Evaluation of a Targeted Drug-Eluting Intravascular Nanotherapy to Prevent Neointimal Hyperplasia in an Atherosclerotic Rat Model

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1. Introduction

Atherosclerosis is the leading cause of death and disability in the United States. In 2016, cardiovascular disease accounted for 17.6 million deaths globally, increasing by 14.5% from 2006.¹ In 2015, cardiovascular disease accounted for annual costs in the United States alone of $351.2 billion.¹ Many of the procedures required for these patients fail secondary to restenosis from neointimal hyperplasia, leading to significant morbidity and mortality following cardiovascular interventions. Over the past several decades, the only therapies that have directly affected restenosis rates following cardiovascular intervention are drug-eluting stents and balloons.² However, it is now known that these drugs (i.e., paclitaxel, sirolimus, or derivatives thereof) indiscriminately inhibit all proliferation, including endothelial cell proliferation, leading to catastrophic late in-stent thrombosis.³,⁴ Furthermore, in 2019, the FDA issued a warning on the use of paclitaxel drug-eluting stents and balloons given the increased mortality reported at 2 and...
5 years.\textsuperscript{[5,6]} Thus, there is a great need for new technology that will improve patient outcomes following all cardiovascular interventions. Specifically, there is a need for a therapy that effectively prevents the formation of neointimal hyperplasia while simultaneously stimulating reendothelialization, thereby promoting overall vascular health.

In prior work, our laboratories created a collagen-targeted, nitric oxide–releasing nanofiber that, when administered systemically after arterial intervention, specifically targets sites of arterial injury and delivers nitric oxide (NO) to inhibit neointimal hyperplasia at 2 weeks in nonatherosclerotic animal models.\textsuperscript{[7]} In addition, this inhibition remained durable out to 7 months.\textsuperscript{[7]} The nanotherapeutic is composed of peptide amphiphile (PA) nanofibers that incorporate a collagen-binding peptide (CBP) for targeting collagen exposed at the site of injury and an S-nitrosylated (SNO) moiety as the therapeutic (CBP-SNO-PA). However, this therapeutic needs to be advanced to the next stage of development and be evaluated in an atherosclerotic

![Figure 1](image_url)

**Figure 1.** Confirmation of atherosclerotic rat model. A) Western blot analysis performed with antibody against apolipoprotein E (ApoE) confirms a lack of ApoE in the Sprague Dawley (SD) ApoE-/- strain compared to wt SD male rats (n = 5). B) Total cholesterol (TC), low-density lipoprotein (LDL), and triglyceride (TG) levels measured in 16-week-old male SD ApoE-/- and wt SD rats were shown to be greater than threefold higher in knockout rats (*p < 0.0001 vs wt values, n = 6–9). C) Plasma levels of malondialdehyde (MDA) and 8-isoprostanone, markers for oxidative stress, showed an upward trend in SD ApoE-/- versus wt SD rats (p = 0.27 and **p = 0.002, respectively). D) Oil Red O (ORO) staining of aorta shows fatty deposition (black arrow) in cross-section of an aortic root of a SD ApoE-/- rat at age 16 weeks at 5\(\times\) magnification. ORO en face staining shows fatty streaks (white arrow) along the aortic trunk in SD ApoE-/- rat.
environment. The atherosclerotic niche brings new challenges to drug delivery, including increased levels of oxidative stress and differing protein expression profiles, which may make it more challenging to use the PA nanofiber technology with our established targets and therapeutics \[^{8-11}\]. In addition, known risk factors for atherosclerosis including hyperlipidemia have been shown to decrease amounts of bioavailable NO, a molecule important for regulating vascular healing \[^{10,12,13}\]. Although our prior studies were conducted in wt Sprague Dawley (SD) rats, until recently there were no atherosclerotic SD models available to advance our work using the same strain. The recent availability and validation of SD apolipoprotein E (ApoE) knockout (-/-) rats now allows our laboratory to advance the study of our nanotherapeutic using the same rat strain, which will allow for direct comparisons to our prior work.

**Figure 2.** Synthesis and characterization of collagen-targeted NO peptide amphiphile (PA) nanofibers. A) Chemical structures of collagen-targeted SNO PA (CBP-SNO-PA) and the nontargeted scrambled SNO PA (scr-SNO-PA). Also shown are chemical structures of the collagen-targeted PA and backbone PA labeled with 5-carboxy tetramethylrhodamine (TAMRA) (CBP-PA + TAMRA and BB-PA + TAMRA, respectively) that were used for fluorescence visualization of coassemblies in vivo. The blue portion of the structures is the hydrophobic region, \(\beta\)-sheet-forming region, and charged sequence that facilitate nanofiber formation. The dark gray region denotes the collagen-targeting sequence (KLWVLPKC), the orange region denotes the scrambled version of the collagen-targeting sequence, the green region is the S-nitrosyl moiety (SNO), and the red region is the fluorescent tag, TAMRA. B) 3D illustration of single peptide amphiphiles (top and middle rows) and nanofiber structures formed when coassemblies are placed in aqueous solution (bottom row). Colors are as in panel A. PAs and ratios are as indicated in panel C. C) Conventional TEM images of all four combinations of PAs used in these studies show formation of nanofibers. Of note, addition of the bulky fluorophore did not significantly change nanofiber formation.
The aim of this study was to evaluate the localization and efficacy of our targeted nanotherapeutic in an atherosclerotic rat model. This is a necessary step to bring this nanotherapeutic closer to the clinical arena. We hypothesize that intravenous delivery of our collagen-targeted NO-releasing nanoﬁber will localize to sites of vascular injury and prevent neointimal hyperplasia at the site of intervention in an atherosclerotic environment.

