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Evaluation of Multimeric Tyrosine-O-Sulfate as a Cytoprotectant in an in vivo Model of Acute Myocardial Infarction in Pigs

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Key Words
Myocardial infarction · Ischemia/reperfusion injury · Coagulation · Complement · Multimeric tyrosine sulfate

Abstract
Objectives: Intracoronary administration of glycosaminoglycan analogs, including the complement inhibitor dextran sulfate, attenuates myocardial ischemia/reperfusion injury (I/R injury). However, dextran sulfate has a distinct anticoagulatory effect, possibly limiting its use in specific situations in vivo. We therefore developed multimeric tyrosine sulfate (sTyr-PAA), a novel, minimally anticoagulatory, fully synthetic non-carbohydrate-containing polyacrylamide conjugate, for in vivo testing in an acute closed-chest porcine model of acute myocardial infarction. ***Methods:*** Following balloon occlusion of the left anterior descending artery just after the first diagonal branch (60-minute ischemia), sTyr-PAA (approx. 10 mg/kg bodyweight, fraction with strongest complement-inhibitory and minimal anticoagulatory properties, n = 11) or phosphate-buffered saline (controls, n = 9) was administered intracoronarily into ischemic myocardium prior to 120 min of reperfusion. ***Results:*** sTyr-PAA significantly reduced infarct size (from 61.0 ± 12.0% of the ischemic area at risk to 39.4 ± 17.0%), plasma creatine kinase, local complement deposition and tissue factor upregulation, without affecting systemic coagulation. Protection was associated with significantly reduced myocardial neutrophil extravasation and translated into a significant improvement of ejection fraction and left ventricular enddiastolic pressure. ***Conclusions:*** sTyr-PAA protected significantly against myocardial I/R injury without substantially affecting systemic coagulation. Local intravascular sTyr-PAA administration may prove advantageous in situations where bleeding complications are likely or are to be avoided at all costs.

Introduction

Activation of the complement and coagulation systems is implicated in initiating endothelial cell activation and subsequently leads to tissue injury following isch-

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emia and reperfusion [1, 2]. Damage to endothelial integrity leads to a loss of thrombo- and immune-regulatory function within the intravascular space [3], with upregulation of endothelial tissue factor (TF), vascular adhesion molecules and cytokine production [2, 4, 5]. We previously developed and described the use of a non-carbohydrate-containing polyacrylamide conjugate of *O*-sulfotyrosine (sTyr-PAA, 80% mol of sTyr) as a potent blocker of P-selectin in vitro and neutrophil extravasation in a rat peritonitis model in vivo [6]. Further studies in vitro revealed that sTyr-PAA compounds, in particular those with a 40% substitution rate (meaning that 40% of all available substitution sites of the polymer-backbone are occupied by tyrosine sulfate), were potent inhibitors of all three pathways of complement activation [7] – all of which have been implicated in ischemia/reperfusion (I/R) injury. In addition to complement inhibition, sTyr-PAA binds to damaged endothelium denuded of its protective heparan sulfate proteoglycan surface layer and prevents human serum-mediated cytotoxicity towards porcine cells [7]. Preceding work in our laboratory has shown that the glycosaminoglycan analog dextran sulfate [DXS; molecular weight (MW) 5,000 Da] significantly ameliorated reperfusion injury in acute myocardial infarction in pigs through association with damaged endothelium and site-specific complement inhibition [8]. Yet DXS, like certain other glycosaminoglycan analogs, including heparin, not only efficiently inhibits complement activation [9–11], but also primary and secondary hemostasis [12–14]. However, in specific clinical situations, including myocardial infarction and cerebral reperfusion injury, (secondary) hemorrhage upon reperfusion may have detrimental consequences. Therefore, as part of our ongoing efforts to develop and characterize novel cytoprotectants, we focused on investigating the potential of sTyr-PAA to associate with damaged endothelium and to reduce the extent of myocardial necrosis in an in vivo pig model of myocardial infarction.

Materials and Methods

Synthesis of sTyr-PAA

All PAA-based conjugates were synthesized by the methods described previously [15, 16]. Briefly, for synthesis of sTyr(40%)-PAA, sTyr powder and triethylamine were added to a solution of poly(4-nitrophenyl acrylate) in dimethyl sulfoxide (DMSO). Following the addition of ethanolamine, the resulting conjugate was purified by gel filtration on a Sephadex LH-20 (3 × 50 cm) column eluted with 1:1 acetonitrile-water. Fractions containing the target conjugate were concentrated in vacuo and divided into four fractions by gel filtration on a Sephadex G-25 (7 × 49.5 cm) column (elution with water): fraction 1 (the heaviest, 2.6%, eluted after the free volume of column), fraction 2 (28.8%), fraction 3 (40.2%) and fraction 4 ('tail', 13.0%). Fraction 4 was not evaluated further. The apparent molecular weights of fractions 1–3 (table 1) were estimated by HPLC on TSK gel G2000SW (TOSOH, Japan, 10 μm, 7.5 × 300 mm) column preliminarily calibrated using a standard kit of poly(acrylic acids), M_n1,250–28,000 Da (elution with 0.2 M NaCl in H_2O, 1 ml/min, at room temperature).

Synthesis of sTyr-PAA-Fluo

For the synthesis of fluorescein-labeled sTyr-PAA (sTyr-PAA-Fluo) 5-[(5-aminopentyl)thioureidyl]fluorescein, dihydrobromide salt, sTyr powder, and triethylamine were added to poly(4-nitrophenyl acrylate) in DMSO. Following the addition of ethanolamine, the resulting conjugate was purified by gel filtration on a Sephadex LH-20 (2 × 35 cm) column eluted with 1:1 acetonitrile-water. Fractions containing the target conjugate were concentrated in vacuo, dissolved in water, and lyophilized to yield the sTyr-PAA-Fluo.

Complement and Coagulation Markers in vitro

Fractions 1–3 of sTyr-PAA with different MWs (table 1), as well as DXS of a low MW (Fluka Chemie Buchs, Switzerland) used as a control, were tested for the inhibition of classic pathway complement activity, determined by standard CH50 assay as previously described [17]. Hemolysis of antibody-sensitized sheep erythrocytes (Biomerieux, Marcy l'Etoile, France, coated with rabbit anti-sheep red cell stroma antiserum, Sigma) by human serum was determined after 60-min incubation at 37°C with or without the addition of sTyr-PAA.

Standard activated partial thromboplastin time (aPTT) tests, measured using Dade Actin FS reagent, were performed using increasing concentrations of sTyr-PAA diluted in veronal-buffered saline.

