Functional Protection by Acute Phase Proteins $\alpha_1$-Acid Glycoprotein and $\alpha_1$-Antitrypsin Against Ischemia/Reperfusion Injury by Preventing Apoptosis and Inflammation

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**Background**—Ischemia followed by reperfusion (I/R) causes apoptosis, inflammation, and tissue damage leading to organ malfunction. Ischemic preconditioning can protect against such injury. This study investigates the contribution of the acute phase proteins $\alpha_1$-acid glycoprotein (AGP) and $\alpha_1$-antitrypsin (AAT) to the protective effect of ischemic preconditioning in the kidney.

**Methods and Results**—Exogenous AGP and AAT inhibited apoptosis and inflammation after 45 minutes of renal I/R in a murine model. AGP and AAT administered at reperfusion prevented apoptosis at 2 hours and 24 hours, as evaluated by the presence of internucleosomal DNA cleavage, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling, and the determination of renal caspase-1– and caspase-3–like activity. AGP and AAT exerted anti-inflammatory effects, as reflected by reduced renal tumor necrosis factor-$\alpha$ expression and neutrophil influx after 24 hours. In general, these agents improved renal function. Similar effects were observed when AGP and AAT were administered 2 hours after reperfusion but to a lesser extent and without functional improvement. Moreover, I/R elicited an acute phase response, as reflected by elevated serum AGP and serum amyloid P (SAP) levels after 24 hours, and increased hepatic acute phase protein mRNA levels after 18 hours of renal reperfusion.

**Conclusions**—We propose that the antiapoptotic and anti-inflammatory effects of AGP and AAT contribute to the delayed type of protection associated with ischemic preconditioning and other insults. This mechanism is potentially involved in the course of many clinical conditions associated with I/R injury. Moreover, exogenous administration of these proteins may provide new therapeutic means of treatment. (*Circulation*. 2000;102:1420-1426.)

**Key Words:** ischemia ■ reperfusion ■ kidney ■ immune system ■ apoptosis

Prolonged ischemia followed by reperfusion (I/R) induces apoptosis and inflammation, leading to organ dysfunction and tissue damage. We recently demonstrated that acute primary apoptosis during early reperfusion is crucial to the initiation of reperfusion-induced inflammation. Conversely, the central inflammatory mediator, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), was shown to contribute to late apoptosis in the course of renal I/R.

TNF-$\alpha$ is also an inducer of the acute phase response (APR), a complex series of reactions executed by the host in the immediate aftermath of injury, trauma, or infection. Protection against reperfusion injury can be induced by various means, including antecedent administration of endotoxin, heat shock, and single or multiple periods of brief antecedent ischemia. Besides such protection, the latter treatments are all expected to induce an APR. During the APR, liver cells and various epithelial cells respond to increasing levels of, among others, TNF-$\alpha$ by producing acute phase proteins, including $\alpha_1$-acid glycoprotein (AGP) and $\alpha_1$-antitrypsin (AAT). These 2 major acute phase proteins exhibit various anti-inflammatory effects and have been shown to prevent hepatocyte apoptosis in a model of TNF-$\alpha$-galactosamine toxicity.

The present study investigates whether AGP and AAT can reduce I/R-induced apoptosis and inflammation and whether renal I/R induces an APR. The results show that physiological serum levels of exogenous AGP and AAT strongly reduce apoptosis and inflammation after I/R and that renal I/R induces an APR.

**Methods**

**Antibodies and Reagents**
Anti-murine neutrophil monoclonal antibody (mAb) Gr-1 was obtained from Pharmingen; rabbit anti-murine AGP polyclonal anti-
bodies were kindly provided by Dr P. Heegaard (Danish Veterinary Laboratory, Copenhagen, Denmark); peroxidase-conjugated goat anti-rat and peroxidase-conjugated goat anti-rabbit IgGs were from Jackson; peroxidase-conjugated and alkaline phosphatase–conjugated sheep anti-digoxigenin and digoxigenin 11-dUTP were from Boehringer-Mannheim; peroxidase-conjugated rabbit anti-sheep IgG was from DAKO; rabbit anti-murine serum amyloid P (SAP) and SAP standard were from Calbiochem-Novabiochem; and Ac-YVAD-amc and Ac-DEVD-amc were from the Peptide Institute. All other reagents were purchased from Sigma Chemical Co.