2. Results

2.1. Conﬁrmation of Atherosclerotic Rat Model

Western blotting analysis comparing wt SD and SD ApoE-/- rats conﬁrmed a lack of ApoE expression in our knockout rat colony (Figure 1A). Hyperlipidemia and hypercholesterolemia were conﬁrmed in the SD ApoE-/- rats by blood work with elevated total cholesterol (291.8 ± 22.3 vs 105.0 ± 3.6 mg dL⁻¹), LDL cholesterol (110.0 ± 8.7 vs 19.2 ± 0.9 mg dL⁻¹), and triglycerides (435.7 ± 25.5 vs 172.5 ± 20.5 mg dL⁻¹) as compared to wt SD rats (Figure 1B, *p < 0.0001 for all). In addition, plasma levels of the oxidative stress markers malondialdehyde and 8-isoprostane were elevated in SD ApoE-/- rats as compared to wt SD rats (32.4 vs 20.2, p = 0.27 and 416.4 vs 86.0, **p = 0.002, respectively) (Figure 1C).

Oil Red O (ORO) staining of the aortic root and en face aorta showed plaque deposition in the aortic root and development of fatty streaks within the aortic wall in SD ApoE-/- (Figure 1D, white arrow) but not wt SD rats. These data suggest an atherosclerotic environment differing from the wt SD rat.

Table 1. Blood work evaluating SD ApoE-/- rats before (control, n = 12 rats) and after CBP-SNO-PA nanoﬁber injection (n = 3–5 rats/time point).

|                         | Control mean ± SEM | 24 h mean ± SEM | 72 h mean ± SEM | 5 days mean ± SEM |
|-------------------------|--------------------|-----------------|-----------------|------------------|
| White blood cells [K μL⁻¹] | 13.4 ± 0.6         | 4.9 ± 0.9⁴      | 6.5 ± 0.4⁴      | 4.7 ± 1.5⁴       |
| Red blood cells [M μL⁻¹]  | 8.3 ± 0.0          | 7.4 ± 0.8       | 7.9 ± 0.3       | 8.3 ± 0.2        |
| Hemoglobin [g dL⁻¹]       | 15.1 ± 0.1         | 13.1 ± 1.3      | 14.4 ± 0.6      | 14.9 ± 0.3       |
| Mean corpuscular volume [fL] | 54.4 ± 0.3       | 52.3 ± 1.0⁴     | 54.0 ± 0.8      | 54.6 ± 0.7       |
| Platelet [K μL⁻¹]         | 783.1 ± 17.3       | 331.2 ± 97.2⁴   | 769.7 ± 75.9    | 831.7 ± 15.7     |
| Differential [%]          |                    |                 |                 |                  |
| Segmented neutrophils     | 8.0 ± 0.5          | 31.9 ± 9.5⁴     | 21.4 ± 4.8      | 12.6 ± 1.4       |
| Lymphocytes               | 85.6 ± 0.8         | 61.4 ± 8.8⁴     | 67.6 ± 5.2⁴     | 77.9 ± 2.5       |
| Monocytes                 | 5.0 ± 0.6          | 3.9 ± 0.3       | 9.6 ± 1.2⁴      | 7.7 ± 1.0        |
| Eosinophils               | 1.3 ± 0.1          | 2.6 ± 1.6       | 1.1 ± 0.1       | 1.3 ± 0.1        |
| Basophils                 | 0.2 ± 0.0          | 0.2 ± 0.1       | 0.2 ± 0.0       | 0.2 ± 0.1        |

*⁴p < 0.05 when compared to control.