In vivo Model of Myocardial Infarction in Pigs

Care and use of animals in this study were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication N0 85-23, revised 1996) as well as the relevant Swiss laws and regulations and were approved by the locally appointed animal experimentation committee.

sTyr-PAA fraction 2 (most favorable in vitro data with respect to inhibition of the complement and coagulation systems) was further tested in a closed chest model of acute myocardial infarction in pigs. The animal experiments were performed as previously described [8, 18]. In brief, 24 large white pigs (29 ± 3 kg), premedicated with ketamine/xylazine, midazolam and atropine, were intubated and mechanically ventilated with a Draeger respirator (O_2/N_2O 1:3, isoflurane 1–1.5 vol%). After the introduction of central venous and arterial lines, a single bolus of unfractionated heparin (2,500 IU) was administered intravenously to prevent clotting after operative intravascular manipulation. Following baseline recordings over 30 min, animals were subjected to 1 h of myocardial ischemia within the area of the left anterior descending coronary artery (LAD; after first diagonal branch) using an over-the-wire percutaneous coronary intervention catheter (diameter 2.5–3 mm, Concerto, Occam, The Netherlands) followed by 2 h of reperfusion. Localization of the balloon and state of inflation was controlled angiographically on a regular basis.

Ten milliliters of fraction 2 of sTyr-PAA (25 mg/ml, equivalent to approx. 10 mg/kg, n = 11) or phosphate-buffered saline (PBS controls, n = 9) were injected intracoronary, through the tip of the catheter, into the ischemic area at risk (AAR) immediately prior to the 2-hour reperfusion phase. The area not at risk (ANR) was distinguished from the area at risk at the end of the experiment by negative demarcation with intravenous Evans Blue (60 ml, 2% wt/vol solution, Sigma) during reocclusion of the balloon, staining all but the AAR, thus essentially leaving a 'negative imprint' of the unstained ischemic area. Animals were sacrificed by intravenous potassium chloride (10 ml, 20 mmol) and the heart excised for further analysis. Viability within the ischemic AAR of the excised heart was determined by staining left myocardial ventricle tissue (3-mm slices) with triphenyl tetrazolium chloride (TTC, Sigma, pH 7.4, 1%) for 20 min at 37°C, as described previously [19]. Myocardial tissue, which stained dark red due to the formation of a formazan complex formed in the presence of active dehydrogenases and cofactors, was defined as viable ischemic tissue (VIT). Unstained tissue, which corresponded to irreversibly damaged myocardium, was defined as necrotic ischemic tissue (NIT). All three tissue sections (Evan's Blue positive area not at risk, ANR; NIT and VIT) were dissected and weighed. Left ventricular mass (LVM, in grams), AAR in grams, AAR as a % of LVM, NIT in grams and NIT as a % of AAR were recorded.

ECG and invasive arterial pressure were recorded with a Hewlett-Packard CMS patient monitor throughout the experiment. Ejection fraction (EF) was determined angiographically and calculated using the area-length-method according to Dodge [20]. Left ventricular enddiastolic pressure (LVEDP) was measured prior to ventriculography. In the case of ventricular fibrillation, a biphasic defibrillator (150 J) was used for defibrillation.

The investigators were blinded to the treatment regimen. All animals were randomized into the 2 groups using a randomization code with a random number generator (SAS, version 9.1.2, SAS Institute Inc., Cary, N.C., USA), prior to beginning the series. The 10-ml samples (PBS or sTyr-PAA fraction 2 in PBS) were prepared according to the randomization output by an independent laboratory technician and stored at –80°C until use. Prior to premedication of the animals, each vial was allocated to the corresponding pig (sequential vial No. = sequential pig No.). Of the 24 consecutively enlisted pigs, 1 died shortly after intubation due to unidentifiable respiratory problems and 3 died during the period of ischemia, prior to administration of PBS or sTyr-PAA following intractable ventricular fibrillation. The remaining 20 pigs were treated according to the above-described protocol. Sample size was determined in advance, estimated from previous work [8, 21] and experience, i.e. not by formal sample-size calculations.

Ischemic Markers in vivo

The levels of creatine kinase MB fraction (CKMB) were determined by enzyme immunoassays (AxSYM microparticle enzyme immunoassay platform, Abbott Laboratories, Abbott Park, Ill., USA). The ELISA was judged sensitive and specific for myocardial injury in pigs.

Measurement of Complement Markers: Plasma and Serum Samples

Blood samples from the central venous catheter access were collected (EDTA plasma/serum), kept on ice until centrifugation (1,750 *g* for 10 min at 4°C) and stored at –80°C until further analysis. Soluble C3a was measured by sandwich ELISA technique. In brief, microtiter plates were coated with a monoclonal antibody (mouse IgG2b) against porcine C3a/C3a(desArg). After washing, plasma samples were incubated at a 1:50 dilution for 2 h at room temperature. Biotinylated monoclonal anti-C3/C3a antibody followed by streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech, Bucks, UK) and 4-nitrophenyl phosphate substrate (Sigma) were used to detect bound C3a. All noncommercial antibodies were kindly provided by Prof. Otto Goetze, Georg-August University, Goettingen, Germany.

Table 1. Molecular weights of fractions 1–3 as estimated by HPLC on TSKgel G2000SW column

	Amount, mg (%)	M_n
Fraction 1	56 (2.6)	approx. 16,000
Fraction 2	612 (28.8)	approx. 4,000
Fraction 3	856 (40.2)	approx. 2,500

In order to quantify the effect of sTyr-PAA on systemic classical pathway complement activity, CH50 values were measured from serum by a standard CH50 assay with antibody-coated sheep erythrocytes, as described for the initial in vitro screening of sTyr-PAA (see previous section).

Histology and Immunostaining

Formaldehyde-fixed, paraffin-embedded myocardial tissue was cut into 3-μm sections. Neutrophil numbers were counted in 10 randomly selected high-power viewing fields (×40 objective, ×400 magnification, 0.57-mm field diameter) from tissue samples (4 samples per area per experiment) of each experiment. The intravascular/interstitial ratio was calculated for each section and experiment and averaged for all experiments in both groups. Five-micrometer sections were cut from all snap-frozen tissue samples, air-dried, acetone-fixed, hydrated and labeled following an indirect immunofluorescence technique. The following antibodies, cross-reactive for porcine antigens, were used: rabbit anti-human C1q, C3b/c and C4b/c (Dako) and mouse monoclonal anti-human C7 (Quidel, Santa Clara, Calif., USA). Secondary antibodies were goat anti-rabbit IgG(H+L)-FITC (Southern Biotechnology Associated, Birmingham, Ala., USA) and rabbit anti-mouse Ig-FITC (Dako). Immunohistochemical staining for TF with polyclonal rabbit anti-TF antibody was carried out as previously described [22, 23]. Binding of sTyr-PAA to the endothelium/myocardium was detected using sTyr-PAA-Fluo instead of the unlabeled sTyr-PAA in 2 further experiments.