**Experimental Protocol**

Male Swiss mice weighing 20 to 25 g from Charles River Breeding Laboratories (Heidelberg, Germany) were housed individually in standard cages with access to food and water ad libitum. The studies were approved by the Institutional Animal Care Committee of the University of Maastricht. Forty-five minutes of unilateral ischemia of the left kidney was followed by contralateral nephrectomy, as described in detail previously. The animals were euthanized at indicated time points. At the time of euthanization, blood was collected by orbital puncture, and the left kidney was harvested.

At reperfusion, mice were administered intraperitoneally (IP) 5 mg bovine AGP (n = 12) or 0.5 mg human AAT (n = 12) in 0.5 mL sterile PBS, resulting in serum levels identical to those observed during the APR. In separate groups, mice received AGP (n = 8) or AAT (n = 8) after 2 hours of reperfusion. To further delineate the therapeutic efficacy of AGP treatment, mice received 1.7 mg AGP (n = 3), 0.5 mg AGP (n = 3), and 0.17 mg AGP (n = 3) in 0.5 mL PBS at reperfusion and were euthanized after 2 hours. A control group received vehicle consisting of 0.5 mL PBS IP (n = 10). A sham-operated group (n = 12) was subjected to the same surgical procedure without clamping of the renal pedicle, treated with PBS, and euthanized at corresponding time points.

In an experiment to investigate whether renal I/R induced an APR, mice were subjected to renal I/R, and blood was collected by orbital puncture at 8, 16, and 24 hours after ischemia (n = 12). Additional animals received 30 μg IP lipopolysaccharide (LPS, Escherichia coli serotype O55:B5) (n = 3) or 0.5 mL of PBS IP (n = 3) and served, respectively, as positive and negative controls for the development of an APR.

**Apoptosis Assays**

Presence of internucleosomal DNA cleavage in kidneys was investigated with a commercial ligase-mediated polymerase chain reaction (LMP-PCR) assay kit (ApoAlert, Clontech) according to the manufacturer’s instructions. Renal caspase-1– and caspase-3–like activities were assessed as described by measuring the release of fluorescent 7-amino-4-methylcoumarin for 1 hour after incubation renal lysates with the fluorogenic substrate Ac-YVAD-amc (caspase-1–like) or Ac-DEVD-amc (caspase-3–like).

**MPO, BUN, and Serum Creatinine**

Renal neutrophil accumulation was quantified by measuring renal myeloperoxidase (MPO) content as described. The MPO activity is expressed per milligram tissue by comparing the optical density of peroxidase-enzymed 4-chloro-1-naphthol to standards. MPO activity is assayed with known quantities of murine MPO.

Kidney specimens were immediately frozen and stored at −70°C or fixed in buffered formalin and embedded in paraffin. Frozen sections (5 μm) were stained for neutrophils with mAb Gr-1 as described. Immunostaining for TUNEL was performed on paraffin sections as described. Histological aspects of apoptosis were studied by standard terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) as described.

**SAP ELISA and Single Radial Immunodiffusion for Serum AGP**

Serum SAP was measured by use of a sandwich ELISA. A 96-well Immunomaxisorp plate (Nunc) was coated with a rabbit anti-mouse SAP IgG. Aspecific binding was blocked with BSA; after washing procedures, samples were diluted, and a standard titration curve of a known quantity of murine SAP was obtained. Detection was performed by use of a biotinylated rabbit anti-mouse SAP IgG, followed by incubation with substrate. Serum AGP levels were determined by single radial immunodiffusion with use of agar gels containing 5% anti-AGP serum. Because purified murine AGP was not readily available, the obtained results were calibrated against a dilution of murine plasma obtained 36 hours after pretreatment with 30 μg LPS, which also served as a positive control.