Table 2. Blood chemistry panel of SD ApoE-/- rats before (control, n = 12 rats) and after CBP-SNO-PA nanoﬁber injection (n = 3–5 rats/time point).

|                           | Control mean ± SEM | 24 h mean ± SEM | 72 h mean ± SEM | 5 days mean ± SEM |
|---------------------------|--------------------|-----------------|-----------------|------------------|
| Alanine aminotransferase (ALT) [U L⁻¹] | 25.5 ± 0.9         | 35.7 ± 2.8⁴     | 22.0 ± 1.2      | 26.7 ± 3.8       |
| Aspartate aminotransferase (AST) [U L⁻¹] | 63.5 ± 1.6         | 130.3 ± 17.5⁴   | 53.7 ± 6.5      | 113.0 ± 38.6     |
| BUN [mg dL⁻¹]             | 22.1 ± 0.4         | 23.8 ± 3.5      | 18.3 ± 0.9      | 17.3 ± 1.5       |
| Cholesterol [mg dL⁻¹]      | 291.5 ± 5.8        | 231.8 ± 37.6    | 270.0 ± 16.8    | 255.7 ± 6.9      |
| Creatinine [mg dL⁻¹]       | 0.5 ± 0.0          | 0.5 ± 0.1       | 0.5 ± 0.0       | 0.4 ± 0.0⁴       |
| Glucose [mg dL⁻¹]          | 283.2 ± 11.7       | 200.3 ± 16.8⁴   | 189.0 ± 27.0⁴   | 187.7 ± 22.6⁴    |
| HDL [mg dL⁻¹]             | 15.9 ± 0.3         | 17.2 ± 1.0      | 16.7 ± 0.3      | 15.7 ± 0.7       |
| Potassium [mmol L⁻¹]       | 5.2 ± 0.1          | 5.8 ± 0.2⁴      | 5.2 ± 0.2       | 5.9 ± 0.4        |
| LDL [mg dL⁻¹]             | 112.8 ± 4.9        | 89.8 ± 6.3⁴     | 106.2 ± 8.6     | 84.7 ± 2.8       |
| Sodium [mmol L⁻¹]          | 142.5 ± 2.1        | 141.9 ± 0.8     | 141.7 ± 1.4     | 135.4 ± 2.2      |
| Phosphorus [mg dL⁻¹]       | 5.9 ± 0.2          | 6.6 ± 0.5       | 6.0 ± 0.0       | 5.4 ± 0.3        |
| Triglycerides [mg dL⁻¹]    | 309.9 ± 15.9       | 343.5 ± 62.0    | 170.0 ± 28.3    | 245.7 ± 38.1     |

*⁴p < 0.05 when compared to control.
2.2. Synthesis and Characterization of Collagen-Targeted NO-Releasing PA Nanofibers

After the PA molecules shown in Figure 2A were synthesized, liquid chromatography mass spectrometry (LCMS) confirmed the purity of component PAs prior to coassembly (Figure S1, Supporting Information). After coassembly of the four PAs (modeled in Figure 2B), transmission electron microscopy (TEM) confirmed nanofiber formation for all PAs that were used for injection (Figure 2C). Safety profiles have been performed previously on these PAs by our laboratory and are published. To evaluate blood work in knockout rats both before and after injection of our PA, which showed a transient mild decrease in platelet count at 24 h and a mild leukopenia out to 5 days post-injection, without clinical significance (Table 1 and 2). These data confirm the biocompatibility of our nanofiber.

2.3. Collagen-Targeted NO-Releasing PA Nanofibers Localize to Areas of Arterial Injury In Vivo for up to 5 Days

To determine if the collagen-binding NO nanofibers localize at the site of arterial injury, male SD ApoE-/- rats underwent balloon angioplasty followed by tail vein injection of the CBP-SNO-PA nanofiber, the scr-SNO-PA nanofiber, or no injection at all. As shown in Figure 3A, we observed localization of the 5-carboxytetramethylrhodamine (TAMRA)-labeled CBP-SNO-PA nanofibers to the injured left carotid artery. Quantification of red pixels in fluorescent microphotographs showed significantly increased fluorescence in the left injured carotid artery versus the right uninjured carotid artery after tail vein injection (2.8 $\pm$ 0.0023 vs 1.5 $\pm$ 0.0023 AU mm$^{-2}$, p < 0.0001; Figure 3B), indicating increased localization of CBP-SNO-PA nanofibers. Although we did observe some signal in the injured carotid artery of rats injected with the scr-SNO-PA nanofiber, this was significantly lower than what we observed with the CBP-SNO-PA nanofiber (3.4 $\pm$ 0.0023 vs 2.8 $\pm$ 0.0023 AU mm$^{-2}$, p < 0.0001). Further evaluation shows initial localization of our CBP-SNO-PA fiber at 20 min, which drops by 24 h, and approaches zero by 5 days after injection (Figure 3C,D). These data confirm our targeting sequence allows for specific localization of our therapeutic to the site of injury with just one injection.