Images of these stainings were analyzed using the ImageJ (version 1.440) software package from the National Institutes of Health (http://rsb.info.nih.gov/ij/). Briefly, all images were adjusted to fixed upper and lower threshold values in an appropriate color channel. Following threshold adjustment, positive areas were selected by the software as square pixels (μm^2). The total signal per section was calculated using the 'measure' function, and the sum of the values of pixels calculated using the raw integrated density. Each calculated average raw integrated density or area derived was from 4–6 independent experiments.

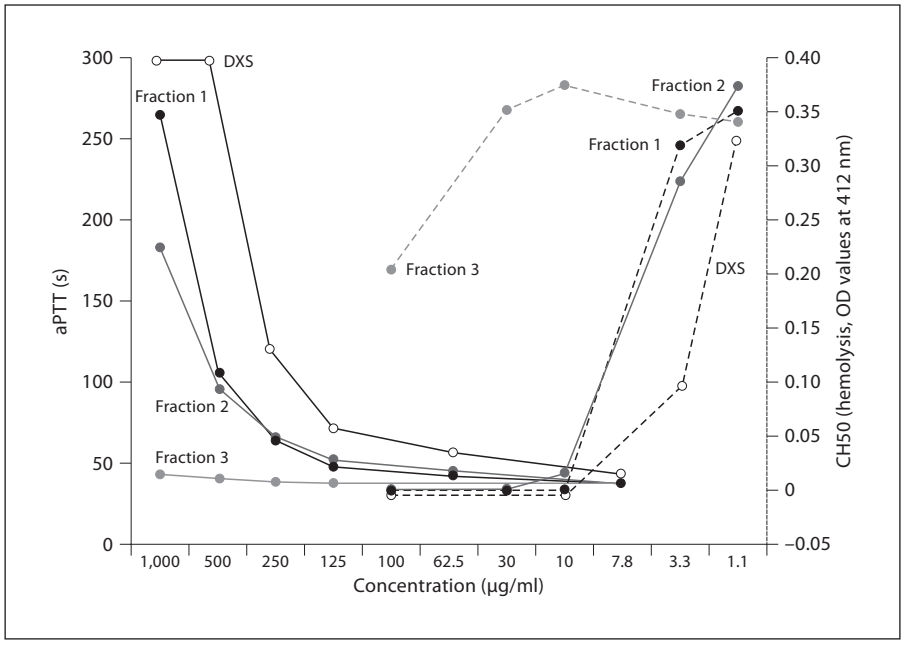


Fig. 1. In vitro inhibitory effects of various fractions of sTyr-PAA and DXS (MW 5,000 Da, a potent inhibitor of complement and coagulation, used a control for comparison) on coagulation (activated partial thromboplastin time in seconds, solid lines) and complement (CH50 and OD412 values, dashed lines).

Statistics

Nonlongitudinal data (TTC staining, neutrophil infiltration) were compared between groups by use of One-way analysis of variance (ANOVA). Comparison of longitudinal data of the reperfusion model between the groups (treatment vs. control) was performed by a mixed linear model (SAS proc mixed), which can be regarded as an extended form of ANOVA. This model assesses between group differences (independent of time), within-group differences (time effect) and the interaction of time effect and group effect (representing the effect of primary interest). It evaluates whether changes over time are different between the 2 groups, corresponding to nonparallel lines in the figures. Presented p values correspond to the test for interaction of group and time. A mixed linear model was preferred over a repeated-measure ANOVA because the latter does not allow heteroscedasticity of data or missing data. A maximum of 7.1% of data (for LVEDP) was missing due to technical measurement issues and was assumed to be missing at random. Statistical significance was defined as $p < 0.05$. SAS version 9.1 (SAS Institute) was used for analyses. Immunostaining data were assessed by means of a 2-tailed unpaired Student t test. Data, unless otherwise specified, are presented as average ± standard deviation in text and figures.

Results

In vitro Inhibition of Complement and Coagulation

sTyr-PAA fractions 1 and 2 (particularly >500 μg/ml) inhibited coagulation more potently than fraction 3, which essentially did not affect coagulation (fig. 1). Corresponding IC_{50} values for fractions 1 and 2 were 636 and 813 μg/ml, respectively. However, coagulation inhibition was still clearly less pronounced than by DXS, which was used as a control (IC_{50} value of 182 μg/ml). Fractions 1 and 2 also proved more potent inhibitors of the classic complement pathway compared to fraction 3 (IC_{50} values of 5.4 and 4.9 μg/ml, respectively, vs. 91.1 μg/ml for fraction 3). The IC_{50} value for complement inhibition for DXS was 2.1 μg/ml.

Myocardial Infarct Size – TTC Staining and CKMB

Evans Blue infusion was used to define the area at risk. In vivo application did not subsequently lead to leakage of Evans Blue dye upon tissue sectioning ex vivo. Equal areas at risk were observed in both experimental groups (mean ± standard deviation/median: 39 ± 8.0/35.5% of LVM for PBS controls, 35.4 ± 8.9/36.2% for sTyr-PAA treatment group, p = 0.239, fig. 2a). Administration of sTyr-PAA fraction 2, however, significantly reduced the total area of necrosis (NIT as a % of the area at risk, measured by TTC staining, 61.0 ± 12.0/66.5% in PBS controls, 39.4 ± 17.0/32.8% in the sTyr-PAA group, p = 0.001, fig. 2a). This result correlated with significantly reduced levels of plasma creatine kinase (CKMB, p = 0.048, fig. 2b).

Hemodynamic Variables

Mean heart rate did not differ significantly between the 2 groups at baseline, during LAD occlusion or reperfusion (p = 0.581, fig. 3a). Mean arterial pressure (MAP)

