Effects of Aprotinin on Gene Expression and Protein Synthesis After Ischemia and Reperfusion in Rats

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Background—Reperfusion injury of ischemic myocardium has been attributed to neutrophil infiltration, inflammatory activation and cardiac necrosis/apoptosis. Serine protease inhibition with aprotinin is cardioprotective, but the mechanism is unknown.

Methods and Results—We studied aprotinin in a rat model of myocardial ischemia for 20 minutes and reperfusion for 20 minutes, 8 hours or 24 hours. Aprotinin (20 000 IU/kg) given 5 minutes before reperfusion significantly reduced leukocyte accumulation (P<0.01), myocardial injury (determined by CK depletion, P<0.01) and myocyte apoptosis (P<0.05) compared with vehicle treated rats. Differential gene expression analysis showed myocardial ischemia plus reperfusion increased expression of proinflammatory genes like P-selectin, E-selectin, intercellular adhesion molecule, tumor necrosis factor-α, tumor necrosis factor-α receptor, interleukin-6, monocyte chemoattractant protein-1, p53, and Fas (CD95). Aprotinin before reperfusion suppressed expression of these inflammatory genes. Finally, differential protein expression analysis demonstrated increased intercellular adhesion molecule-1, tumor necrosis factor-α, and p53 after myocardial ischemia plus reperfusion, and this effect was diminished by aprotinin.

Conclusions—We demonstrated myocardial ischemia plus reperfusion induced leukocyte accumulation, inflammation, gene expression, protein expression and finally tissue injury and showed aprotinin limiting reperfusion injury through each of these stages, even after 24 hours of reperfusion. This effect seems partly attributable to suppression of proinflammatory genes and leukocyte accumulation. This work casts further light on the complex signaling of ischemia and reperfusion. (Circulation. 2007;116[suppl I]:I-121–I-126.)

Key Words: ischemia-reperfusion injury ■ leukocytes ■ gene expression profiling ■ protein synthesis

Myocardial ischemia without reperfusion results in severe myocyte necrosis and cardiac dysfunction. Thus, early reperfusion of the ischemic myocardium is a therapeutic goal. Unfortunately, reperfusion itself contributes to injury by triggering a cascade of events including neutrophil infiltration, free radical generation, cytokine release and complement activation.1 Central to these cascades are serine proteases. These are widespread enzymes with triggering and signaling roles (eg, complement C1s and thrombin) and ‘effector’ roles (eg, elastase, trypsin and kinins).

Strategies to limit reperfusion injury have been directed at many points in this complex cascade, but the use of serine protease inhibitors such as aprotinin is particularly promising. Aprotinin is a naturally occurring broad spectrum protease inhibitor obtained from bovine lung. Notwithstanding recent debate over its use sparked by 2 recent articles reporting an association with renal failure and other complications,2,3 aprotinin has found widespread use in cardiac surgery for its benefits in reducing perioperative bleeding,4 myocardial ischemia,5,6 and cerebral events.6,7 Animal studies have revealed a variety of effects including cardioprotection, reduced endothelial dysfunction and leukocyte migration.7,8

Exactly how aprotinin and the other serine proteases mediate antiinflammatory effects is unknown. There is mounting evidence that reperfusion triggers ‘early’ components such as release of stored factors (like P-selectin and cytokines), and subsequent ‘late’ components are more likely to be mediated by changes in de novo protein synthesis and expression. The predominance of each component depends on the protein type involved.9 Aprotinin may affect both of these signaling modalities.10,11

Whether and how serine proteases affect protein release or gene expression (and hence de novo protein synthesis) in myocardial ischemia and reperfusion and inflammatory states needs further evaluation. Therefore, we determined the effect of aprotinin on myocardial gene and protein expression in a

References

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well established \textsuperscript{12} rat model of myocardial ischemia and long-term reperfusion.

### Materials and Methods

#### Experimental Protocol

A detailed description of our protocol is published elsewhere, \textsuperscript{12} but briefly, rats anesthetized with ether underwent a minithoracotomy and myocardial ischemia (MI) produced by left anterior descending artery occlusion with a reversible suture. Rats were recovered and continued to breathe spontaneously. The suture was released (without reopening the chest) 20 minutes later, thus initiating myocardial reperfusion (R). Rats were studied for different reperfusion periods of 20 minutes, 8 hours, and 24 hours. Blood samples were taken during reperfusion and hearts were harvested after reperfusion for analysis. These reperfusion durations were chosen to allow study of early events (20 minutes to 8 hours) eg, P-selectin expression and gene array analysis as well as later events (24 hours) eg, eventual injury and apoptosis. Animals were randomly divided into 3 major groups consisting of (1) sham-operated rats (n=5) receiving aprotinin (Trasylol, Bayer Leverkusen), 20 000 IU/kg, (2) MI+reperfusion rats (n=9) receiving vehicle (saline 0.9%, 0.3 mL) and (3) MI+reperfusion rats (n=9) receiving 20 000 IU/kg aprotinin. Aprotinin or vehicle was administered by a single intravenous bolus injection 5 minutes before reperfusion (or equivalent time point in sham-operated animals). In subsequent experiments we varied the timing of aprotinin (or vehicle) administration to test efficacy when given before MI and 5 minutes after reperfusion (n=9 for each group).