2.4. Biodistribution of Collagen-Targeted NO-Releasing PA Nanofibers In Vivo

To determine off-target localization of our CBP-SNO-PA nanofibers, fluorescent microscopy was used to quantify fluorescence in other internal organs 20 min, 24 h, 72 h, and 5 days after injection of the targeted nanofiber, including the contralateral carotid artery, heart, kidney, liver, lung, and spleen. There was minimal fluorescence in the evaluated internal organs compared to our injured left carotid target, with a small increase seen in the liver at 72 h that resolves by 5 days (Figure 4A). Representative images show minimal fluorescence (red pixels) throughout the organs, indicating

![Figure 3. Collagen-targeted NO-releasing PA nanofibers localize to areas of arterial injury in vivo for up to 5 days. A) Carotid artery balloon injury was performed and immediately followed by injection of 5.0 mg of a nanofiber coassembly containing 5 mole% CBP-T PA and either 95 mole% CBP-SNO-PA or 95 mole% scr-SNO-PA. This was allowed to circulate for 20 min prior to thoracotomy, perfusion, and fixation. Cross-sections of rat carotid artery imaged via fluorescence microscopy (scale bar = 100 nm). Green = autofluorescence of elastic lamina, red = TAMRA signal. B) Red pixel quantification of each treatment group was performed showing greater fluorescence in the injured carotid of rats injected with TAMRA-labeled CBP-SNO-PA nanofibers versus the uninjured artery and rats injected with TAMRA-labeled scr-SNO-PA nanofibers (p < 0.0001, n = 4-8 rats/group, 7 technical replicates). There was no statistical difference between rats injected with scr-SNO-PA nanofiber and the uninjured contralateral artery (p = 0.29). C) Duration of localization was evaluated in injured carotid cross-sections harvested at 20 min, 24 h, 72 h, and 5 days after tail-vein injection (scale bar = 100 nm). Green = autofluorescence of elastic lamina, red = TAMRA signal. D) Red pixel quantification showed a significant decrease at 24 h with minimal fluorescence seen at 5 days (n = 3-5 rats/group). Fluorescence at 20 min was increased versus all other time points (#p < 0.001). Fluorescence at 5 days was decreased versus 24 and 72 h (**p < 0.02).](https://www.advancedsciencenews.com/content/2000093)
minimal off-target localization of the CBP-SNO-PA nanofibers (Figure 4B). Stark differences were seen between localization in uninjured and injured vasculature as seen by representative images of the uninjured right carotid and the injured left carotid after CBP-SNO-PA nanofiber injection (Figure 3A, B and 4A, B). Overall, these data suggest that the CBP-SNO-PA nanofiber serves as an intravenously administered, targeted, localized therapy that is likely metabolized in the liver.

2.5. Collagen-Targeted NO-Releasing PA Nanofibers Decrease Inflammation at the Site of Injury In Vivo

To determine immediate antiinflammatory effects of CBP-SNO-PA nanofibers, we performed immunofluorescence staining to evaluate for CD45⁺, ED1⁺, and MPO⁺ cells 72 h after injury (Figure 5A). Treatment with the CBP-SNO-PA nanofiber decreased CD45⁺, ED1⁺, and MPO⁺ cells at the site of arterial
First, we showed that there was a 64.6% decrease in the intimal area and a 69.3% decrease in percent occlusion in the injury plus CBP-SNO-PA group compared to the injury alone group (*p < 0.0001; Figure 5B). Interestingly, the CBP-SNO-PA nanofiber decreased the intimal area by 64.6%, and the percent occlusion by 69.3% compared to injury alone, with no significant changes in media area (*p < 0.0001; Figure 6B, Table 3). This effect was durable out to 3 months, as seen by the 60.0% reduction in intimal area and 58.9% reduction in I/M ratios in rats treated with CBP-SNO-PA nanofiber compared to injury alone (*p < 0.0001; Figure 6C,D and Table 3). Of note, these localization and efficacy effects were similar to those seen in the IZ ApoE-/- strain, demonstrating a therapy that reproducibly inhibits neointimal hyperplasia in other rat strains (Figure S3, Supporting Information).

3. Conclusion

In this study we show that the localization and efficacy of a collagen-targeted, NO-releasing nanofiber was not impaired by the atherosclerotic milieu in a rat model of atherosclerosis. These findings build upon our previously published work showing localization to the site of injury and inhibition of neointimal formation in a nonatherosclerotic rat model.[7] We first showed that SD ApoE-/- rats maintained on regular chow can develop an atherosclerosis-prone environment by 16 weeks of age. Second, we demonstrated nanofiber localization in the atherosclerotic environment to areas of arterial injury. Third, we further validated the specific targeting by showing limited nonspecific, off-target localization in our biodistribution analysis. Finally, we showed efficacy against neointimal hyperplasia development in two different atherosclerotic-prone rat strains that was similar to what we previously showed in the nonatherosclerotic environment, and this effect was durable out to 3 months in the SD ApoE-/- model. Therefore, this current study significantly advances upon prior work and is necessary to proceed with further translation to the clinical arena.

