Catalytic peroxynitrite decomposition improves reperfusion injury after heart transplantation

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Objective: Peroxynitrite, a reactive nitrogen species, has been implicated in the development of ischemia–reperfusion injury. The present study investigated the effects of the potent peroxynitrite decomposition catalyst FP15 on myocardial and endothelial function after hypothermic ischemia–reperfusion in a heterotopic rat heart transplantation model.

Methods: After a 1-hour ischemic preservation and implantation of donor hearts, reperfusion was started after application of vehicle (5% glucose solution) or FP15 (0.3 mg/kg). The assessment of left ventricular pressure–volume relations, total coronary blood flow, endothelial function, immunohistochemical markers of nitro-oxidative stress, and myocardial high-energy phosphates was performed at 1 and 24 hours of reperfusion.

Results: After 1 hour of reperfusion, myocardial contractility (maximal slope of systolic pressure increment at 140 μ L left ventricular volume: 5435 ± 508 mm Hg/s vs 2346 ± 263 mm Hg/s), coronary blood flow (3.98 ± 0.33 mL/min/g vs 2.74 ± 0.29 mL/min/g), and endothelial function were significantly improved, nitro-oxidative stress was reduced, and myocardial high-energy phosphate content was preserved in the FP15-treated animals compared with controls.

Conclusions: Pharmacologic peroxynitrite decomposition reduces reperfusion injury after heart transplantation as the result of reduction of nitro-oxidative stress and prevention of energy depletion and exerts a beneficial effect against reperfusion-induced graft cardiac and coronary endothelial dysfunction. (*J Thorac Cardiovasc Surg* 2012;143:1443-9)

Ischemia–reperfusion injury is a common problem in cardiac surgery. Myocardial performance and long-term outcome are determined by the level of ischemia–reperfusion injury, especially after heart transplantation with an extended time of ischemia. Most studies on the effects of myocardial ischemia–reperfusion focus on myocardial injury and the recovery of contractile function. Recent studies show the importance of protecting the microvasculature to attenuate reperfusion injury. Therefore, novel therapeutic strategies concentrate on management modalities that prevent both myocardial and endothelial injury during reperfusion.^{1,2}

The double-faced role of endothelial nitric oxide (NO) in cardiovascular (patho)physiology has been increasingly

recognized. Triggering the synthesis of cyclic guanosine monophosphate, NO exerts beneficial effects in the cardiovascular system, such as vasorelaxation, inhibition of platelet aggregation, or protection/preconditioning of the myocardium.^{3,4} On the other hand, emerging evidence supports the role of harmful NO in pathophysiologic conditions associated with increased nitro-oxidative stress. Ischemia–reperfusion injury initiates a pathophysiologic cascade, including an inflammatory response with liberation of reactive oxygen and nitrogen species.^{1,5} An extremely toxic nitrogen species, peroxynitrite is formed by the reaction of NO and superoxide anion radical. Potential biological actions of peroxynitrite include cardiac depression, lipid peroxidation, nitration of tyrosine residues on proteins, inducing DNA strand breaks, and subsequent activation of the poly(ADP-ribose) polymerase (PARP) enzyme. PARP initiates an energy-consuming metabolic cycle by transferring adenosine diphosphate (ADP)-ribose units from NAD⁺ to nuclear proteins. This process results in rapid depletion of intracellular adenosine triphosphate (ATP) pools, eventually leading to cellular dysfunction and death.^{6,7}

Increased nitrotyrosine (NT) staining (as a footprint of peroxynitrite) has been reported in experimental cardiac rejection in rats⁸ and humans,⁹ suggesting that reactive nitrogen species (NO or peroxynitrite) may contribute to graft failure.¹⁰ Recent studies have shown that neutralizing NO¹¹ or peroxynitrite¹² directly limits the extent of protein

