Polyethylene-Oxide (PEO) Linear Nano-Polymer Directly Stimulates Endothelium and Protects Myocardium from Focal Ischemia-Reperfusion Injury via eNOS Pathway in Rats

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ABSTRACT

Medical grade Nano-polymers (NP) used to enhance circulation, drug and gene delivery. Injection of Polyethylene-oxide (PEO) increase tissue perfusion and decrease mean blood pressure in animal models. Polyethylene-oxide (PEO) NP's ultra high molecular weight (2-5 x 10^6 Da) and rheological properties allow it to interact with endothelial cells to increase endothelial nitric oxide (eNO) and its attendant enzyme (eNOS). eNOS over-expression and eNO induction is known to provide cardioprotection. We hypothesized that injection of PEO will have cardioprotective properties in a model of myocardial ischemia reperfusion injury (I/R), via induction of eNOS due to direct stimulation of endothelium. Male rats (310±9 g, n=16) were randomized to IV injection of 10 ppm of a saline-PEO solution (PEO) or equal volume of saline (CONT). Focal I/R injury was induced by LAD ligation for 30 minutes followed by 60 min of reperfusion. ECG, and aortic pressure monitored continuously and infarct size calculated as % area at risk. eNOS and its active phosphorylated form (p-eNOS) were detected on Western blots in left ventricle protein extracts. Mean blood pressure was 78±10 and 75±6 in PEO compared to 58±12 and 66±4 in CONT, at 30 min of Ischemia and 60 min reperfusion respectively ($p<0.05$ PEO vs. CONT). PEO had 28% and 47% higher eNOS and p-eNOS vs. CONT. PEO had a 28% infarct sparing effect compared to CONT. PEO prior to myocardial infarction significantly reduces infarct size and induces activation and up regulation of eNOS pathway. This is a possible novel mechanism for PEO NP cardioprotective effect and behavior in blood circulation. These properties of PEO warrant further investigation.

Keywords: polyethylene-oxide, nano-polymer, endothelium, heart, myocardium, ischemia, eNOS, nitric oxide

1 BACKGROUND

Nanotechnology offers a broad range of opportunities for improving diagnostic and therapeutic interventions in medicine. The mechanisms by which nano-compounds comprised of a Polyethylene oxide (PEO) and co-polymers affect vascular endothelium are not yet understood but appear to have clinical relevance in protection from ischemia reperfusion injury.

Injection of high molecular weight (>1000 kDa) nanopolymers into the blood stream enhance blood flow and tissue perfusion. Because of its water and blood soluble properties Polyethylene-oxide (PEO) has gained interest. [1-5] Blood soluble nanopolymers can integrate into the systemic circulation and modify characteristics of blood flow. [6-11]

Nanopolymers cause an increase in circulation and decrease in peripheral vascular resistance. [12, 13] In experiments using mice, rats and dogs PEO has been shown to promote better recovery from hemorrhagic and cardiogenic shock. [7, 3-5, 8, 11]

To achieve efficient increase in blood circulation it is important to understand interactions of nanopolymers with the biological environment, specifically the endothelium. Endothelial cells have been shown to be able to mediate opposite effects of shear stress (protective laminar shear stress vs. pathogenic disturbed shear stress). [14, 15]

Shear stress stimulation of endothelial cells can lead to phosphorylation of AKT and eNOS which in turn induces NO production. [16, 17] Shear stress affects cellular structure and function, vascular tone and diameter, wall remodeling, hemostasis, and inflammatory response through PI3K/eNOS pathway and NO. [18-20] NO appears to be critical in myocardial ischemia protection as an antioxidant molecule scavenging reactive oxygen species and as a signaling molecule initiating a variety of cell mediators. [21]

Viscoelastic properties of PEO were reported as possible factors in the improved survival under low flow states, but the circulatory effects of nano-polymer compositions in vasculature are poorly understood. We hypothesized that a polymer-endothelial interaction exists to achieve improved circulation through endothelial shear stress stimulation and primary activation of eNOS pathway.

2 MATERIALS AND METHODS

Adult male laboratory rats (310±9 g, n=16) were anesthetized with administration of 90 mg/kg Ketamine and 10 mg/kg Xylazine, placed on a heating pad to prevent hypothermia. A tracheostomy tube was installed to provide an airway. A rodent ventilator (Harvard Apparatus) and room air/O2 mixture were connected to the animal. Right carotid artery and tail vein were cannulated. Tail vein catheter was connected to the syringe pump (Kent Scientific) to provide an IV fluid infusion and for timed

administration of PEO/Saline solution. A 22G arterial/ventricular pressure catheter (Millar) was inserted into the carotid artery, and pressure continuously monitored. Physiological parameters were recorded on a data acquisition system PowerLab (ADInstruments).

