Activated Protein C Protects Against Myocardial Ischemia/Reperfusion Injury via Inhibition of Apoptosis and Inflammation

Sarah T.B.G. Loubele, C. Arnold Spek, Peter Leenders, René van Oerle, Hella L. Aberson, Karly Hamulyák, Gary Ferrell, Charles T. Esmon, Henri M.H. Spronk, Hugo ten Cate

Objective—In spite of major advances in reperfusion therapy for patients presenting with acute coronary syndrome, long-term morbidity is still substantial. A limitation of initial treatment of myocardial ischemia is the lack of prevention of ischemia/reperfusion (I/R) injury. Activated protein C (APC), a crucial mediator in the coagulation process, plays a prominent role in the crosstalk between coagulation and inflammation and provides cytoprotective effects via inhibition of apoptosis and inflammation in several human and animal studies.

Methods and Results—APC was administered in an animal model for myocardial I/R. APC largely inhibited early myocardial I/R injury after varying reperfusion times, an effect that was absent on administration of heparin, a nonspecific anticoagulant agent. The protective effects of APC were absent in case of absence or blockade of protease activated receptor-1 (PAR-1), indicating a critical role for PAR-1 in this process. Furthermore, we showed a strong antiapoptotic effect of APC in the early phase of reperfusion combined with an antiinflammatory effect at an early stage (IL-6), as well as at a later stage (leukocyte infiltration).

Conclusions—APC exerts strong protective effects on early myocardial I/R injury, primarily via inhibition of apoptosis and inflammation, which are regulated via PAR-1. (Arterioscler Thromb Vasc Biol. 2009;29:1087-1092.)

Key Words: activated protein C • myocardial ischemia/reperfusion • inflammation • apoptosis • protease activated receptor-1

Acute myocardial infarction (AMI) is an important contributor to cardiovascular mortality. Although mortality attributable to the acute coronary event has declined substantially with current improved reperfusion therapies, long-term morbidity is increased because of secondary heart failure in survivors of AMI. Unfortunately, the shift from acute to long-term morbidity after acute ischemic injury of the heart reveals other therapeutic shortcomings. Whereas revascularization in the acute phase of ischemia or thrombolysis has caused a major improvement in survival, there are no agents that limit ischemia/reperfusion (I/R) injury, contributing to apoptosis, inflammation, and subsequent heart failure.1

The natural anticoagulant protein C, which is converted by thrombin into activated protein C (APC),2 plays a crucial role in the process of coagulation but is also prominent in the crosstalk between coagulation and inflammation.3 The anti-inflammatory role of APC emerged from the PROWESS study where APC was shown to reduce mortality in patients with severe sepsis.4 Animal studies indicate that APC offers protection against I/R injury in in vivo models of ischemic stroke, spinal cord ischemia, and renal injury.5–8 Hence, we hypothesized that APC might also reduce myocardial injury after ischemic damage of the heart.

To explore the potential beneficial effects of APC on myocardial I/R injury, we applied mouse APC in an experimental mouse model of myocardial I/R. The effects of APC on myocardial I/R injury, inflammation, as well as apoptosis were explored to gain a better insight into the specific mechanisms of APC on cardiac myocytes. We especially focused on the cell signaling pathways on several time points of reperfusion and the effects on coagulation, inflammation, and apoptosis related processes.