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Abbreviations and Acronyms

ADP	= adenosine diphosphate
ATP	= adenosine triphosphate
CBF	= coronary blood flow
dP/dt _{max}	= maximal slope of systolic pressure increment
LV	= left ventricular
LVEDP	= left ventricular end-diastolic pressure
LVSP	= left ventricular systolic pressure
NO	= nitric oxide
NT	= nitrotyrosine
PAR	= poly(ADP-ribose)
PARP	= poly(ADP-ribose) polymerase
PTFE	= polytetrafluoroethylene

nitration, prevents PARP activation in rat cardiac transplants, and prolongs graft survival. However, to date there are few data on the effects of pharmacologic peroxynitrite decomposition on myocardial and coronary endothelial function after heart transplantation. Peroxynitrite reacts rapidly and efficiently with synthetic metalloporphyrins. One of them, FP15, an N-PEGylated-2-pyridyl iron porphyrin, has shown a superior performance as a peroxynitrite decomposition catalyst.⁶ The current study investigated the effect of FP15 on ischemia-reperfusion injury in our well-established rat model of heterotopic heart transplantation.¹³

MATERIALS AND METHODS**Rat Model of Heterotopic Heart Transplantation**

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (Publication No. 86-23, revised 1996). The experiments were approved by the Ethical Committee of the Land Baden-Württemberg for Animal Experimentation.

According to the model described previously,¹³ donor rats (young adult male Lewis rats, 300-350 g) were anesthetized with a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (3 mg/kg). After the abdominal cavity was opened, 400 IU/kg sodium heparin was injected into the inferior caval vein. The abdominal aorta was exposed, and a 1-cm segment of the infrarenal aorta was occluded by small vessel forceps. A single intravenous cannula with a polytetrafluoroethylene (PTFE) catheter (Vygon GmbH & Co, Aachen, Germany) was advanced into the aorta straight to the aortic arch by shortly opening the upper vessel forceps. The cannula was removed from the PTFE catheter. Bilateral thoracotomy was performed, and the heart was exposed. Afterward, cardiac arrest was induced by injection of 40 mL of cold (4°C) histidine-tryptophan-ketoglutarate solution (Custodiol, Dr Franz Köhler Chemie GmbH, Alsbach-Hähnlein, Germany) at a rate of 20 mL/min using the PTFE catheter. To reduce the load, the abdominal inferior caval vein was cut. After successful induction of cardiac arrest, the superior and inferior caval veins and pulmonary veins were tied en masse with a 4-0 single silk suture. The aorta and pulmonary artery were divided, and the heart was immediately placed into cold histidine-tryptophan-ketoglutarate solution (4°C). The recipient rats (same strain, age, and weight as donors) were

anesthetized with a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (3 mg/kg) and heparinized with 400 IU/kg. The abdomen was opened by a midline incision, and the aorta and the inferior caval vein were exposed by reflecting the intestines to the left side. Two-centimeter segments of the infrarenal aorta and the inferior caval vein were isolated and occluded by small vessel forceps. The aorta and pulmonary artery of the donor heart were anastomosed end-to-side to the abdominal aorta and the inferior caval vein of the recipient rat, respectively. This was achieved using a 9-0 monofilament polyamide sutures operating under a 16-power magnification microscope. To minimize variability between experiments, the duration of the ischemic period was standardized at 1 hour. After completion of the anastomoses, the vessels were released and the heart was then reperfused with blood in situ for 1 or 24 hours.

Nonischemic Heterotopic Heart Transplantation

The technique of ischemic heterotopic heart transplantation was slightly modified to implant the donor heart without a significant ischemic time. To ensure a quick implantation of the donor heart, the recipients were specially prepared before explantation. The animals were heparinized with 400 IU/kg intravenously. The abdomen was opened by a midline incision, and the aorta and vena cava were exposed. One-centimeter segments of the infrarenal aorta and vena cava were isolated and occluded by small vessel forceps. Thin polyethylene tubes were inserted via a small incision into the abdominal aorta and inferior caval vein, respectively. The connections were tightened by local application of tissue glue. Afterward, the donor hearts were explanted as described above. The donor aorta and pulmonary artery were attached immediately to the corresponding aortic and caval polyethylene tubes and fixed with 5-0 single silk surgical thread. Then the recipient vessels were released, and the heart was perfused with blood via the polyethylene tubes for a 1-hour period.