Previous work has demonstrated the potential of intravenously administered therapeutics targeting and inhibiting areas of neointimal hyperplasia after vascular intervention. Zhang et al. used a rapamycin-containing spherical nanoparticle physiologically targeted to areas of low pH and high reactive oxygen species (ROS) with additional cell targeting moieties to exposed collagen IV.[14] Although they were able to show a decrease in neointimal hyperplasia development at 2 weeks similar to our results here, this experiment was done in the setting of wild type, nonatherosclerotic SD males and required multiple injections. The pH probe is an interesting approach of targeting that takes advantage of the increased ROS present after arterial injury, but this may prove difficult to advance to an atherosclerotic environment. Numerous studies have found that atherosclerosis increases the oxidative environment, which may be nonuniform as Liu et al. found in their study in ApoE-/- mice.[15] In addition, many individuals presenting with atherosclerosis often have multiple areas of disease as atherosclerosis is a chronic, systemic illness, which may lead to altered biodistribution in this setting.[16,17]

Given the increased oxidative stress present in the atherosclerotic environment, we were surprised that our CBP-SNO-PA nanofibers were so effective at inhibiting neointimal hyperplasia. We previously reported a decrease in I/M ratio of 46% and decrease in percent occlusion of 56% with the CBP-SNO-PA nanofiber compared to the scr-SNO-nanofiber.[7] In the current study, we observed a decrease in I/M ratio of 64% and decrease...
Although we recognize that there can be slight differences in the injury model sustained from surgeon to surgeon, we take these data to indicate that the CBP-SNO-PA was effective at reducing neointimal hyperplasia even in an atherosclerotic milieu. We know from previous work that increased oxidative stress from cardiovascular risk factors and reactive oxygen species causes decreased eNOS activity and resultant endothelial cell dysfunction. Perhaps this NO-poor environment is more responsive to NO therapy than in a nonoxidative stress environment that may have increased levels of basal nitric oxide. In addition, it is difficult to assess if (and which) components of our PA are cellularly internalized or if mere proximity of nitric oxide to cells of interest is adequate for producing positive downstream effects. This, however, would need to be studied further. Studies evaluating downstream NO effects and directly comparing to wt SD rats will give us more

Figure 6. Collagen-targeted NO-releasing PA nanofibers inhibit neointimal hyperplasia at short and long time points in vivo. A) Hematoxylin and eosin (H&E)-stained arterial cross-sections of carotid arteries in SD ApoE-/- rats 2 weeks after balloon injury (n = 4 rats/group, scale bar = 100 nm). Treatment groups received 5 mg of 100 mole% CBP-SNO-PA nanofibers via tail vein injection. B) Analysis of cross-sections taken with 5× objective to evaluate intima/media area (I/M) ratio and percent occlusion shows significant decreases in all areas after injection of CBP-SNO-PA nanofibers. The I/M ratio was decreased by 68.2% when compared to injury with no treatment and by 70.2% when compared to scr-SNO-PA nanofibers (*p < 0.0001). Percent occlusion showed a 69.3% and a 56.0% decrease when comparing CBP-SNO-PA nanofiber treatment to injury alone and scr-SNO-PA nanofiber treatment (*p < 0.0001, #p < 0.05, respectively). C) H&E-stained cross-sections of carotid arteries harvested 3 months after injury (scale bar = 100 nm). D) Quantification of neointimal hyperplasia at 3 months after injury showed 58.9% and 55.2% reductions in I/M ratios and percent occlusion respectively in rats treated with CBP-SNO-PA nanofibers compared to injury-alone (n = 2–4 rats/group, *p < 0.0001).
Table 3. Morphometric analysis of carotid arteries 14 days and 3 months after balloon injury (n = 3–5 rats/time point). Data are presented as mean ± /− SEM.

|                | Lumen area [μm²] | Intima area [μm²] | Media area [μm²] | I/M | Percent occlusion |
|----------------|------------------|-------------------|------------------|-----|------------------|
| 2 weeks        |                  |                   |                  |     |                  |
| Injury alone   | 441103.0 ± 25725.1 | 119296.3 ± 22806.4 | 112050.4 ± 6326.6 | 0.99 ± 0.20 | 21.1 ± 3.9 |
| scr-SNO PA     | 521217.6 ± 8859.3 | 90354.9 ± 7428.8  | 89827.2 ± 3087.1 | 1 ± 0.01 | 14.7 ± 1.1  |
| CBP-SNO-PA     | 614096.6 ± 14424.6 6 | 42230.3 ± 7052.8 6 | 121529.3 ± 9873.7 | 0.36 ± 0.01 6 | 6.5 ± 1.1 6 |
| 3 months       |                  |                   |                  |     |                  |
| Injury alone   | 682579.5 ± 10562.2 | 187974.3 ± 10692.4 | 133998.9 ± 1590.9 | 1.43 ± 0.10 | 21.4 ± 1.0 |
| CBP-SNO-PA     | 706723.7 ± 6873.2  | 75546.2 ± 6983.4  | 129649.3 ± 6956.9 | 0.59 ± 0.04 | 9.6 ± 0.8  |

*p < 0.0001 compared to injury alone within the same time point, mean ± SEM.