**Measurement of Hepatic Acute Phase Protein mRNA Content**

Total RNA was extracted from livers and transcribed into cDNA, of which the concentration was subsequently standardized on the basis of the β-actin cDNA fraction. To determine hepatic AGP, AAT, and SAP mRNA content, three 2-fold serial dilutions of cDNA were amplified with specific primers. Murine AGP mRNA–specific primers, designed on the basis of sequence homology with an acute phase–inducible gene in Mus caroli, were sense primer 5′-GGCCGCTGTCTAAACCCT-3′ and antisense primer 5′-CAAGTCAAAAGGACAGATG-3′; murine AAT mRNA–specific primers were sense primer 5′-TCCCATGAGATCGCTACAAAC-3′ and antisense primer 5′-TGATAATGGTTCTTGGCCTCT-3′; murine SAP mRNA–specific primers were sense primer 5′-CCATGAAAC-3′ and antisense primer 5′-ACGGACTGTGACTTTTGATTGT-3′. Additional primers were sense primer 5′-TGAAATCCTGGACCTTGGCCAT-3′ and antisense primer 5′-GGAGACCTGATCTTTTCTTGCATGAAAC-3′. After separation on a 1.5% agarose gel, band proportions were estimated by measuring the intensity of ethidium bromide fluorescence with a digital camera (Imagemaster VDS, Pharmacia) by using commercial gel analysis software (Sigma Gel, SPSS).

**Statistics**

Data are expressed as mean±SEM, and statistical analysis was performed by Student t test. A value of P<0.05 was taken to denote statistical significance.

**Results**

**AGP and AAT Reduce Early and Delayed Apoptosis Induced by Renal I/R**

Apoptosis contributes to I/R-induced organ dysfunction and may serve as a target for the protective effects of acute phase proteins. No apparent internucleosomal DNA cleavage was detected at 2 hours of reperfusion in kidneys obtained from mice treated with either AGP or AAT compared with kidneys obtained from PBS-treated mice (Figure 1). These early effects of AGP and AAT suggest direct inhibition of apoptosis, inasmuch as early primary apoptosis precedes the first signs of inflammation in this model. In line, as indicated by the absence of apparent internucleosomal DNA cleavage (Figure 1), decreased numbers of TUNEL-positive nuclei (Figure 2), and attenuated caspase-1– and caspase-3–like activities (Figure 3), apoptosis was reduced after 24 hours in mice treated with either AGP or AAT compared with PBS-treated control mice, possibly because of a combination of antiapoptotic and anti-inflammatory effects.

We previously demonstrated that inhibition of early apoptosis prevents the initiation of inflammation as well as...
secondary apoptosis caused by inflammation in our model. The present study indicates that abrogation of inflammation does not occur when apoptosis inhibitors are administered after 2 hours of reperfusion. Hence, apoptosis precedes the inflammatory response at 2 hours of reperfusion. Therefore, administration of AGP or AAT at 2 hours of reperfusion enabled us to differentiate primary apoptosis from secondary apoptosis and to study the possible involvement of an anti-inflammatory effect of these acute phase proteins. Compared with PBS treatment, AAT administered at 2 hours of reperfusion decreased caspase-1– and caspase-3–like activities after 24 hours of reperfusion (Figure 3). However, AAT did not reduce internucleosomal DNA cleavage (Figure 1), whereas AGP reduced caspase-1– and caspase-3–like activities (Figure 3) and prevented internucleosomal DNA cleavage after 24 hours (Figure 1). These data suggest that reduced secondary apoptosis is a result of the anti-inflammatory effect of AGP and AAT, although we cannot exclude a contribution via direct inhibition of secondary apoptosis.

To investigate the therapeutic efficacy of AGP treatment, the effect of a dose range of AGP on internucleosomal DNA cleavage after 2 hours of reperfusion was studied. A single dose of 1.7 mg AGP at reperfusion sufficed to reduce renal internucleosomal DNA cleavage (Figure 1). This therapeutic effect gradually declined when dosages of 0.5 of 0.17 mg of AGP were used (Figure 1). Rodent acute phase plasma has been reported to contain up to 3.5 mg/mL AGP compared with almost undetectable constitutive levels, indicating that systemic rises in endogenous AGP during an APR are potentially protective against I/R-induced apoptosis.