Functional Measurements in the Graft

After 1 or 24 hours of reperfusion, a latex balloon was introduced into the left ventricle of the graft via the apex and connected to a precision calibrated syringe for administration or withdrawal of fluid to determine left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) and maximal slope of systolic pressure increment (dP/dt_{max}) at different left ventricular (LV) volumes by a Millar micromanometer (Millar Instruments, Inc, Houston, Tex). From these data, LV pressure-volume relationships were constructed.¹³ Total coronary blood flow (CBF) was measured by a perivascular ultrasonic flow probe on the donor aorta. After baseline measurement, the endothelium-dependent vasodilator acetylcholine (1 nM, 0.2 mL) and the endothelium-independent vasodilator sodium nitroprusside (10 nM, 0.2 mL) were administered directly into the coronary arteries of the graft via the donor aorta. Between the infusions, CBF was allowed to return to baseline levels. Vasodilator response was expressed as maximum percent change of CBF from baseline.¹³

Immunohistochemical Analysis

LV myocardial sections of the grafts were excised for histologic processing immediately after completing functional measurements. The tissue samples were fixed in buffered paraformaldehyde solution (4%) for 1 day and embedded in paraffin. Adjacent sections were processed for both of the following types of immunohistochemical labeling. According to the methods previously described,¹⁴ we performed immunohistochemical staining for NT (marker of nitrosative stress) and poly(ADP-ribose) (PAR, the enzymatic product of PARP). Primary antibodies used for the stainings were polyclonal sheep anti-NT antibody (Upstate, Chicago, Ill) and mouse monoclonal anti-PAR antibody (Calbiochem, San Diego, Calif). Quantitative histomorphologic assessment of NT staining was performed by the COLIM software package (Pictron Ltd, Budapest, Hungary) according to the intensity of labeling. The results were expressed with a grading system of 0 (no staining) to 4 (extensive staining) based on the measured intensity of positive labeling as described in detail previously.¹³

Determination of High-Energy Phosphates

Transplanted hearts from a separate series of experiments (after 1- or 24-hour reperfusion) were excised and immediately immersed in fluid nitrogen (-196°C) and stored at -80°C . Myocardial tissue was homogenized in 3.5% HClO_4 and centrifuged at 20,000 U/min. The supernatant was neutralized with triethanolamine- $\text{HCl}/\text{K}_2\text{CO}_3$ solution. ATP degradation was assessed with standard photometry using enzyme kinetic assay containing glyceraldehyde-3-phosphate-dehydrogenase, 3-phosphoglycerate-kinase, glycerin-3-phosphate-dehydrogenase, and triosephosphate-isomerase.¹³ ATP, ADP, and adenosine monophosphate contents were expressed as micromoles/gram dry weight.

Groups and Experimental Protocol

Four transplant groups were studied ($n = 6$ /each group). Immediately before releasing the aortic clamp, the slow intravenous injection of 5% glucose solution vehicle (control group) or FP15 (0.3 mg/kg body weight), a dose comparable to that used in previous studies,^{5,15} was started and continued during the first 5 minutes of the reperfusion period. In groups A (control 1 hour) and B (FP15 1 hour), the measurements of systolic and diastolic function and CBF were performed after 1 hour of reperfusion. In groups C (control 24 hours) and D (FP15 24 hours), the abdominal cavity was closed and the animals were allowed to recover from the anesthesia. During the following 24 hours, the animals of both groups received the same standard diet and normal drinking water. After 24 hours, the animals were reanesthetized and the abdominal cavity was reopened. The grafts were instrumented, and the measurements were performed as in groups A and B.

In a separate series of experiments, 4 groups ($n = 6$ /each group) of hearts were transplanted and treated with FP15 or saline vehicle similarly to the above-mentioned protocol. After 1 or 24 hours of reperfusion, the grafts were excised to determine high-energy phosphate contents.

The nature of the model and the protocol above allowed the characterization of temporal changes in heart function during reperfusion; however, they did not allow an absolute comparison with preischemic values. To address this issue, a modified nonischemic sham-operated transplant group was investigated (group NI, $n = 6$) (see above).

Materials

All materials used were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless specified otherwise. FP15 (FeCl tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin) was dissolved in 5% glucose solution vehicle.