Methods

Procedure Myocardial I/R
All animal experiments were approved by the Animal Ethics Committee of Maastricht University. C57/B6 male mice (Charles River Laboratories, Maastricht, The Netherlands), 6 weeks old and...
25 g, were used for all experiments. Male PAR-1 knockout mice (PAR-1<sup>−/−</sup>) were obtained from Prof S. Coughlin (San Francisco, Calif). Per group, a total of 6 animals was used. Myocardial I/R was induced according to the method of Jong et al.<sup>6</sup> Mice were anesthetized with isoflurane (induced with 3 to 4% and maintained with 1.5 to 2.5%) and were ventilated (frequency: 210/min; volume: 250 μL) during the ischemic period. Ischemia was induced for 60 minutes followed by 3 different reperfusion times: 2, 6, or 24 hours. A sham group was included in which all surgical procedures were performed but no I/R was induced. Sham animals were euthanized after 90 minutes of anesthesia comparable to the experimental group. Mouse APC in a dose of 0.4 mg/kg, or placebo (0.9% NaCl) was administered (i.v. via the vena jugularis) 15 minutes after induction of ischemia and a second time 5 minutes after induction of reperfusion. Unfractionated heparin (Heparin Leo, Leo Pharma BV) was administered (i.v. via the vena jugularis) twice at the same time points in a dose of 5 U each. The protease activated receptor-1 (PAR-1) inhibitor P1pal12 was administered 30 minutes before induction of reperfusion in a 10 μmol/L dose (200 μL i.p.) according to Slofstra et al.<sup>9</sup> After the requested reperfusion time, the animals were anesthetized as described earlier. Sodium citrate (3.2%) was injected directly into the vena cava and plasma was collected. In one group, heart tissue was collected for RNA and protein analysis and for obtaining paraffin tissue sections. In a distinct group, I/R injury was determined by means of the Evans Blue/TTC staining as described by Erlich et al.<sup>11</sup> In brief, after the requested reperfusion time, the surface was removed and Evans Blue dye (500 μL) was injected into the vena cava and allowed to circulate to mark the area at risk (AAR). After excision of the heart, it was cut into 0.5-mm-thick slices and TTC was added to mark the viable cells within the AAR (AAR). Afterward a percentage was made of AOI (white tissue) to the AAR (red and white tissue). Slight modifications were applied to the protocol to calculate the size of the areas as pixel counts were used to determine the size of the areas.

**Coagulation**

Thrombin generation in mouse plasma was determined using the Calibrated Automated Thrombogram (CAT). The CAT was obtained from Thrombinscope BV (Maastricht, The Netherlands), and all plasma samples were analyzed in a 96-well plate fluorometer (Ascent Reader, Thermolabsystems OY, Helsinki, Finland) according to the method as described by Tchaikovski et al.<sup>12</sup> APC was added in varying concentrations of 0 to 250 nmol/L, and heparin was added in concentrations of 0 to 5 U/mL to mouse plasma. Lag time, time to peak, peak height, endogenous thrombin potential (ETP or the area under the curve), and time to tail were determined.

**Gene Expression**

Apoptosis and cell signaling–related gene expression levels were determined in tissue homogenates containing 1 mg/mL protein using an ELISA reagent set. All analyses were performed according to the manufacturer’s manual (eBioscience). Paraffin sections were stained for CD 45 using a rat antimouse CD 45 antibody (BD Pharmingen). The number of CD 45-positive cells per surface area was determined in 3 fields within the infarcted area using a 40× magnification.

**Statistical Analysis**

Data analysis was performed with GraphPad Prism version 5.00. Values are means±SEM. Differences between groups were tested using a Mann–Whitney t test, and probability values <0.05 were considered statistically significant.

**Results**

APC Diminishes Myocardial I/R Injury via PAR-1

In a model of myocardial I/R injury, administration of mouse recombinant APC decreased, as compared to placebo, the amount of I/R injury (depicted as a percentage of AOI to AAR) with a trend observed at 2 hours and obtaining a maximal and statistically significant protective effect after 6 hours, which sustained after 24 hours of reperfusion (Figure 1A). As a control, AAR ratios to the whole heart were determined in the placebo-treated animals, and these remained equal over time (data not shown). To assess the effects of APC against an active comparator agent, the anticoagulant unfractionated heparin was administered. Heparin administration at a peak level of 2.5 U/mL had no effect on myocardial I/R injury as the % AOI/AAR after heparin administration was comparable to placebo administration after 6 hours of reperfusion, suggesting specific protective effects of APC not involving its anticoagulant action (Figure 1B). PAR-1 was recently shown to influence cardiac remodeling, hypertrophy, and myocardial ischemia/reperfusion injury.<sup>14,15</sup> Furthermore, blocking PAR-1 reversed the protective effects of APC on apoptosis in hypoxic cells. To reveal the role of PAR-1 in the protective mechanism of APC in the myocardium, APC was administered in combination with the specific PAR-1 blocker P1Pal12 and was administered to PAR-1<sup>−/−</sup> mice. P1Pal12 reversed the protective effects of APC on myocardial I/R injury because the % AOI/AAR remained equal to placebo administration after a 6-hour reperfusion period (Figure 1C). Furthermore, administration of APC to PAR-1<sup>−/−</sup> mice confirmed these results as no protective effect of APC was observed in PAR-1<sup>−/−</sup> mice (Figure 1C).