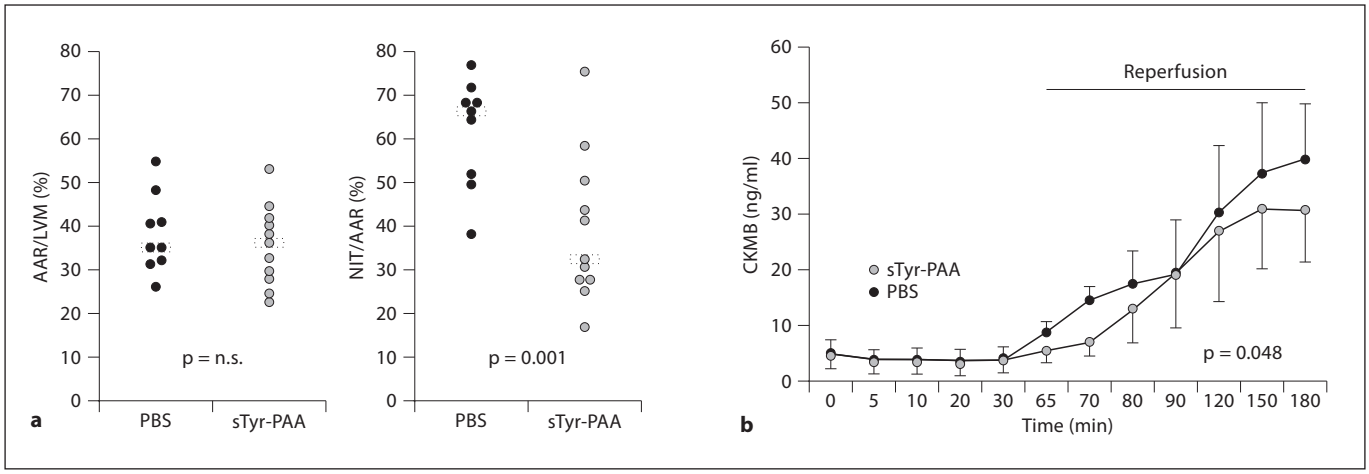


Fig. 2. In vivo experiments. **a** In the left panel, the ischemic AAR is represented as a % of the total LVM [p = 0.239, difference between groups was not significant (n.s.)]. The right panel shows NIT (reduction from 61.0 ± 12.0 to 39.4 ± 17.0%, p = 0.001 between groups). Individual data of each experiment are shown with median values indicated as bars. **b** Plasma levels of CKMB. Significant reduction of CKMB values in the sTyr-PAA-treated group, p = 0.048 compared to the PBS controls. Data are mean ± standard deviation.

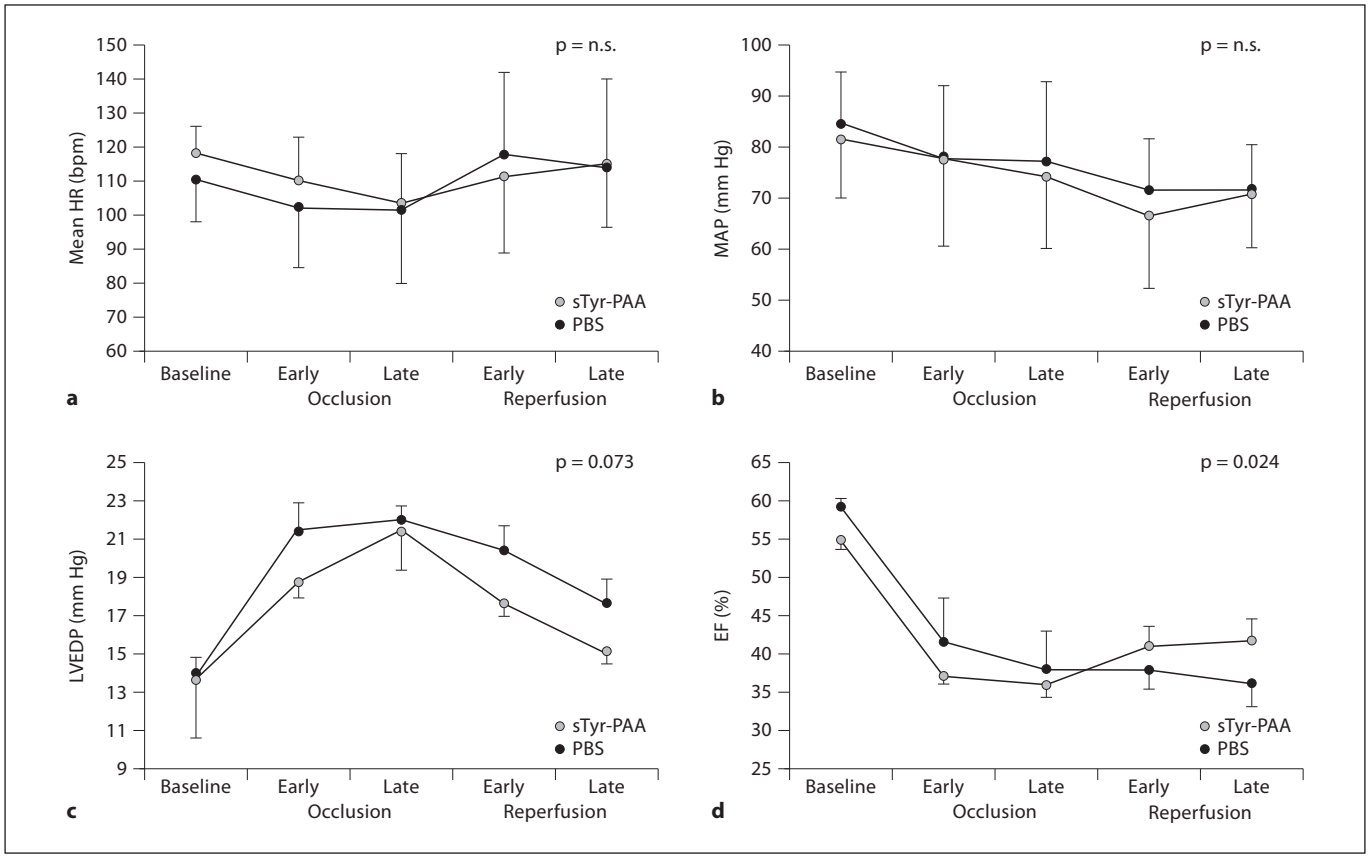


Fig. 3. Hemodynamic monitoring before occlusion (baseline) and during the early (1st half hour) and late (2nd half hour) ischemia and reperfusion phases in vivo. **a** Mean heart rate (HR). **b** MAP. **c** LVEDP. **d** EF. No significant changes in HR and MAP in the sTyr-PAA-treated versus the PBS control group (p = n.s.). Trend towards better LVEDP recovery with sTyr-PAA (p = 0.073). Significant improvement of EF following sTyr-PAA treatment (p = 0.024). Data are presented as mean ± standard deviation.