Rats were randomized to receive 2 ml tail vein injection of 10 ppm (final blood concentration) saline-5000 kDa PEO solution (PEO) (Dow Chemicals) or equal volume of saline (CONT) after 30 min of ischemia 5 min before start of reperfusion. Focal I/R injury was induced by LAD ligation for 30 minutes followed by 60 min of reperfusion. ECG, and aortic pressure monitored continuously and infarct size calculated as % area at risk. Area at Risk and Infarcted Area will be assessed through double-staining technique at the time of death or 60 min after reperfusion. [22] Areas at risk/myocardial infarction were visualized by BPB and TTC respectively and stereoscopically measured using the point-counting method of Weibel. Percentile share of each area were calculated.

eNOS, its active phosphorylated form (p-eNOS) and p-AKT were detected on Western blots in left ventricle protein extracts. Harvested cells and tissues were processed for total protein extraction with Protein extraction kit (Chemicon). Total protein concentration has been determined with BCA Protein Assay Kit (Pierce), SpectraMax plate reader and SoftMax Pro software (Molecular Devices). Western blot was performed using NuPAGE electrophoresis system (Invitrogen). Primary antibodies against eNOS(S1177)/AKT(T308) and GAPDH (Santa Cruz) and ECL Advance Western Blotting Detection Kit (Amersham) and Storm imager were used for identification of protein bands. Protein levels were calculated using ImageQuant PC qantitation software (Amersham).

This model of acute coronary syndrome with Injection of PEO at the start of reperfusion reflects current strategies for treatment with focus on a combination of pharmacologically decreasing myocardial oxygen demand and restoring blood flow.

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Institutional Animal Care and Use Committee. [23, 24]

3 STATISTICAL ANALYSIS

The data analyzed using parametric and non parametric test. Length/Number of treatment response analyzed using ANOVA followed by Bonferroni correction for post hoc t test with correction for repeated measures. Probabilities values of <0.05 is considered to be statistically significant. The dependent variable is time and independent variable is the hemodynamic parameters. Endpoints of survival and infarct size analyzed using Klaplan-Mier survival curves and non parametric analysis. [25, 26] Statistical computations were done using Statistica Software (StatSoft Inc.Tulsa, OK).

4 RESULTS

Rats injected with PEO before reperfusion demonstrated better survival and restoration of hemodynamic parameters (Table 1) and smaller infarct size (Figure 1). Mean blood pressure was 78±10 and 75±6 in PEO compared to 58±12 and 66±4 in CONT, at 30 min of Ischemia and 60 min reperfusion respectively ($p<0.05$ PEO vs. CONT).

	Mean Arterial Pressure (mmHg)		Survival time after reperfusion (min)
	Baseline	Reperfusion	
CONT	112±8	66±4	45±26
PEO	109±7	75±6	60

Table 1: Mean arterial pressure (MAP) and survival time in Control (CONT) and rat subjected to PEO treatment (PEO) after 30 min of myocardial ischemia at the start of 60 min of reperfusion. ($p<0.05$)

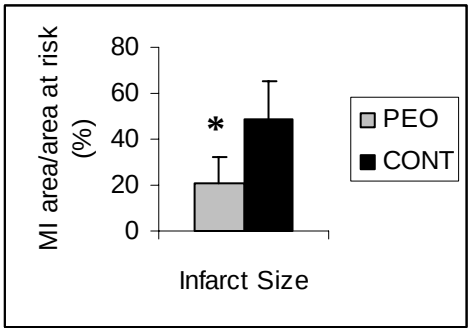

Figure 1: Decrease in infarct size in rat left ventricle subjected to PEO treatment. Representative chart of area of infarct/area at risk (%). Data obtained from quantitative pixelometry. *Significantly different from CONT ($p<0.05$)

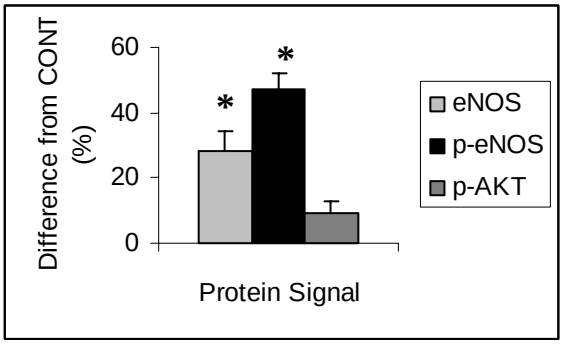

Figure 2: Changes in eNOS, p-eNOS, p-AKT levels in left ventricle after ischemia reperfusion with PEO treatment. Data obtained from quantitative blots densitometry. *Significantly different from Control ($p<0.05$)