There are several important limitations to our study. First, this study was done only in male rats, despite females making up a large percentage of the vascular patient population. As we translate this to a large animal model, we will be able to include both sexes, whereas here we were limited in our ability to perform the balloon injury model by the size of the 16-week-old ApoE−/− rats (<250 g). Of note, we have studied our therapy in nonatherosclerotic male and female rat models in the past. Second, this study does not include a treatment group consisting of collagen-targeted nontherapeutic nanoparticle injections as a control; however, it was previously established in our prior publication that localization without nitric oxide did not translate to efficacy in the nonatherosclerotic environment. Third, although TAMRA labeling was used to identify localization of our nanoparticle, we recognize that the fluorophore could dissociate from the PA during the study time course. However, TAMRA-labeled scr-SNO-PA did not localize to the injured carotid (Figure 3A), suggesting that it is unlikely that TAMRA itself is responsible for the localization. In addition, we previously confirmed short-term downstream effects of our targeted nitric oxide nanoparticle (increased nitrotyrosine and S-nitrosocysteine) at 72 h postinjection compared to injury alone, suggesting localization of our nitric oxide therapy rather than any combination of fluorophore alone or fluorophore plus CBP. Fourth, while we did not perform confocal microscopy to determine intracellular localization of PAs, we have observed that CBP-PA is preferentially taken up by vascular smooth muscle cells over fibroblasts and endothelial cells (data not shown). Although this difficult to visualize and confirm from in vivo tissue sections, it may not be necessary for the PA to be internalized by cells. Given the diffusible nature of nitric oxide, proximity to the cells of interest provided by CBP localization likely allows for cellular interaction and favorable downstream effects.

In conclusion, we characterized the recently developed SD ApoE−/− rat as an atherosclerotic model to systemically administer a collagen-binding NO-releasing nanoparticle to target areas of arterial injury after vascular intervention. Not only did we find specific localization, but we also showed the continued and reproducible efficacy of this therapeutic in the atherosclerotic milieu. These data represent the second phase of development of an injectable therapy to improve the durability of vascular interventions. The next steps will be translating to an atherosclerotic, diabetic model, followed by further translation to a large animal model.

4. Experimental Section

Study Approval: All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the University of North Carolina at Chapel Hill Animal Care and Use Committee. The number of rats randomized to each treatment group (n = 4) was calculated using a power of 0.8, difference in means of 0.3, standard deviation of 0.1, and p-value of 0.05.

PA Synthesis: Peptide amphiphile (PA) molecules were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide chemistry on Rink amide methybenzhydryl amine resin using a CEM Liberty Blue automated microwave peptide synthesizer (CEM Corp.; Matthews, NC) as described previously. The collagen-targeting sequence (KLWLPK) reported by Chan et al. was incorporated into a PA sequence to yield the CBP-PA: KLWLPKCKAAAVK(C12) where lauric acid (C12) is attached to the ε-amino of the C-terminal lysine. Cleavage was performed using a standard solution of 90% trifluoroacetic acid, 2.5% triisopropylsilane (TIPS), 2.5% water, and 5% 2,2′-(ethyleneoxy) diethanol. To visualize nanoparticle localization, PAs were synthesized to contain a fluorescent molecule, TAMRA. All PAs were purified by high-performance liquid chromatography (HPLC) and characterized by LCMS.

S-nitrosylation of the cysteine residue was achieved utilizing methods previously described. Briefly, 1 mmol L−1 PA was dissolved in an acidic solution (pH 3) with 1 equivalent of NaNO2 and 50 μmol L−1 pentelic acid for 1 h and protected from light. The SNO-PA was high performance liquid chromatography (HPLC) purified, lyophilized, and stored at −80 °C.

Coassembly of PAs was achieved by dissolving combined molar ratios of each PA component in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at 10 mg mL−1 followed by horn sonication for 3 × 1 s intervals and water bath sonication for 15 min. The following combinations were required for PA localization assessment: 1) 95 mole% collagen-targeted SNO-PA (CBP-SNO-PA; sequence KLWLPK(C(SNO)KAAAVV-K(C12))) + 5 mole%...
TamRA-labeled collagen-targeted PA (KLWVLPKC(TAMRA)KAAVVK(C12)) and 2) as a control, 95 mole% scrambled SNO-PA (sc-SNOR/SNO; SNO-C(SNO)KAAVVK(C12) – 5 mole% labeled backbone (sequence [TAMRA]KAAVVK(C12)). For PA efficacy assessment at 2 weeks, 100% CBP-SNO-PA and 100% scr-SNO-PA were used for treatment and control groups, respectively. HIFIP was removed under high vacuum using a Schlenk line and PAs were resuspended in deionized water to make 5 mg aliquots prior to freezing in liquid nitrogen and lyophilizing to dryness. Aliquots were stored at –80°C until use.