To compare the anticoagulant effects of both heparin and APC, both anticoagulants were added in a thrombin genera-
tion measurement in a dose varying around the dose administered to the mice. Both APC and heparin already inhibited thrombin generation at a dose (Heparin: 0.052 U/mL; APC: 10 nmol/L) that was much lower than the dose administered in the mice (Heparin: 5 U/mL; APC: 214 nmol/L), indicating that both heparin and APC exert an equally strong anticoagulant function within the experiment, suggesting that other functions of APC besides its anticoagulant function are necessary for its protective effect in myocardial I/R injury (Figure 2A). Because APC has been shown to inhibit the NF-κB transcription complex in specific cells including mononuclear cells,\(^{16}\) we determined whether the administration of APC would also diminish protein levels that result from immediate gene translation of the NF-κB pathway, including tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1). Both TF activity and PAI-1 antigen levels in heart tissue homogenates increased on I/R after 2, 6, or 24 hours of reperfusion compared to the sham group. Remarkably, APC administration increased TF activity levels as well as PAI-1 antigen concentrations after 2 hours of reperfusion compared to the placebo treated animals. TF activity levels were no longer increased on APC administration in combination with P1Pal12. After 6 and 24 hours of reperfusion neither TF activity nor PAI-1 antigen levels were different between APC and placebo treated animals (Figure 2B and 2C).

**APC Attenuates Apoptosis**

APC was previously shown to reduce apoptosis in hypoxic cells and to be neuroprotective in a mice stroke model,\(^{5,6}\) as well as to reduce apoptosis in the heart in a I/R model in rats.\(^{17}\) To assess the effects of mouse specific APC on I/R-induced apoptosis, cell death was analyzed by means of apoptosis staining. Staining with a chromogenic as well as a fluorogenic detection method revealed a reduced number of apoptotic cells on APC administration, showing the most pronounced effect after 2 and 24 hours of reperfusion (Figure 3A). Scoring of the stained sections for the number of positive cells revealed a decrease after 2 and 24 hours of reperfusion on APC administration compared to placebo, a trend which was also visible after 6 hours of reperfusion (Figure 3B). Thus, APC has a strong antiapoptotic effect on myocardial I/R injury at an early onset in the reperfusion phase.

**APC Reduces Inflammation Regulated via PAR-1**

Besides its antiapoptotic effects, APC also exerts antiinflammatory properties in patients with sepsis as well as in experimental animal studies.\(^{4–8}\) Within the placebo group, IL-6 levels increased compared to the sham group showing maximal levels at 2 hours of reperfusion. IL-6 levels remained slightly increased after 6 hours of reperfusion, and returned to baseline sham levels after 24 hours of reperfusion. Administration of APC caused an early reduction in IL-6 protein levels at 2 hours of reperfusion (Figure 4A). Blocking PAR-1 counteracts the antiinflammatory effect of APC as IL-6 levels remained equal to placebo administration (Figure 4A). IL-1β and TNF-α values in the left ventricle remained below the detection limit at all reperfusion times. As an important hallmark of I/R injury, leukocyte infiltration was determined by means of CD 45 staining. Leukocyte infiltration was attenuated on APC administration after a prolonged reperfusion time (Figure 4B). The number of CD 45+ cells per surface area increased after 6 and 24 hours of reperfusion compared to a 2 hours reperfusion period in placebo treated mice, whereas on APC administration the number of infiltrating leukocytes decreased after 6 and 24 hours of reperfusion (Figure 4C). These results show marked antiinflammatory properties of APC in association with a diminished myocardial I/R injury, primarily by inhibition of leukocyte influx and IL-6 levels, probably mediated via PAR-1. However, proteins transcribed in a NF-κB dependent manner including TF and PAI-1 do not seem to contribute to the protective effect of APC.

**APC Reduces Apoptotic Related Gene Expression**

The effect of APC on gene expression relating apoptosis and NF-κB-mediated genes were analyzed using Multiplex Ligation Dependent Probe Amplification (MLPA). From the
apoptosis gene panel, Bfl-1 and Bcl-xl expression levels were increased on I/R compared to the sham animals. Administration of APC decreased Bfl-1 and Bcl-2 expression levels after 6 hours of reperfusion. Bcl-w and Bcl-xl were not influenced on APC administration. The Bax-like proapoptotic related genes Bak1, Bax, and Mcl-1 increased on I/R whereas the expression level of Bcl-Rambo decreased on I/R compared to sham. The RNA expression levels of Bax, Bcl-Rambo, and Mcl-1 decreased on APC administration. The expression level of Bak1 showed a trend toward a decreased expression on APC administration after 6 hours of reperfusion, but the results were not significantly different. The BH3-only proapoptotic related gene Bid was increased after I/R at 6 and 24 hours of reperfusion, whereas Bik expression levels were decreased after I/R at 2 hours of reperfusion. Administration of APC decreased Bik. Bim, and Map-1 levels after 2 and 6 hours of reperfusion, whereas the expression level of Bid was increased after APC administration on 2 hours of reperfusion. The expression levels of Bad were not influenced by the administration of APC (Figure 5). These results showed a downregulating effect of APC on a number of proapoptotic genes, indicating an early effect on the BH3-only related genes at early reperfusion times and a downregulating effect of Bax-like related genes at later stages of reperfusion. The differences in RNA expression levels of other apoptosis related genes are shown in supplemental Table I. MLPA analysis on NF-κB related cell signaling proteins did not reveal a contributing effect of APC on these pathways (data not shown).