#### Leukocyte Accumulation and Reperfusion Injury After Ischemia and Reperfusion

Leukocyte accumulation and myocardial CK analysis was performed on animals reperfused for 24 hours as described previously. \textsuperscript{13} Samples of left ventricular free wall (LVFW, ie, ischemic/reperfused myocardium) and intraventricular septum (ie, nonischemic control myocardium) were removed and homogenized. The LVFW sample was central to the area supplied by the left anterior descending artery to ensure maximal ischemia-reperfusion injury. Any nonischemic myocardium included would lessen the observed differences. We assumed that this area underwent MI+reperfusion. This was confirmed with the results of the subsequent homogenate assays. The septum is known to be supplied by the right coronary artery in rats \textsuperscript{14} allowing its use as nonischemic control myocardium.

Septum and LVFW samples were analyzed for myeloperoxidase activity (MPO) as a marker of leukocyte accumulation. One unit of MPO hydrolyzed 1 mmol of peroxide/min at 25°C. The MPO increase in the LVFW was calculated by subtracting MPO-septum from MPO-LVFW and expressed as MPO difference (U/100 mg wet weight tissue). Creatine kinase activity (CK Assay Sigma) was assessed because the CK washout from the LVFW is a useful index of tissue injury after ischemia/reperfusion and is known to be significant after 24 hours of reperfusion. \textsuperscript{15} The CK-LVFW was subtracted from CK-septum and expressed as CK difference (IU/g protein).

#### mRNA Expression After Ischemia and Reperfusion

LVFW mRNA expression after 8 hours of reperfusion was assessed in the sham, vehicle and aprotinin (5 minutes before reperfusion) groups. The amount of mRNA was determined and subsequent cDNA was synthesized as described previously. \textsuperscript{16} cDNA was p32 radioisotope-labeled and hybridized against the Atlas Rat cDNA Expression Array (Clontech). With software analysis we assessed semiquantitative differences in mRNA expression between the 3 groups and expressed results as a change in relative expression. There were 5 animals in each of these groups.

#### Protein Expression After Ischemia and Reperfusion

To detect changes in cell adhesion molecule, p53 expression, and serum tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) levels, after the 3 different reperfusion durations, we used immunohistochemical techniques and an ELISA as described previously. \textsuperscript{13} Sections of LVFW and septal myocardium from sham, vehicle and aprotinin (given 5 minutes before reperfusion) groups were incubated with primary antibody for P-selectin, intercellular adhesion molecule (ICAM)-1 and p53 (Phar-mingen, Germany and Santacruz, Germany). After staining, the proportion of P-selectin or ICAM-1-positive staining venules was counted using microscopy and expressed as a percentage of the total number of venules seen. An intensity score for the proportion of p53-positive staining myocytes is presented for p53 results and serum concentration (pg/mL) is presented for TNF\(\alpha\) results.

#### Apoptosis After Ischemia and Reperfusion

Reperfusion injury results not only in an inflammatory necrosis but also in increased programmed cell death (apoptosis). Apoptosis after reperfusion for 24 hours was assessed as described previously. \textsuperscript{13} LVFW from sham, vehicle and aprotinin (given 5 minutes before reperfusion) groups was examined for the induction of apoptosis by

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**TABLE 1. Aprotinin Effects on Leukocyte Accumulation**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI+R+Vehicle</th>
<th>MI+R+ Aprotinin Before I</th>
<th>MI+R+ Aprotinin Before R</th>
<th>MI+R+ Aprotinin 5 Minutes After R</th>
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<tbody>
<tr>
<td>MPO difference</td>
<td>0.1±0.07</td>
<td>1.44±0.27</td>
<td>0.21±0.12*</td>
<td>0.35±0.13*</td>
<td>0.95±0.21</td>
</tr>
</tbody>
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Mean LVFW-septum MPO difference (U/100 mg wet weight tissue)±SEM, n=5 (sham group) and 9 (all other groups).