AGP and AAT Reduce Inflammation After I/R
We studied the effects of AGP and AAT on I/R-induced inflammation by assessing renal TNF-α expression and neutrophil influx. At 24 hours after I/R, AGP and AAT administered at reperfusion limited TNF-α expression (Figure 5D and 5E) and neutrophil influx (Figure 4). These effects are most likely the result of direct inhibition of early primary apoptosis, implicated in the subsequent induction of inflammation. Alternatively, direct anti-inflammatory effects may also be involved. Nevertheless, these findings explain the

Figure 1. Renal apoptosis is reflected by fragmented DNA amplified by ligase-mediated polymerase chain reaction and visualized on ethidium bromide–stained gel. In PBS-treated animals, internucleosomal DNA cleavage became apparent after 2 hours of reperfusion. Treatment with AGP or AAT prevented internucleosomal DNA cleavage, except for AAT administered after 2 hours of reperfusion. M indicates molecular weight markers (range, 100 to 2000 bp).

Figure 2. Representative light micrographs showing evident in situ detection of DNA nick ends by TUNEL histology (magnification ×200). In contrast to cells from sham-operated mice (A), tubular epithelial cells of outer medulla of biopsies obtained from PBS-treated mice after 24 hours of reperfusion showed extensive apoptosis coinciding with influx of inflammatory cells (B, upper left corner). Treatment with AGP on reperfusion apparently decreased numbers of TUNEL-positive nuclei after 24 hours (C), similar to numbers associated with AAT treatment on reperfusion (D).

Figure 3. In homogenates of tissue obtained after 1 day of reperfusion, renal caspase-like activities were determined kinetically by fluorogenic substrate assay in which Ac-YVAD-amc (caspase-1–like) or Ac-DEVD-amc (caspase-3–like) served as substrates. Data are expressed as increase in fluorescence as a function of time, normalized against data obtained from sham-operated group. Groups with t=2 on x-axis received treatment after 2 hours of reperfusion. All other groups were treated on reperfusion. Compared with PBS-treated group, all groups showed significantly decreased caspase-like activities (P<0.05, not indicated). Statistical significance compared with animals that received similar treatment on reperfusion was denoted at #P<0.05. Data are mean±SEM.

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observed inhibition of secondary apoptosis in mice treated at reperfusion.

AGP and AAT given after 2 hours of reperfusion attenuated the inflammation at 24 hours to a lesser extent than did treatment given at reperfusion (Figure 4). However, treatments at both time points decreased inflammation compared with PBS treatment at 24 hours of reperfusion (Figure 4). The PBS-treated control mice showed significant renal inflammation as reflected by TNF-α expression in the outer stripe of the outer medulla, along the damaged tubular epithelium (Figure 5B), and in infiltrating leukocytes (Figure 5C). Also, significant renal neutrophil accumulation was reflected by an enhanced MPO content (Figure 4) and evident accumulation of Gr-1-positive cells (Figure 6). AGP and AAT administered at 2 hours prevented inflammation as reflected by these parameters, revealing a direct anti-inflammatory potential of AGP and AAT.

![Figure 4. Neutrophil influx was assessed quantitatively by determination of MPO increase after 24 hours of reperfusion. Values are presented relative to amount of MPO present in contralateral kidney harvested immediately after reperfusion and normalized with respect to MPO increase after 24 hours of reperfusion in PBS-treated mice. The groups with t=2 received treatment after 2 hours of reperfusion. *P<0.05 vs control treated animals; #P<0.05 vs animals that received similar treatment on reperfusion. Data are mean±SEM.](image)

**Figure 4.** Neutrophil influx was assessed quantitatively by determination of MPO increase after 24 hours of reperfusion. Values are presented relative to amount of MPO present in contralateral kidney harvested immediately after reperfusion and normalized with respect to MPO increase after 24 hours of reperfusion in PBS-treated mice. The groups with t=2 received treatment after 2 hours of reperfusion. *P<0.05 vs control treated animals; #P<0.05 vs animals that received similar treatment on reperfusion. Data are mean±SEM.