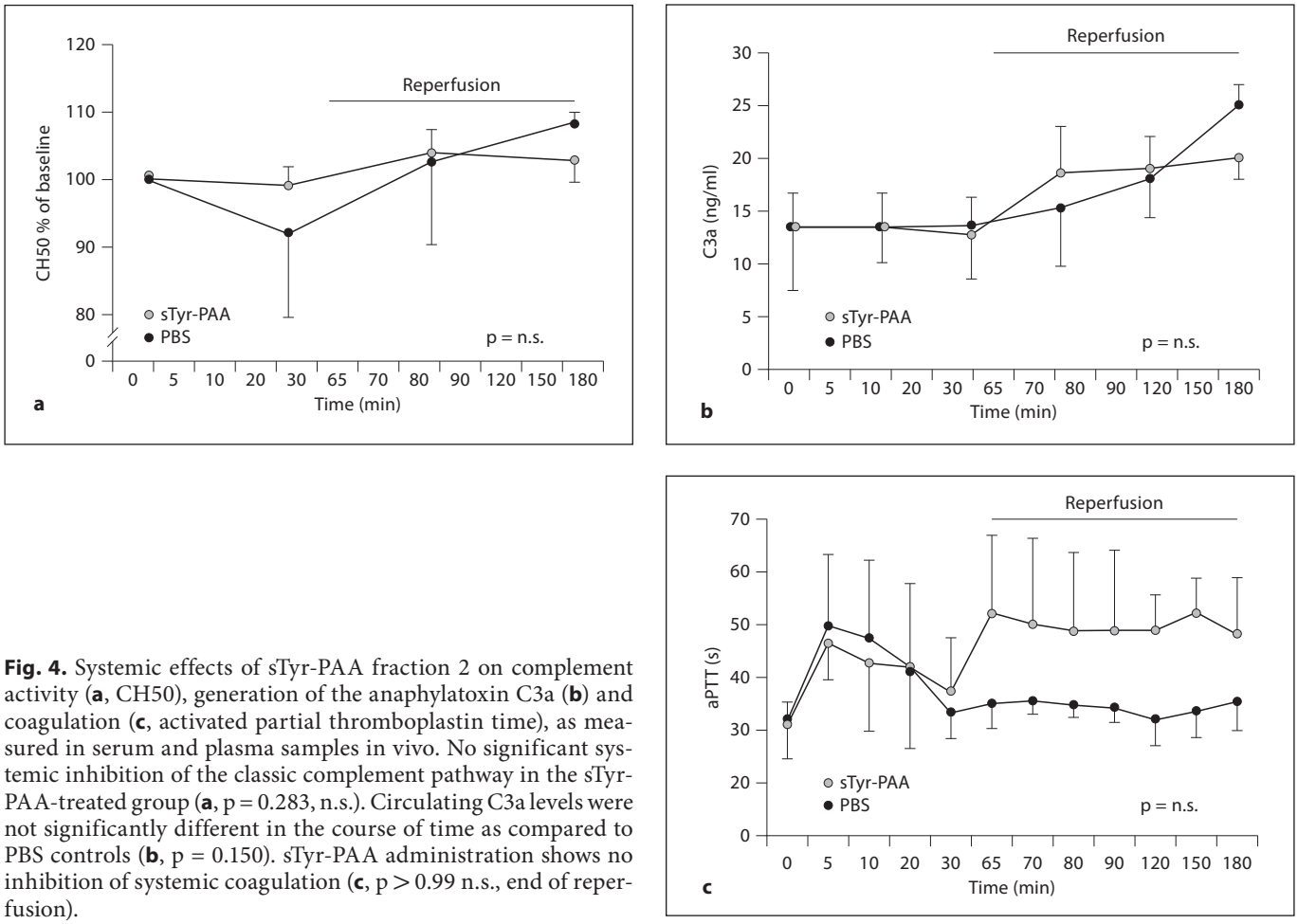


Fig. 4. Systemic effects of sTyr-PAA fraction 2 on complement activity (**a**, CH50), generation of the anaphylatoxin C3a (**b**) and coagulation (**c**, activated partial thromboplastin time), as measured in serum and plasma samples in vivo. No significant systemic inhibition of the classic complement pathway in the sTyr-PAA-treated group (**a**, p = 0.283, n.s.). Circulating C3a levels were not significantly different in the course of time as compared to PBS controls (**b**, p = 0.150). sTyr-PAA administration shows no inhibition of systemic coagulation (**c**, p > 0.99 n.s., end of reperfusion).

dropped during LAD occlusion from an average of 85 ± 12 mm Hg (PBS) and 82 ± 10 mm Hg (sTyr-PAA) to 76 ± 18 mm Hg and 74 ± 14 mm Hg, respectively, after 60-minute ischemia (fig. 3b). There was a slight trend towards stabilization/recovery of MAP at the end of the 2-hour reperfusion period, but differences between the groups were not significant at the end of reperfusion (p = 0.461). In contrast, LVEDP (fig. 3c) increased markedly in both groups during LAD occlusion, dropping upon reperfusion. Recovery in the sTyr-PAA group showed a trend towards improvement within the observed 2-hour reperfusion phase (p = 0.073). EFs were similar in the 2 experimental groups at baseline (58.1 ± 0.4% in PBS controls, 54.6 ± 0.2% in the sTyr-PAA group). sTyr-PAA administration lead to significantly improved EF at the end of the experiment compared to the PBS controls (p = 0.024, fig. 3d)

Arrhythmias

Of the 20 pigs included in the study, no fatalities occurred due to arrhythmias following the administration of either PBS or sTyr-PAA. In total, there were 12 episodes of ventricular fibrillation in each of both groups, all successfully treated with cardioconversion using a biphasic defibrillator (150 J). No significant differences in frequency of ventricular fibrillation was noted between the groups (1.3 episodes/pig for PBS vs. 1.09/pig for sTyr-PAA, n = 0.357).

Complement and Coagulation Variables

sTyr-PAA did not significantly affect activity of the systemic classic complement pathway (CH50 test, p = 0.584, PBS vs. sTyr-PAA group, fig. 4a). Systemic levels of C3a were not reduced following sTyr-PAA administration within the 2-hour reperfusion period (p = 0.150, fig. 4b). Surprisingly, for both the sTyr-PAA group as well

as the PBS controls, there was no significant increase in fluid-phase C5a levels in peripheral blood samples during the 2-hour reperfusion period and there were no significant differences at the measured time points between the 2 groups (results not shown).

Systemic coagulation was not significantly altered by the intracoronary infusion of sTyr-PAA (aPTT, $p > 0.99$, PBS vs. sTyr-PAA group, fig. 4c). The small increase in aPTT was observed in both groups in the first 20–30 min of LAD occlusion and is attributable to the initial intravenous administration of 2,500 IU to prevent clogging of central venous and arterial lines. However, despite its limited anticoagulant effect in the fluid phase, microthrombi formation within the ischemic area, as evaluated in tissue sections, was not observed more frequently in sTyr-PAA-treated animals compared with the PBS controls (not shown).

Histology and Tissue Neutrophil Granulocytes

Reperfused, vital myocardial tissue samples (VIT, TTC red areas) showed focal, minimal signs of ischemic damage, including the presence of wavy fibers. Characteristic signs of reperfusion damage including contraction bands and coagulation necrosis were observed in the infarcted area (NIT, TTC unstained areas) from both the sTyr-PAA PBS groups. No marked differences were observed between the groups. Gross hemorrhage was detected in one animal of the PBS group and 2 animals of the sTyr-PAA group. Foci of microscopic hemorrhage were equally detected (n = 2 in both groups) and ANR samples showed normal histological findings in both groups.