Statistical Analysis

All values were expressed as mean $\pm$ standard error of the mean. Individual means between the groups were compared by 1-way analysis of variance followed by an unpaired t test with a Bonferroni correction for multiple comparisons and the post hoc Scheffe's test.

RESULTS

Early Reperfusion, 1-Hour, and "Nonischemic" Transplants

Systolic functional recovery was significantly better in the FP15 group compared with control. LVSP and $\text{dP}/\text{dt}_{\text{max}}$ were significantly ($P < .05$) higher in the FP15 group. Systolic cardiac function curves showed a significant leftward shift in the FP15 group compared with the vehicle-treated group (Figure 1). The values of the "nonischemic" group were significantly higher compared with the vehicle-treated transplant group; however, there were no differences compared with the FP15-treated transplant group. LVEDP did not differ between the groups. The diastolic compliance curves (end-diastolic pressure–volume relationships) were similar in all groups (Figure 1). CBF was significantly higher ($P < .05$) in the FP15 group compared with control after 1 hour (Figure 2, A). Endothelium-independent vasodilatation after sodium nitroprusside (Figure 2, C) was similar in both groups. In contrast, endothelium-dependent vasodilatation after acetylcholine was significantly

FIGURE 1. LVSP–LVV (A), $\text{dP}/\text{dt}_{\text{max}}$ –LVV (B), and LVEDP–LVV (C) relationships after heart transplantation and 1 and 24 hours of reperfusion in all groups. All values are given as mean $\pm$ standard error of the mean (SEM). * $P < .05$ control at 1 hour versus every other group. LVSP, Left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVV, left ventricular volume.

FIGURE 2. CBF (A) and vasodilator responses after application of the endothelium-dependent vasodilator acetylcholine (1 nM 0.2 mL, B) and the endothelium-independent vasodilator sodium nitroprusside (10 nM 0.2 mL, C) after heart transplantation and 1 and 24 hours of reperfusion in all groups. All values are given as mean $\pm$ SEM. * $P < .05$ versus control at a given time point. † $P < .05$ versus 1 hour. ‡ $P < .05$ nonischemic versus FP15. ACh, Acetylcholine; CBF, coronary blood flow; SNP, sodium nitroprusside.

($P < .05$) better in the FP15 group than in the vehicle-treated transplant group (Figure 2, B). Myocardial high-energy phosphate content was preserved by FP15 treatment during heart transplantation (Figure 3), especially ATP content.

Immunohistochemical staining showed increased immunoreactivity for NT (diffuse brown staining) and PAR (dark brown staining in cell nuclei), indicative of nitrosative stress and enhanced activation of PARP, in the wall of

intramyocardial blood vessels (especially in the intima layer) and in the LV myocardium of transplanted hearts of the control group (Figure 4). FP15 treatment notably decreased NT immunoreactivity (NT intensity score: 3.1 ± 0.4 control vs 1.2 ± 0.3 FP15, $P < .01$) in the myocardium. No PAR immunoreactivity could be detected in the FP15 treatment group, indicating that FP15 could completely prevent PARP activation and PAR formation in the transplanted myocardium. As expected, no immunoreactivity for NT or PAR could be observed in the “nonischemic” group. Figure 4 shows representative stainings for NT and PAR in the control and FP15 treatment groups after 1 hour of reperfusion.

FIGURE 3. Myocardial total adenylate pool (ATP, ADP, adenosine monophosphate) after heart transplantation and 1 and 24 hours of reperfusion in the control and FP15 treatment groups. All values are given as mean $\pm$ SEM. * $P < .05$ versus control at a given time point. AMP, Adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Late Reperfusion: 24 Hours

After 24 hours of reperfusion, there were no differences in LVSP (Figure 1, A), dP/dt_{max} (Figure 1, B), and LVEDP (Figure 1, C) between the vehicle and the FP15-treated transplant groups. In the vehicle-treated transplant group, all these parameters showed a significant improvement compared with the values after 1 hour of reperfusion ($P < .05$). In the FP15-treated transplant group, there were no significant differences compared with the values of 1 hour of reperfusion. Systolic cardiac function curves (Figure 1, A and B) and diastolic compliance curves (Figure 1, C) of the control and FP15 groups were nearly identical. After 24 hours of reperfusion, CBF was also similar in both groups. After 24 hours, endothelium-dependent