Rats injected with PEO had increased levels of eNOS, p-eNOS and p-AKT levels in the heart. (Figure 2). PEO had 28% and 47% higher eNOS and p-eNOS vs. CONT. PEO had a 28% infarct sparing effect compared to CONT. There was no difference in left ventricle total AKT levels between PEO and CONT.

PEO prior to myocardial infarction significantly reduces infarct size and induces activation and up regulation of eNOS pathway.

5 DISCUSSION

Studies in our laboratory using PEO nano-polymer suggest that nano-polymers induction of eNOS/AKT is important for their physiological effects. PEO can cause increase in local near-wall space viscosity and subsequently wall shear stress, inducing shear stress-dependent mechanisms of the endothelium.

PEO in our study through shear stress can cause eNOS activation via phosphorylation or de-phosphorylation of specific sites, and enhance NO production and vasodilatation. It therefore would increase myocardial perfusion and protection after ischemia reperfusion injury.

Straight portions of arteries are exposed to relatively laminar flows and thus well protected. In contrast, branched, bifurcated, and curved arteries such as the left descending coronary arteries and precapillary network experience "disturbed" shear stress conditions. [27, 28] Because of complex arterial geometry combined with pulsatile blood flow during cardiac cycle, these areas experience disturbed shear conditions, including temporal and spatial gradients of wall shear stress over relatively short distances, flow reversal, and flow separation, leading to a low level of shear stress. [28]

Phosphorylation/de-phosphorylation of eNOS and AKT has been recognized as a critical regulatory mechanism controlling eNOS activity. eNOS(S1177)/AKT(T308) sites have been recognized as primary targets for shear stress induced activation. [29]

eNOS shear stress induced phosphorylation involves several specific sites. We have investigated eNOS-S1179(S1177) phosphorylation site. It plays an important role in stimulation of eNOS activity in response to shear stress. [30, 31]

Further testing with inhibition of AKT/eNOS by Wortmannin/L-NAME could provide additional evidence of eNOS pathway importance for shear stress dependent and cardioprotective effects of PEO nano-polymer.

It is possible that this is not the only mechanism of nano-polymers effect on the endothelium, and thus other factors may need to be explored. The prostaglandin pathway is another plausible alternative and can be differentiated through pharmacological inhibition using selective and non selective Cyclooxygenase inhibitors.

Therefore we provide novel evidence that in vivo administration of nano-polymers can results in eNOS dependent NO increase.

6 CONCLUSIONS

Treatment of acute myocardial ischemia with PEO nanopolymer is clinically relevant and has potentially broad applications. Therefore we examined PEO effect on endothelial function in a clinically relevant in vivo rat model of focal myocardial ischemia reperfusion and provided the mechanistic basis for its action through shear stress dependent eNOS. We showed that PEO nano-polymer can non-pharmacologically stimulate the endothelium and improve circulation via shear stress dependent mechanism. This mechanism is most likely to involve activation of endothelial Nitric Oxide Synthase (eNOS) and PI3-kinase–AkT (PI3/AkT) pathways. Our observations suggest that eNOS activation is important for its physiological effects of PEO nanopolymer.

Due to its standardized properties and prolonged non-pharmacological, non-metabolic stimulation of the endothelium PEO nano-polymer could potentially be used as a novel agent to investigate shear stress dependent endothelial pathways, and provide a method for cardio protection and amelioration of ischemia reperfusion induced injury.

7 REFERENCES

[1] Buxton, D., The promise of nanotechnology for heart, lung and blood diseases. Expert Opin Drug Deliv, 2006. 3(2): p. 173-5.

[2] Buxton, D.B., et al., Recommendations of the National Heart, Lung, and Blood Institute Nanotechnology Working Group. Circulation, 2003. 108(22): p. 2737-42.

[3] Nadia Antonova and Z. Lazarov, Hemorheological and hemodynamic effects of high molecular weight polyethylene oxide solutions. Clinical Hemorheology and Microcirculation, 2004. 30(3-4): p. 381-390.