![Figure 5. Representative light micrographs showing immunohistochemical staining of TNF-α by mAb 52B83 (magnification ×100). Although absent in kidneys obtained from sham-operated controls (A), TNF-α was expressed in outer stripe of outer medulla and distributed in and along damaged tubular epithelium (B) as well as in infiltrating leukocytes (C) after 24 hours of reperfusion. Treatment with AGP on reperfusion decreased intrarenal TNF-α expression after 24 hours (D), similar to AAT treatment on reperfusion (E).](image)

**Figure 5.** Representative light micrographs showing immunohistochemical staining of TNF-α by mAb 52B83 (magnification ×100). Although absent in kidneys obtained from sham-operated controls (A), TNF-α was expressed in outer stripe of outer medulla and distributed in and along damaged tubular epithelium (B) as well as in infiltrating leukocytes (C) after 24 hours of reperfusion. Treatment with AGP on reperfusion decreased intrarenal TNF-α expression after 24 hours (D), similar to AAT treatment on reperfusion (E).

![Figure 6. Representative light micrographs showing immunohistochemical staining by mAb Gr-1 of infiltrating neutrophils (magnification ×100). Neutrophils were scarce in kidneys obtained from sham-operated animals (A). In contrast, marked renal neutrophil accumulation (B) was observed after 24 hours of reperfusion.](image)

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**AGP and AAT Prevent I/R-Induced Renal Dysfunction**

Renal dysfunction was reflected by increased BUN content and serum creatinine levels after 24 hours of reperfusion (Figure 7). Compared with PBS, both AGP and AAT administered at reperfusion lowered BUN content and serum creatinine levels. However, compared with PBS, AGP and AAT administered after 2 hours of reperfusion failed to significantly decrease BUN or serum creatinine. These findings illustrate the necessity for the prevention of primary apoptosis-induced inflammation in addition to the direct prevention of inflammation after I/R for optimal therapeutic effects.

**Renal I/R Induces an APR**

To investigate whether renal I/R induces an APR, serum SAP and AGP levels and hepatic acute phase protein mRNA content were measured. SAP was used as a marker for the APR because it is coreleased with AGP and AAT during the APR. Renal I/R induced an elevation in plasma...
SAP levels at 16 and 24 hours after ischemia (Table). LPS administration served as a positive control and increased SAP levels after 36 hours. Serum AGP levels increased to a similar extent (an approximate factor 10 compared with constitutive levels) after 24 hours of reperfusion and 36 hours after LPS (data not shown). In contrast to previous reports,9,10 our results show no apparent rise in hepatic acute phase protein mRNA content after renal I/R or LPS administration (Figure 8). Conversely, after 16 hours of reperfusion, hepatic AGP and SAP mRNA levels increased compared with levels in sham-operated control mice or mice that received only PBS (Figure 8). This rise in hepatic acute phase protein mRNA was similar to that observed in mice 16 hours after 30 μg LPS (Figure 8.). These data clearly demonstrate that renal I/R induces an APR.

**Discussion**

The effects of the acute phase proteins AGP and AAT on early (2-hour) and late (24-hour) apoptosis and inflammation after renal I/R were investigated. Both AGP and AAT administered at reperfusion decrease early as well as late apoptosis, as reflected by renal internucleosomal DNA cleavage, TUNEL histology, and caspase-like activities. In line, we previously demonstrated that abrogating acute early apoptosis with selective antiapoptotic agents prevents subsequent inflammation as well as secondary apoptosis caused by inflammation, whereas antiapoptotic treatment that is initiated after the onset of apoptosis does not reduce I/R-induced inflammation.1 In contrast, in the present study, treatment after 2 hours of reperfusion inhibited inflammation, which is supported by reports of anti-inflammatory effects mediated by AGP and AAT.12,13