FIGURE 4. Representative immunohistochemical stainings for NT (A, B; brown staining) and (ADP ribose) (C, D; dark brown staining mainly in cell nuclei) in the myocardium of transplanted hearts after 1 hour of reperfusion in the control (A, C) and FP15 treatment groups (B, D). Magnification: 400×, scale bar: 50 μm.

vasodilatation was significantly increased ($P < .05$) in both groups compared with the 1 hour-reperfusion values. Endothelium-dependent vasodilatation after acetylcholine was significantly higher in the FP15 group compared with the vehicle-treated transplant group (Figure 2, B). After 24 hours, total adenylate pool and ATP content showed no significant differences between the groups (Figure 3).

DISCUSSION

In this study, the benefits of the application of the peroxynitrite decomposition catalyst FP15 during reperfusion were assessed after reversible hypothermic ischemia in a heterotopic rat heart transplantation model. Heterotopic heart transplantation was used to simulate the clinical conditions in terms of whole blood reperfusion and to allow an observation time of 24 hours, which is strongly limited in isolated organ models. Furthermore, the heterotopic situation also allows the assessment of myocardial function independently of the actual loading conditions. This is the first study to show that pharmacologic peroxynitrite decomposition improves myocardial and endothelial functional recovery during early reperfusion and attenuates nitro-oxidative stress and energy depletion in a clinically relevant heart transplant model.

During reperfusion after global myocardial ischemia, high levels of reactive oxidants and free radicals are produced and are central mediators of reperfusion injury. A potent oxidant species, peroxynitrite (ONOO^-), is formed by the reaction of superoxide anion radical (O_2^-) and the vascular mediator NO and has been established as a pathophysiologic relevant endogenous trigger of DNA single-strand breakage. Peroxynitrite-induced DNA-damage leads to the activation of nuclear enzyme PARP, which initiates an

energy-consuming metabolic cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins. This process results in rapid depletion of intracellular ATP pools, eventually leading to cellular dysfunction and death.⁷ Pharmacologic inhibition of PARP has been demonstrated to improve myocardial and endothelial dysfunction after heart transplantation in different animal models,^{13,16} showing the involvement of the peroxynitrite-PARP pathway in the pathophysiology of ischemia-reperfusion injury during heart transplantation. Pharmacologic catalytic decomposition of peroxynitrite with FP15 has been demonstrated to effectively eliminate peroxynitrite and prevent PARP activation both in vitro and in vivo.^{6,15}

As demonstrated in other studies,^{12,13,17} the present study showed intensive immunoreactivity for NT and activation of PARP in the LV myocardium after heart transplantation (Figure 4), which confirms the increased nitro-oxidative stress and the activation of the peroxynitrite-PARP pathway at 2 characteristic levels and is consistent with the above discussed pathophysiologic processes occurring during ischemia-reperfusion. Our immunohistochemical data after FP15 treatment (markedly reduced NT and PAR formation in the myocardium (Figure 4) indicate the inhibition of the peroxynitrite-PARP pathway, the main downstream mechanism of nitro-oxidative stress. WW85 (another peroxynitrite decomposition catalyst) also has been shown to decrease nitrosative stress and prevent PARP activation in an experimental model of acute cardiac rejection,¹² which is consistent with the current study.

On the basis of the results of the present study, we propose that the decomposition of peroxynitrite by FP15 during reperfusion may contribute to a better recovery of the cellular ATP (Figure 3) and to improved myocardial

contractility. Moreover, it was shown that energy depletion mediated by the peroxynitrite-PARP pathway significantly contributes to endothelial injury in endotoxin shock¹⁸ and diabetes mellitus.¹⁹ Thus, improved endothelial function seen in the FP15 treatment groups might also be explained by the improved energetic balance of the endothelium.