The absolute numbers as well as intravascular/interstitial ratios of neutrophils in tissue samples are shown in table 2. Evident neutrophil extravasation was particularly blocked in the VIT following sTyr-PAA administration, as shown by the reduction in absolute numbers as well as the ratio of intravascular to interstitial neutrophil granulocytes ($p = 0.008$ for absolute numbers, ratio: 1.02 ± 0.25, $p = 0.013$ vs. PBS). In the ANR and NIT, the absolute numbers of intravascular or interstitial neutrophils and the ratios between the groups were not significantly different ($p = 0.420$ ANR, $p = 0.464$ NIT for ratios).

Tissue Factor Staining

sTyr-PAA treatment markedly decreased TF expression in blood vessels within the NIT, as also confirmed by ImageJ software analysis (fig. 5, $p = 0.0310$). Upregulation of TF within the injured vasculature (mainly endothelial surface) in samples from PBS experiments was mainly associated with the infarct areas. Some weak focal TF staining was observed in certain vessels obtained from VIT and select vessels in the ANR (not shown, not significant). Minimal expression of TF was observed within the native cardiac vasculature of healthy pigs (not shown).

Table 2. Representation of absolute number and intravascular/interstitial ratio of tissue neutrophils in myocardial samples

Group	ANR	VIT	NIT
sTyr-PAA	7.4 ± 2.2 iv 8.1 ± 0.9 is (0.95 ± 0.29)	8.7 ± 1.3 iv 8.9 ± 1.3 is (1.02 ± 0.25)*	9.9 ± 2.4 iv 36.9 ± 2.3 is (0.27 ± 0.01)
PBS	6.2 ± 1.7 iv 6.4 ± 0.9 is (0.99 ± 0.24)	8.4 ± 1.8 iv 11.3 ± 1.3 is (0.74 ± 0.24)	10.1 ± 1.9 iv 38.5 ± 2.3 is (0.26 ± 0.03)

Four samples per area and experiment were examined and an average calculated from these. There were significantly more interstitial extravasated neutrophils in the VIT of PBS controls versus the sTyr-PAA group. * $p = 0.013$. There were no significant differences in the ANR and NIT ($p = 0.420$ and $p = 0.464$). Results are mean ± standard deviation (ratio in parentheses).

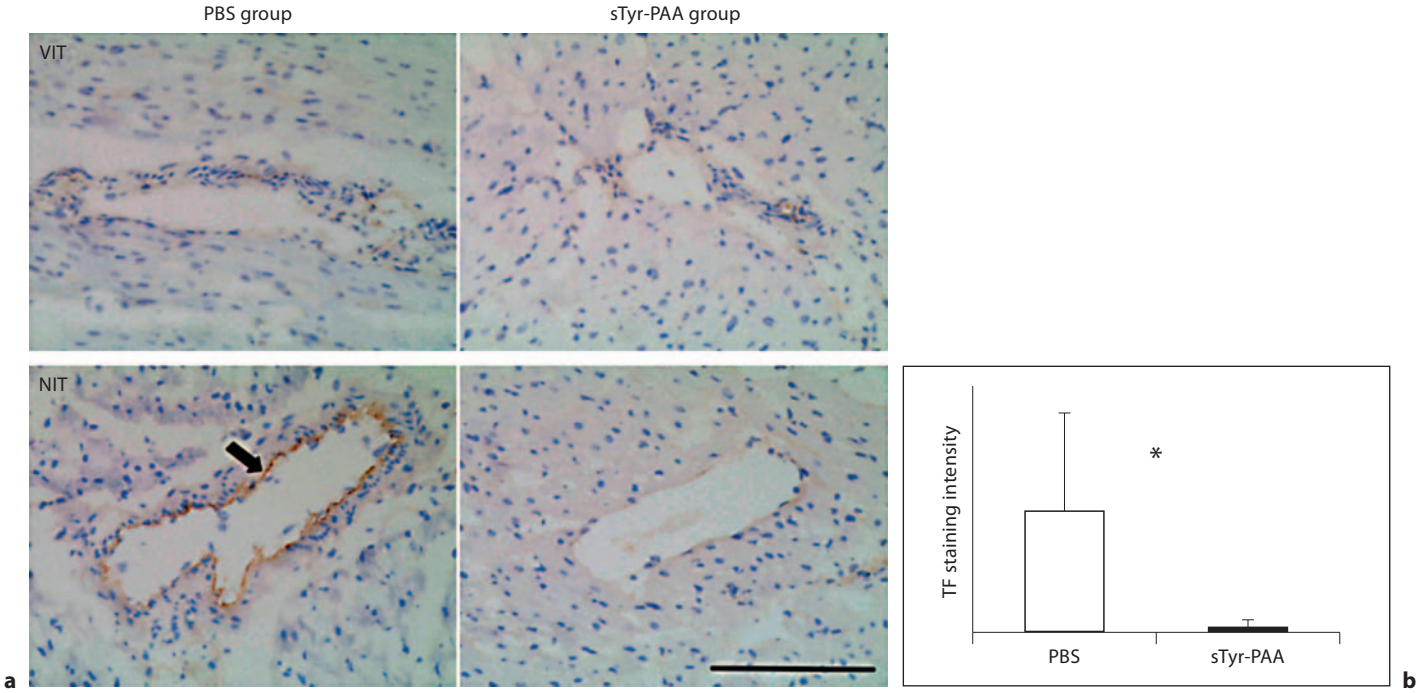
is = Interstitial extravasated neutrophils; iv = intravascular neutrophils.

Complement Staining

In the in vivo model, the complement-inhibitory effect of sTyr-PAA fraction 2 translated to a significantly reduced complement deposition within the NIT compared to the PBS controls (immunofluorescence staining and results of analysis with ImageJ software) for C1q ($p = 0.0472$), C3b/c ($p = 0.0395$), C4b/c ($p = 0.0413$) and C6 ($p = 0.1655$) (terminal complement complex, fig. 6a). Comparatively little complement deposition was observed within the VIT in both experiments, without any significant differences being observed (not shown).