*\(P<0.001\) vs MI+R+vehicle

R indicates reperfusion; I, ischemia.

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**Figure 1. Effect of aprotinin on CK assessed reperfusion injury.**

CK difference between septum and LVFW after reperfusion for 24 hours in sham-, vehicle- and aprotinin-treated rats (with aprotinin or vehicle given at 3 timepoints). MI+reperfusion plus vehicle-treated animals had a substantial difference in tissue CK levels in the ischemic region (LVFW) compared with the septum. This change was significantly attenuated by aprotinin. The effect was more pronounced the earlier the aprotinin was given in the MI+reperfusion sequence. Mean CK difference (IU/g protein) ± SEM, n=5 for sham and 9 for all other groups, **\(P<0.001\) versus MI+reperfusion and vehicle group. R indicates reperfusion.

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caused by substantial injury to the LVFW compared with the nonischemic septum. When animals were treated with 20 000 IU/kg aprotinin, depletion of CK decreased significantly. Interestingly, when aprotinin was administrated before ischemia or reperfusion, we observed a significant reduction in reperfusion injury. When aprotinin was administered 5 minutes after reperfusion the degree of cardioprotection was decreased.

Table 1 shows accumulation of leukocytes (after 24 hours reperfusion) into the LVFW as the difference in MPO activity between LVFW and septum. Sham-operated animals had no significant differences in MPO activity between these territories. Vehicle-treated animals underwent a profound MPO activity increase indicating accumulation of leukocytes in the LVFW attributable to MI+reperfusion. Aprotinin treatment before ischemia or reperfusion produced a similar and significant reduction of leukocyte accumulation. However, aprotinin administered 5 minutes after reperfusion had a smaller effect on leukocyte accumulation.

Effects of Ischemia and Reperfusion and Aprotinin on Gene Expression

We found an increase in expression of proinflammatory genes like P-selectin, E-selectin, ICAM, TNFα, TNFα receptor, interleukin-6, monocyte chemoattractant protein-1, p53, Fas (CD59), MAK-2, JNK, STAT3, c-myc, and c-jun attributable to MI+reperfusion when we compared LVFW gene expression between sham-operated and vehicle-treated rats (data not shown).

Figure 2 shows changes in LVFW mRNA (after reperfusion for 8 hours) in animals given aprotinin (5 minutes before reperfusion) relative to vehicle-treated animals. Interestingly, aprotinin produced a significant reduction in expression of various proinflammatory genes, such as those of the adhesion molecule group (P-selectin and ICAM,) the cytokine/proinflammatory gene group (TNFα, iNOS, nuclear factor κB, TNF-receptor, interleukin-6 receptor) and the apoptosis group (p53 and fas). The effect of aprotinin in reducing expression of these inflammatory genes was substantial and ranged from a relative reduction (versus MI+reperfusion plus vehicle) of −1.5 to −4.2.

Effect of Aprotinin on Adhesion Molecule Cell Surface Expression

Table 2 shows P-selectin underwent a profound increase in surface expression after MI and reperfusion of only 20 minutes. This increase was partly sustained after 8 hours reperfusion but was substantially lower after 24 hours reperfusion. Aprotinin significantly reduced this MI+reperfusion
induced P-selectin cell surface translocation at all time points. MI+reperfusion (±aprotinin) produced a similar pattern of ICAM-1 expression (Figure 3), but the timecourse of ICAM-1 changes was later (peaking after 8 hours reperfusion) than P-selectin.

**Effect of Aprotinin on Serum TNFα and Myocardial p53 Expression**

Serum TNFα (Figure 4) increased to 7.3±2.9 pg/mL after MI and reperfusion (20 minutes), rising further to 55.3±6.9 pg/mL after MI+reperfusion (8 hours) and remaining significantly elevated after MI+reperfusion (24 hours) in vehicle-treated rats. Aprotinin significantly reduced TNFα levels.

p53 expression (Figure 5) rose substantially after MI+reperfusion (24 hours) in vehicle-treated animals. This increase was significantly attenuated by aprotinin (given 5 minutes before reperfusion).

**Effect of Aprotinin on Apoptosis**

Table 3 data shows MI+reperfusion resulted in a significant and substantial increase in LVFW myocyte apoptosis from 2% to 3% (a background rate of apoptosis measured in this model) to 28.3±3.7% in vehicle-treated animals. Nonischemic septal tissue from the same animals did not exhibit this increase in apoptosis. Aprotinin administered before reperfusion produced a significant reduction in LVFW apoptosis.