**Material Characterization:** In addition to characterization performed during synthesis, further studies were done to assess adequate nanofiber formation prior to injection. PA samples were resuspended in Hanks balanced salt solution (HBSS, 1 mg mL⁻¹) and conventional TEM images were taken using an FEI Tecnai T-12 TEM (ThermoFisher Scientific; Hillsboro, OR) at 80 kV with an Orius 2 k x 2 k CCD camera (Gatan, Inc.; Pleasanton, CA). Negative staining was used to prepare the samples for TEM by incubating 8 µL samples on 400-mesh copper grids covered with a thin carbon film treated with glow discharge. Following this, samples were stained with 2% uranyl acetate for 2–3 min, washed with deionized water, and air dried prior to imaging. Pictures were taken at 6500 x, 11 000 x, and 15 000 x magnification.

**Confirming Atherosclerotic Rat Model:** Male and female SD ApoE-/- rats and an appropriate breeding license were obtained (Horizon Discovery; St. Louis, MO) and used to initiate and maintain a colony. Rats were weighed and blood work, including total cholesterol (TC), low-density lipoprotein (LDL), and triglyceride (TG) levels, were determined using an antibody against ApoE (abcam, catalog #:20874; Ann Arbor, MI). Serum and plasma obtained from wt SD or SD ApoE-/- rats were isolated. The external carotid artery and superior thyroid artery were divided, and atrumatic clamps placed on the common carotid and internal carotid arteries prior to making an arteriotomy in the external carotid artery. A 2F embolectomy balloon (Edwards Lifesciences; Raleigh, NC) was placed in the common carotid artery via the arteriotomy and inflated to 5 atmospheres of pressure for 5 min. After removal of the balloon, clamps were flashed, the external carotid artery ligated, and clamps removed. PA solutions prepared by dissolving 5 mg of PA in 1 mL of HBSS were injected via tail vein following reestablishment of carotid artery blood flow. This dose was chosen based prior work performed in wt SD rats with this PA.[7] Rats were euthanized 20 min, 24 h, 72 h, 5 days, 2 weeks, and 3 months following injection to assess targeting, duration of localization, and neointimal hyperplasia development.

To confirm reproducibility and translatability of this therapy, localization (20 min) and efficacy (2 weeks) were assessed in the Lean Zucker (LZ) ApoE-/- rat, a kind gift from Dr. Edward Bahnson with similar levels of LDL, total cholesterol, and triglycerides as SD ApoE-/- rats (Figure S2, Supporting Information). Unfortunately, due to COVID-19 restrictions, we were not able to perform the localization duration studies or long-term efficacy (3 month) studies in the male LZ ApoE-/- model to allow for direct comparison with the SD ApoE-/- model.

**Tissue Processing:** In situ perfusion with 300 mL of PBS solution followed by 300 mL of 2% paraformaldehyde was performed after euthanasia via anesthetic overdose and bilateral thoracotomies. Organs, including bilateral carotids, heart, kidney, liver, lung, and spleen, were collected for tissue processing. Samples were fixed with 2% paraformaldehyde for 1 h followed by 30% sucrose overnight, except aortas for en face staining (see earlier). Samples were then frozen in O.C.T. over liquid nitrogen and stored at –80°C. Samples were cut into 5 µm sections using a CryoStar NX70 (ThermoScientific) cryostat and stored at –80°C. Targeting and duration of localization time points (20 min, 24 h, 72 h, and 5 days) were evaluated via fluorescent microscopy and efficacy time points (2 weeks and 3 months) were evaluated via brightfield microscopy for neointimal hyperplasia using routine hematoxylin and eosin staining.

**Fluorescence Microscopy:** Digital images were acquired using a Zeiss Axio Imager.A2 microscope (Hallebergoos, Germany) with the 5 x and 20 x objectives. The HE CY3 filter (Zeiss filter #43) with excitation and emission wavelengths of 545 and 605 nm, respectively, was used to assess TAMRA fluorescence. The green fluorescent protein filter (Zeiss filter #38) with excitation and emission wavelengths of 470 and 525 nm, respectively, was used to assess tissue autofluorescence. Fluorescence quantification for targeting, biodistribution, and duration of localization used images of the tissue taken at 5 x magnification with exposure times adjusted to eliminate any background autofluorescence. Area of fluorescence, expressed in arbitrary units (AU) per tissue area (µm²), was measured using Image software from seven evenly spaced sections per organ per animal. During imaging analysis, a standard threshold was set for all tissues. Comparisons of AU per tissue area were made among injured carotid arteries harvested from rats injected with CBP-SNO-PA nanofibers, sc-SNO-PA nanofibers, and no solution (injury alone). Biodistribution compared the injured carotid artery to the following off-target organs: tracheal carotid artery, heart, kidney, liver, lung, and spleen.

