usually subjected to heparinization during donor and recipient procedures in transplantation medicine, heparinase pretreatment of RNA (or DNA) samples should be considered as an essential step before performing RT-PCR (or PCR) in order to obtain accurate and meaningful results.

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