Binding of sTyr-PAA

sTyr-PAA-Fluo was used to characterize binding of sTyr-PAA to myocardial tissue samples. Binding of sTyr-PAA-Fluo was mainly localized to the inner lining of blood vessels within the myocardium as well as the outer layers of larger vessels, with occasional, focal binding to groups of cardiomyocytes (fig. 6b) with no binding observed in distant organs (not shown).



a b

Fig. 5. Immunohistochemical staining for (endothelial) TF on myocardial tissue sections in controls versus the sTyr-PAA-treated group ($p = 0.0310$). **a** Minimal staining for TF within the VIT (top panels). Increased staining (brown, indicated by the arrow) within the NIT in the PBS (lower left) but not the sTyr-PAA group (lower right). Scale bar represents 50 μm. **b** Staining of NIT was analyzed by ImageJ software. Results are shown in a simple graph where the y-axis represents staining intensity. Data are mean ± standard deviation. * $p < 0.05$.

Discussion

The results of this work reinforce the previously observed beneficial effects of sTyr-PAA [7] in vitro and show that it also protects from reperfusion injury in vivo. The initial in vitro evaluation enabled choosing a fraction with minimal systemic anticoagulatory effects whilst retaining optimal complement inhibitory properties. Thus, the primary end point of reduced tissue damage (reduced NIT, reduced CKMB release) could be achieved with a single bolus administration of sTyr-PAA.

The cytoprotective effect of sTyr-PAA correlates with binding of sTyr-PAA to the site of injury, visualized using sTyr-PAA-Fluo. Indeed, this localized binding of sTyr-PAA – in part restoring an anti-inflammatory, complement-inhibitory local environment – was in itself paralleled by a clearcut reduction in local tissue-bound myocardial complement deposition without systemically affecting circulating complement levels or activity, as revealed by C3a and C5a ELISA (results not shown) as well as by CH50 assay. These results point towards a role for tissue-focused complement inhibition and underline the importance of complement-mediated damage in this I/R model, shown extensively in previous studies [24, 25]. In our own previous study, cell surface- and locally-targeted complement inhibition with Mirococept, a membrane-targeted complement inhibitor derived from human CR1, has already been shown to effectively reduce myocardial reperfusion injury in vivo [18].

Of particular clinical relevance in this model is modulation of the immediate postreperfusion phase – a time vital for tissue salvage, as has been shown in other postreperfusion strategies, such as postconditioning [26] or intracoronary acid infusion as an alternative [27].

The activated endothelial state induced during I/R injury may directly contribute to postreperfusion complications, including plugging of the capillaries by polymorph nuclear cells as well as microvascular thrombosis, inevitable sequelae of the procoagulant endothelial surface after reperfusion. Therefore, systemic inhibition of the coagulation system routinely accompanies reperfusion of coronary arteries [28]. In animal models, various anticoagulants have provided tissue salvage [5, 29], partly through reducing inflammation. In our model, an initial

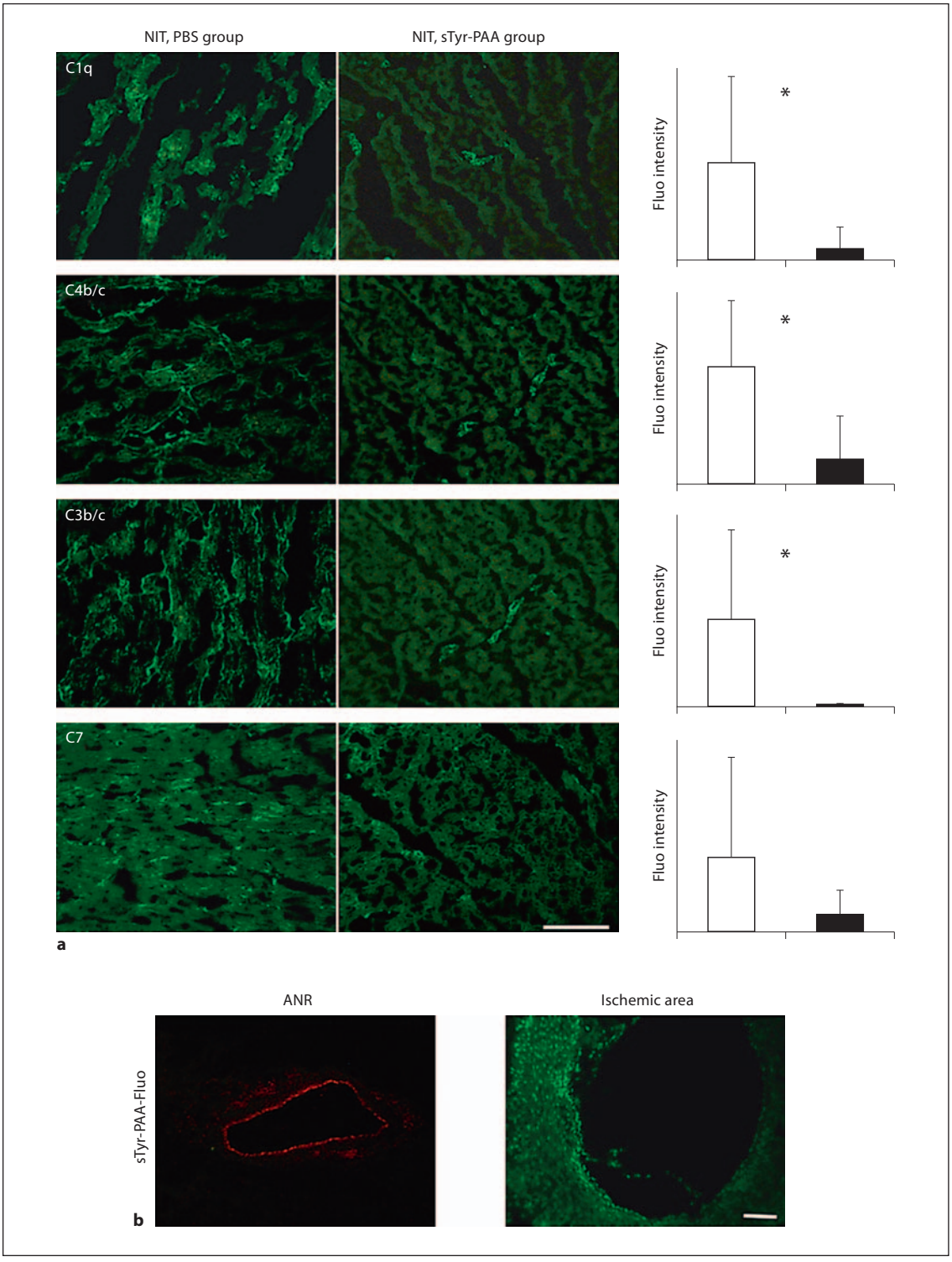


Fig. 6. a Immunofluorescence staining for complement deposition (C1q, C4b/c, C3b/c and C6) in the NIT. Graphs: results of staining as analyzed by ImageJ software. White bar = PBS, black bar = sTyr-PAA, y-axis represents fluorescence (Fluo) intensity. Data are mean ± standard deviation. * $p < 0.05$. sTyr-PAA reduces complement binding within the NIT (C1q p = 0.0472; C4b/c p = 0.0413; C3b/c p = 0.0395; C5b-9 p = 0.1655). **b** sTyr-PAA fraction 2 binding to myocardial tissue in vivo using fluorescein-labeled sTyr-PAA (sTyr-PAA-Fluo). Note sTyr-PAA binding to blood vessels within the myocardium and a certain background red fluorescence caused by Evans Blue dye bound to vessels in the ANR. Scale bars = 50 μm.