**Discussion**

The present study demonstrates the cardioprotective qualities of aprotinin. The mechanisms of these effects are likely to include reduced protease induced expression of stored proteins such as P-selectin. In addition, this study provides novel evidence that aprotinin reduces inflammatory gene expression and hence de novo inflammatory protein synthesis. These effects of aprotinin on myocardial protein expression are also associated with a profound reduction of leukocyte accumulation, myocardial necrosis and myocardial apoptosis.

The timing of aprotinin administration is important because myocardial injury and leukocyte accumulation were substantially greater when administration was delayed until after reperfusion. This finding lends weight to the notion that the earliest stages of tissue injury involve the expression of proinflammatory mediators whose release is activated by the action of ‘triggering proteases’17 such as complement, coagulation factors (eg, thrombin and plasmin) and kinins. The relatively short therapeutic window of aprotinin implies that...
this triggering mechanism will have been initiated if the aprotinin administration is delayed, thus resulting in attenuation of its effects.

The effect of aprotinin in reducing leukocyte accumulation is in keeping with work from several groups\textsuperscript{7,11,18} although the Asimakopoulos group has also shown aprotinin limits leukocyte accumulation independently of leukocyte adhesion.\textsuperscript{18} Thus, there remains the possibility of leukocyte extravasation by 2 mechanisms (one involving P-selectin-mediated rolling and the other without). Alternatively, this disparity may arise from the differences in cell adhesion, migration and inflammatory mediator release seen between in vitro and in vivo models.

Although an effect of aprotinin on cerebral endothelial ICAM-1 synthesis\textsuperscript{19} and umbilical vein endothelial cell VCAM expression\textsuperscript{20} is recognized, to our knowledge this is the first demonstration of an effect of aprotinin via changes in mRNA transcription in vivo. By comparing the timecourse of observed changes in mRNA expression and the timecourse of expression of the relevant protein, we can infer whether the protein expression was a result of release from storage or perhaps de novo synthesis. Aprotinin affected P-selectin levels after only 20 minutes of reperfusion, and this effect is likely to be mediated by the release of P-selectin from Weibel-Palade bodies as suggested by Lorant et al.\textsuperscript{21} Previous work shows aprotinin inhibits the action of thrombin, (a known ligand for protease-activated receptor–1), from initiating the translocation of P-selectin from Weibel-Palade bodies to the cell surface.\textsuperscript{22} This may represent one of the ways that serine protease inhibitors such as aprotinin control the trigger mechanisms that proteases exert on the inflammatory cascade. Interestingly, in addition to influencing these early reperfusion events, we also found aprotinin reduced P-selectin mRNA transcription after reperfusion for 8 hours. This may have contributed to the reduction in P-selectin protein expression that we observed (after 8 and 24 hours reperfusion) by affecting de novo P-selectin synthesis. The later time-course of ICAM-1 cell surface expression is in keeping with previous work\textsuperscript{23} and may have arisen primarily through a mechanism involving changes in de novo expression, either as a direct consequence of aprotinin’s actions or indirectly through the observed reduction in serum TNFα levels.

The finding of reduced myocardial TNFα mRNA expression suggests a suppressive effect of aprotinin on de novo synthesis of TNFα by one of the cellular components of the myocardial homogenates. The observed suppression of serum TNFα levels may have arisen by an effect of aprotinin on intramyocardial TNFα production or in suppression of TNFα release by circulating monocytes. Other data\textsuperscript{8} supports a reduction of TNFα by aprotinin although this effect has not been borne out consistently, particularly in human studies.\textsuperscript{24}

Our study shows cardioprotection by aprotinin occurs not only through an antinecrotic but also through an antiapoptotic mechanism. In the present study, we illustrate a potential genetic mechanism by which aprotinin may influence apoptosis. We found aprotinin suppressed Fas and p53 mRNA transcription and showed a corresponding reduction in p53 and TNFα protein expression. Because p53, fas and TNFα have been mechanistically linked to apoptosis,\textsuperscript{25} the suppression of these factors may explain how aprotinin limits apoptosis.

Aprotinin’s established role in clinical practice has been questioned by 2 recent nonrandomized studies describing significant complications.\textsuperscript{2,3} The present study adds further evidence showing antiinflammatory, antinecrotic, antiapoptotic and cardioprotective effects of aprotinin. We provide novel data regarding the gene expression modifying mechanisms behind aprotinin’s cardioprotective effects. These effects may be mediated by a combined reduction of the early ‘triggering’ effects of proteases in the release of adhesion molecules and other inflammatory mediators as well as the control of later events in reperfusion injury mediated via changes in mRNA transcription and de novo protein synthesis.

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### Disclosures

I. Friedrich has received consultation fees from Bayer AG. There are no other conflict of interest disclosures.

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