Discussion

This study reveals a role for APC in the protection against myocardial I/R injury via a combined effect on inflammation and apoptosis and mediated via PAR-1. Although the absence of PAR-1 was previously shown to be protective against myocardial I/R injury, the current study highlights the need for PAR-1 in the protective actions of APC in myocardial I/R injury, consistent with results in an ischemic stroke model. A great part of cardiomyocyte cell death occurs via the process of apoptosis, which is preceded by cell shrinkage leading to cell fragmentation. Whether apoptosis is induced during the ischemic or the reperfusion phase remains contradictory. Several pathways are shown to be involved in the induction of I/R in the heart, including: activation of TNF-α, p53, Bcl-2, Bax up-, or downregulation, and infiltration of neutrophils. Here we show a strong antiapoptotic effect of APC on apoptosis staining at an early time point of reperfusion combined with an effect on BH3
only and Bax-like proapoptotic genes at early and later stages of reperfusion. The most striking effects of APC were visible on genes involved in the Bcl-2 family, which was also revealed in brain endothelial cells.24 A study by Pirat et al also revealed an antiapoptotic effect of human APC after 20 minutes of ischemia and 2 hours of reperfusion, but an anti-inflammatory effect on C-reactive protein or TNF-α levels was not visible possibly because of the short ischemic period used.17 Direct effects of APC on neutrophil migration have been demonstrated in experimental models under conditions of inflammation.25 In I/R injury the process of apoptosis is thought to trigger an inflammatory response, including the influx of leukocytes, a process which is confirmed in the kidney but likely also applies to other organs.26 Excess inflammatory activity is also thought to contribute to lethal cardiomyocyte injury and inhibition of this pathway by APC may in part explain the protective effect of this agent. The latter is also illustrated by an early inhibitory effect of APC on IL-6 production in the heart. It is unknown whether APC inhibits IL-6 production by infiltrating leukocytes or by cardiomyocytes, but the latter is more likely to occur early in the reperfusion phase given that leukocyte influx occurs beyond 2 hours, the time point when the anti–IL-6 effect of APC is evident.

Figure 4. A, IL-6 protein in heart tissue. B, Representative pictures of the CD45 staining for leukocytes. C, Counting the numbers of CD45 positively stained cells per surface area. All values are mean ± SEM, n=6. *2 hours vs 6 hours, **6 hours vs 24 hours, ***2 hours vs 24 hours for placebo or APC; #2 hours R, ##6 hours R, ###24 hours R between placebo and APC; $2 hours R, $$6 hours R, $$$24 hours R between placebo and P1Pal12, P<0.05.

Figure 5. The RNA expression levels of a number of Bcl-2 antiapoptotic, Bax-like proapoptotic, and BH3 only proapoptotic genes. *difference between placebo and APC administration, n=6, P<0.05.
Thrombin generation analysis in plasma revealed reduced thrombin levels on APC administration in a dose comparable to the dose administered in the mice. Remarkably, TF activity and PAI-1 antigen levels in the heart increased on APC administration after a 2-hour reperfusion time. Given the temporary reduction in IL-6 levels in the heart, a link between the increased TF and PAI-1 levels to an unexpected proinflammatory effect of APC, such as described in endothelial cells in vitro could not be made, leaving it rather difficult to explain this observation. Our gene analysis data do not provide evidence for additional NF-κB mediated effects of APC in this model.

Based on the above data we propose that intervention with APC could indicate a new and important pharmacological tool in the prevention of early myocardial I/R injury. This might ultimately provide an intervention that would reduce heart failure arising from MI.

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**Disclosures**

None.

**References**

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**Table I:** The difference in RNA expression level between placebo or APC treated animals of several apoptosis related genes not involved in the Bcl-2 family.

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