[4] Kameneva, M.V., et al., Blood soluble drag-reducing polymers prevent lethality from hemorrhagic shock in acute animal experiments. Biorheology, 2004. 41(1): p. 53-64.

[5] Pacella, J.J., et al., A novel hydrodynamic approach to the treatment of coronary artery disease. Eur Heart J, 2006. 27(19): p. 2362-9.

[6] Sakai, T., et al., I.V. infusion of a drag-reducing polymer extracted from aloe vera prolonged survival time in a rat model of acute myocardial ischaemia. Br J Anaesth, 2007. 98(1): p. 23-8.

[7] Liu, J., F. Zeng, and C. Allen, In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration. Eur J Pharm Biopharm, 2007. 65(3): p. 309-19.

[8] McCloskey, C.A., et al., Tissue hypoxia activates JNK in the liver during hemorrhagic shock. Shock, 2004. 22(4): p. 380-6.

[9] Gutierrez, G. and S.P. Fuller, Of hemorrhagic shock, spherical cows and Aloe vera. Crit Care, 2004. 8(6): p. 406-7.
[10] Cotoia A, K.M., Marascalco PJ, Fink MP, Delude RL, Drug-reducing hyaluronic acid increases survival in profoundly hemorrhagic rats. Shock, 2008.
[11] Polimeni, P.I., B. Ottenbreit, and P. Coleman, Enhancement of aortic blood flow with a linear anionic macropolymer of extraordinary molecular length. J Mol Cell Cardiol, 1985. 17(7): p. 721-4.
[12] Gillissen, J.J.J., Polymer flexibility and turbulent drag reduction. Phys. Rev. E, 2008. 78(4): p. 046311.
[13] Singh, S. and H.S. Nalwa, Nanotechnology and health safety--toxicity and risk assessments of nanostructured materials on human health. J Nanosci Nanotechnol, 2007. 7(9): p. 3048-70.
[14] Gao, F., et al., Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation, 2002. 105(12): p. 1497-502.
[15] Nishimatsu, H., et al., Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. Circ Res, 2001. 89(1): p. 63-70.
[16] Bene, L., et al., Nanoparticle energy transfer on the cell surface. J Mol Recognit, 2005. 18(3): p. 236-53.
[17] Wu H, Jin Y, Arias J, Bassuk J, Uryash A, Kurlansky P, Webster K, Adams JA. In vivo upregulation of nitric oxide synthases in healthy rats. Nitric Oxide, 2009.Aug;21(1): p. 63-8.
[18] Davies PF. Flow-mediated endothelial echanotransduction. Physiol Rev, 1995. 75: p. 519–560.
[19] Uryash A, Wu H, Bassuk J, Kurlansky P, Sackner MA, Adams JA. Low-amplitude pulses to the circulation through periodic acceleration induce endothelial-dependent vasodilatation. J Appl Physiol, 2009. Jun 106(6): p. 1840-7.
[20] Tsao PS, Lewis NP, Alpert S, and Cooke JP. Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide. Circulation, 1995. 92: p. 3513–3519
[21] Adams JA, Wu H, Bassuk JA, Arias J, Uryash A, Jorapur V, Lamas GA, Kurlansky P. Periodic acceleration (pGz) prior to whole body ischemia reperfusion injury provides early cardioprotective preconditioning. Life Sci, 2010. May 86(19-20): p. 707-15.
[22] Lecour, S., et al., Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. J Mol Cell Cardiol, 2002. 34(5): p. 509-18.
[23] IACUC, Institutional Animal Care and Use Committee 2001 Guidebook. 2001.
[24] Waynforth, H.B. and P.A. Flecknell, Experimental and Surgical Technique in the Rat. 1992, San Diego, CA: Academic Press.
[25] Abdi, H., The Bonferonni and Šidák Corrections for Multiple Comparisons, in Encyclopedia of Measurement and Statistics, N. Salkind, Editor. 2007, Sage: Thousand Oaks (CA).
[26] Hassard, T.H., Understanding Biostatistics. 1991, St. Louis, MI: Mosby Year Book.
[27] Davies PF. Flow-mediated endothelial mechanotransduction. Physiol Rev, 1995. 75: p. 519–560
[28] Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, and Garcia-Cardena G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann NY Acad Sci, 2000. 902: p. 230–240
[29] Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, and Murphy TJ. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest, 1992. 90: p. 2092–2096
[30] Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, and Jo H. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. J Biol Chem, 2002. 277: p. 3388–3396
[31] Dimmeler S, Assmus B, Hermann C, Haendeler J, and Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. Circ Res, 1998. 83: p. 334–341