intravenous bolus of 2,500 IU heparin was administered in all animals – a minimal dose to prevent possible clotting events following early surgical manipulation. Consequently, however, the minimal (and not significant) systemic effect of sTyr-PAA did not appear to confer disadvantages in this acute in vivo model. These data fit in with results from other studies, where the use of non-anticoagulant heparin and sulodexide, a mixture of glycosaminoglycans with limited anticoagulant activity, proved effective in minimizing tissue injury [30, 31]. It is possible that direct endothelial association and localized complement inhibitory effect, rather than systemic anticoagulation, provided protection [32]. Although no prominent systemic effect on coagulation was observed with sTyr-PAA, the local procoagulant intravascular environment during ischemia was importantly controlled, with a reduced expression of TF. As has been highlighted in a recent review by Mitroulis et al. [33], the beneficial effect of inhibiting TF, for instance in I/R injury, may derive from blocking its multivalent role in initiating procoagulatory and proinflammatory events. Indeed, one mechanism of action of sTyr-PAA may be through the inhibition of TF upregulation and ensuing neutrophil-mediated injury; increased TF activity has been shown to lead to such injury though thrombin-dependent signaling [34].

This current in vivo data on sTyr-PAA fits in with earlier work, which supports the observation that sTyr-PAA modifies neutrophil extravasation. Polymers bearing densely situated tyrosine-sulfate residues constitute potent P-selectin inhibitors in vitro, and block neutrophil extravasation in vivo in a model of peritonitis [6]. We also previously demonstrated that sTyr-PAA is a potent P-selectin blocker [7]. The interaction between polymorph nuclear cells and the endothelium critically contributes to tissue injury [35]. In this model, it still has to be investigated in more detail whether alterations in P-selectin-mediated neutrophil extravasation are of importance or whether the reduced neutrophil numbers are attributable to indirect effects via local complement inhibition, site-specific reduction of activated complement proteins (such as C5a) within the ischemic tissue and the subsequent reduction of neutrophil numbers. The sTyr-PAA-induced reduction in extravasated neutrophils was primarily observed within the VIT myocardial area and not in the NIT. However, neutrophil numbers in the tissue in general were not very high, which may be attributable to the early postinfarct period in which the tissue samples were analyzed. sTyr-PAA also decreased thrombotic events within small intramyocardial blood vessels. Although this reduction points towards ameliorated tissue perfusion and subsequently a potential for influencing the so-called no-reflow phenomenon [36], further experiments are needed to investigate if adequately restored epicardial blood flow equated with improved microvascular perfusion. Overall, results in this model point towards the fact that the binding of sTyr-PAA to damaged endothelium and the local modulation of the proinflammatory and procoagulant environment were possibly more critical than systemic anticoagulation. However, in the clinical setting with frequent implantation of coronary stents, additional systemic anticoagulation is clearly unavoidable and is part of the current standard of care in the longer postinfarction course; whether its application could be reduced in favor of local regulatory strategies is still to be investigated.

sTyr-PAA did not have a negative impact upon basic hemodynamic variables following its administration. Within the short observed period of reperfusion, it improved EF significantly and showed a trend towards a reduction of LVEDP after ischemia. Whilst this improvement within such a limited phase of reperfusion is very encouraging, it is unclear whether it is of prognostic value, as clinical studies indicate that early functional analysis following Q-wave myocardial infarction has only a very limited competence to predict the recovery of cardiac function. Owing to the limited 2 h of reperfusion, further follow-up studies are needed to investigate whether sTyr-PAA administration favorably improves performance in the period no longer affected by myocardial stunning. Furthermore, in subsequent studies sTyr-PAA may be used intravenously in addition to local application in an effort to further improve functional outcome.

Limitations of the Model

The precise extent of vascular plugging and no reflow within the myocardial tissue was not evaluated in detail. However, our main aim was to establish whether sTyr-PAA would be suitable as a candidate substance for local administration to reduce myocardial I/R injury with only limited systemic effects on coagulation. The key message lies in the clear reduction of infarct size in the sTyr-PAA-treated group. Although TTC staining to discriminate between VIT and NIT may require a certain period of reperfusion [37], it does allow for sensitive and specific determination of infarct size [38]. We and other authors have previously shown the validity of TTC staining in this and other models [18, 39, 40]. Isoflurane is known to have a protective effect in terms of preconditioning [25]. However, the animals of both groups were treated according to the same anesthetic regime. Any precondi-

tioning, subsequent reduction of reperfusion injury and of associated complement activation would therefore be expected to occur equally in both groups.

The results of complement inhibition in clinical trials are currently equivocal. In the COMMA trial, pexelizumab, a monoclonal anti-C5 antibody, did not measurably influence infarct size, but significantly reduced 90-day mortality [41]; in the COMPLY trial, it blocked complement activity but neither reduced infarct size nor adverse clinical outcomes [42]. The reasons for the partly disappointing results in clinical trials are diverse. Reperfusion injury is complex and complement activation only represents one mechanism of tissue damage. To some extent, thrombolytic agents, used for the noninterventional treatment of myocardial infarction, activate complement themselves. Indeed, recently published important reviews discuss the problems of the translation of bench to bedside in I/R research in general, including in some cases the inadequacy of the models used and the difficulties in setting up and implementing an appropriate study design [43]. It therefore remains to be confirmed how the results obtained in animal models can be translated into successful use of the reagents in the complex clinical setting.

In summary, the use of sTyr-PAA fraction 2 in the current dose of up to 10mg/kg is safe and effective in vivo to reduce myocardial I/R injury. Clearly, further studies with an increased observational period after ischemia will be needed to confirm the promising short-term effects. Whilst previous studies have focused on a more systemic approach of complement inhibition to reduce reperfusion injury, these current data suggest that carefully controlled local complement inhibition – the proposed mode of action of sTyr-PAA – may be just as important and effective in damage control in post-reperfusion treatment.

The future use of sTyr-PAA may be envisaged in situations which necessitate local modification of a proinflammatory and procoagulant vascular environment, without systemically influencing the coagulation or complement system. Indeed, this property may prove advantageous for envisaged application in situations where bleeding complications are likely or are to be avoided at all costs, such as in cerebral I/R injury.

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Conflict of Interest

There was none declared.

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