Early primary19,20 as well as late secondary2,21 apoptosis after I/R has been reported to be caused by various means. The present results, showing that AGP and (to a lesser extent) AAT protect against TNF-α—dependent late apoptosis,2 are in line with data from Van Molle et al.14 They showed that AGP protects against TNF-α—induced liver apoptosis in both galactosamine-pretreated and actinomycin D—pretreated mice, whereas AAT conferred protection in only the galactosamine model. Because inflammation is not involved in the process of primary apoptosis after I/R, our results show that the antiapoptotic potential of AGP and AAT is not limited to TNF-α—induced apoptosis.

AGP has been reported to exhibit anti-inflammatory properties, such as inhibition of neutrophil activation and induction of macrophage-derived interleukin-1 receptor antagonist release.11,12 AGP also binds to bacterial endotoxin and protects mice from endotoxin-induced septic and hypovolemic shock.22,23 Currently, no clear antiapoptotic property can be deduced from the AGP molecule. AAT can inhibit neutrophil superoxide production,24 induce macrophage-derived interleukin-1 receptor antagonist release,11 and reduce TNF-α—induced lethality.25 AAT additionally inhibits elastase26 and, as a consequence, elastasedependent synthesis and release of platelet-activating factor,26 a mediator of I/R—induced inflammation.27 Elastase has been reported to cleave tyrosyl-tRNA synthetase during apoptosis to fragments with interleukin-8—like chemotactic properties,28 a process potentially involved in primary I/R—induced apoptosis.1 AGP or AAT may interact with the proteolytic cascade of enzymes involved in apoptosis. However, both acute phase proteins lack inhibitory effects on caspases in vitro (Dr C. Libert, personal communication, 1999). Further studies are needed to establish the molecular mechanisms by which AGP and AAT prevent I/R—induced apoptosis and inflammation.

Antecedent ischemia can induce protection from I/R—induced injury in a biphasic pattern.8 Such protection lasts for 30 minutes to 2 hours and is followed by a second window of protection appearing 12 to 24 hours later. This so-called ischemic preconditioning has been attributed to local protective mechanisms, including induction of heat-shock proteins and adenosine formation.30 However, brief ischemia in remote organs11,12 or endotoxin pretreatment4—6 has been shown to confer similar protection, suggesting involvement of other than local mechanisms. Indeed, the observed I/R—induced SAP and AGP increase and elevated hepatic acute phase protein mRNA content...
show that renal I/R induces an APR. Our data also indicate that the serum AGP increase during the APR is of potentially sufficient extent to protect against I/R-induced apoptosis. Thus, an APR likely confers systemic protection against I/R injury, which could contribute to the second window of protection associated with ischemic preconditioning.

The physiological mode of protection outlined above may determine the natural history of clinical conditions associated with I/R as observed during severe trauma or septic shock. For instance, sepsis mortality is highest during the initial stages of the disease, and the chances of survival increase with disease duration. This increased survival may well be a consequence of protection against shocklike complications because systemic levels of acute phase proteins increase as the disease progresses. Moreover, protective acute phase proteins such as AGP and AAT may provide new means to treat clinical conditions associated with I/R injury.

We conclude that the APR may be part of a physiological protection mechanism against I/R injury and show that protection conferred by acute phase proteins such as AGP and AAT is mediated by distinct antiapoptotic as well as anti-inflammatory effects. We additionally demonstrate that I/R itself induces an APR, which may explain the second window of protection induced by ischemic preconditioning.

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References


Figure 8. Hepatic acute phase protein mRNA expression 16 hours after either renal I/R, sham operation, administration of 30 μg of LPS IP, or PBS only. Shown are representative samples, of which cDNA was amplified in three 2-fold serial dilutions and calibrated against identical levels of β-actin mRNA. Band proportions are indicated and expressed as pixel units, calibrated according to raw pixel values of the digital capture image. Background (black) corresponds to a pixel value of 0; amplified DNA (white) corresponds to a pixel value of 255.

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