This is the first study to investigate the effects of the peroxynitrite decomposition catalyst FP15 on global hypothermic ischemia–reperfusion injury. By comparing our data after 1 and 24 hours of reperfusion and the data of nonischemic transplants, we can conclude that the administration of FP15 in the given dose was able to markedly reduce reperfusion injury after a short period of hypothermic preservation. The reported beneficial cardiac effects of FP15 on global cardiac ischemia–reperfusion are in agreement with those of Lauzier and colleagues,²⁰ who demonstrated the cardioprotective effects of a peroxynitrite decomposition catalyst after cardioplegic arrest in a working isolated rat heart model. Likewise, reduced infarct size and ameliorated myocardial damage have been observed after pharmacologic peroxynitrite decomposition in rodent²¹ and porcine⁵ coronary artery ligation (regional ischemia–reperfusion) models. On the other hand, our control group showed a recovery after 24 hours, with similar values to the FP15 group, suggesting that the applied cardiac preservation time and reperfusion only lead to reversible changes of functional status of the heart. We have compared the data with previous rat heart transplant studies with similar ischemia–reperfusion protocols.^{22–24} On summarizing the data of these studies, a biphasic recovery pattern is characteristic for this model with an early phase (<1 hour) followed by a further improvement during the next 24 hours.

Although enhanced formation of reactive oxidants during ischemia–reperfusion occurs in both cardiomyocytes and endothelial cells, leukocyte–endothelial cell interactions and increased release of oxidants from leukocytes affect first and foremost mainly the endothelium, resulting in endothelial dysfunction. The damaged dysfunctional coronary endothelium is responsible for the impaired endothelial vasodilatory function of coronary arteries, which limits CBF leading to impaired cardiac performance. As in our previous studies, endothelial function was severely impaired after 1 hour of reperfusion and was still depressed in the control group after 24 hours, as indicated by the lower vasodilatory response to acetylcholine (Figure 2). The slower recovery of endothelial function indicates that the coronary endothelium is more vulnerable to reperfusion injury than the myocardium. Accordingly, a previous work demonstrated that after normothermic ischemia and reperfusion, myocardial and endothelial function can be dissociated. Although myocardial function showed a full recovery in their model, endothelial function remained impaired.²⁵ The present study is the first to demonstrate that catalytic peroxynitrite decomposition improves not only myocardial but also

endothelial function after heart transplantation. Furthermore, the initial treatment with FP15 has a persisting long-term beneficial effect on endothelial function (Figure 2). The observed endothelial effect is comparable to that with application of NO precursors,²³ inosine,²⁴ or synthetic PARP inhibitors.¹³ Because pharmacologic elimination of peroxynitrite restores ATP levels, this may contribute to improved endothelial function.

In regard to the improved cardiac and endothelial function, rapid catalytic decomposition of peroxynitrite by FP15 in our model seems to be comparable to or better than the efficacy of blocking the pathway by PARP inhibition. By eliminating peroxynitrite, nitro-oxidative stress can be reduced (as demonstrated by our immunohistochemical data) and severe damage of DNA can be effectively prevented. In addition, by using this concept, peroxynitrite-induced modifications of enzymes, receptors, and structural proteins can be avoided and may help to restore the normal bioavailability of the crucial physiologic mediator NO (as confirmed by the improved endothelium-dependent vasodilatation).

On the basis of the data presented in the current report, we propose that pharmacologic decomposition of peroxynitrite represents a potential therapy approach to reduce ischemia–reperfusion injury during heart transplantation.

Limitations

According to the end points assessed, ischemia–reperfusion injury is a reversible phenomenon in the present model. However, the recovery rates of myocardial and endothelial damage are different. A complete recovery (to the level of nonischemic controls) of myocardial but not endothelial function has been observed after 24 hours of reperfusion, which indicates that the coronary endothelium is more vulnerable to reperfusion injury than the myocardium. Although the protective effects of peroxynitrite decomposition at early reperfusion were unequivocally confirmed, the nature of the present model allows the assessment of longer-term benefits of FP15 only in case of endothelial damage. A more robust model (eg, longer ischemic storage of the donor organ) would be needed to investigate the long-term effects of FP15 on myocardial ischemia–reperfusion injury.

CONCLUSIONS

Further studies are warranted to provide evidence for mechanisms on how FP15 may alter endothelial cell activation, inflammatory cell recruitment, and leukocyte infiltration.

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