**Immunofluorescence Staining:** To evaluate inflammatory markers, three evenly spaced frozen carotid artery sections were taken from each animal in the CBP-SNO-PA-treated and injury-alone group 72 h postinjury and stained with antibodies against ED1, MPO, and CD45 (n = 3–5 animals/group). Slides were permeabilized using 0.3% Triton X-100 in Chemical; Ann Arbor, MI) and the 8-isoprostane enzyme-linked immuno-}


PBS for 10 min, incubated with primary antibody (anti-ED1, 1:600 dilution; Bio Rad MCA341GA, Hercules, CA; anti-MPO, 1:100 dilution; Abcam ab9535, Cambridge, MA; anti-CD45, 1:300 dilution; Abcam ab10558) in IHC-TEK antibody diluent (IHC World, Woodstock, MD) and incubated at 4 °C overnight. The following day, slides were washed three times with PBS and stained with goat antirabbit 647 secondary antibody (1:1000 dilution; Fisher Scientific, Rockford, IL) for 1 h in the dark at room temperature. After washing away excess secondary antibody with 1 × PBS three times, slides were quickly rinsed with deionized water. Coverslips were added to the slides and mounted using ProLong Gold antifade mountant with DAPI (Fisher Scientific). Slides were cured overnight and imaged using the Axio Imager.A2 fluorescent microscope with a 20× objective and the HE CY5 filter (Zeiss filter #50), using excitation and emission wavelengths of 640 and 690 nm, respectively. Cells were counted and reported as positive cells/total number of cells for each slide.

**Brightfield Microscopy:** Digital images were acquired using the Axio Imager.A2 microscope with the 5 × 20× objectives. Lower magnification images were used for quantification of neointimal hyperplasia by measuring the lumen area, intima area (I), and media area (M) using Axiovision software (release 4.8.2, 06-2010; Pleasanton, CA). The intima/media (I/M) area ratio was calculated as an additional measure of neointimal hyperplasia. Percent occlusion, another metric of neointimal hyperplasia development, was calculated as follows: \((1 - \frac{\text{lumen area}}{\text{lumen + intima area}}) \times 100\).

**Statistical Analysis:** Results were expressed as mean ± the standard error of the mean (SEM). Origin (OriginPro 2018b, OriginLab Corporation; Wellesley Hills, MA) was used to determine differences between treatment groups using one- or two-factor analysis of variance (ANOVA) followed by a post hoc Student’s-t test. Origin was also used to calculate mean ± SEM and determine differences between rat groups for blood work analysis in characterization of the atherosclerotic model.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

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**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

arterial restenosis, collagen, nanofibers, neointimal hyperplasia, nitric oxide, targeted therapeutic

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### References

[1] E. J. Benjamin, P. Muntner, A. Alonso, M. S. Bittencourt, C. W. Callaway, A. P. Carson, A. M. Chamberlain, A. R. Chang, S. Cheng, S. R. Das, F. N. Delling, L. Djurosse, M. S. V. Elkind, J. F. Ferguson, M. Fornage, L. C. Jordan, S. S. Khan, B. M. Kissela, K. L. Knutson, T. W. Kwan, D. T. Lackland, T. T. Lewis, J. H. Lichtman, C. T. Longenecker, M. S. Loop, P. L. Lutssey, S. S. Martin, K. Matsushita, A. E. Moran, M. E. Mussolino, et al., *Circulation*, 2019, 139, e56.

[2] C. Stettler, S. Wandel, S. Alleman, A. Kastrati, M. C. Morice, A. Schomig, M. E. Pfrister, G. W. Stone, M. B. Leon, J. S. De Lezo, J. Goy, S. J. Park, M. Sabate, M. J. Sutterp, H. Kelbaek, C. Spaulding, M. Menichelli, P. Vermeersch, M. T. Dirksen, P. Cervinka, A. S. Petronio, A. J. Nordmann, P. Diem, B. Meier, M. Zwahlen, S. Reichenbach, S. Trelle, S. Windecker, P. Juni, *Lancet*, 2007, 370, 937.

[3] S. Y. Lee, M. K. Hong, Y. Jang, *Korean Circ. J.*, 2017, 47, 823.

[4] M. Joner, A. V. Finn, A. Farb, E. K. Mont, F. D. Koldodjie, E. Ladichi, R. Kutys, K. Skorjia, H. K. Gold, R. Virmani, *J. Am. Coll. Cardiol.*, 2006, 48, 193.

[5] U. S. FDA, FDA Executive Summary: Paclitaxel-Coated Drug Coated Balloon and Drug-Eluting Stent Late Mortality Panel, https://www.fda.gov/media/127698/download (accessed: January 2020).

[6] K. Katsanos, S. Sipiliopoulos, P. Kitrou, M. Krokidis, D. Karnabatisid, *J. Am. Heart Assoc.*, 2018, 7, e011245.

[7] E. S. Bahnson, H. A. Kassam, T. J. Moyer, W. Jiang, C. E. Morgan, J. M. Vercammen, Q. Jiang, M. E. Flynn, S. I. Stupp, M. R. Kibbe, *Antioxid. Redox Signal.*, 2016, 24, 401.

[8] A. J. Katkoor, N. V. K. Pothineni, D. Palagiri, J. L. Mehta, *Curr. Atheroscler. Rep.*, 2017, 19, 42.

[9] T. J. Guzik, J. Sadowski, B. Guzik, A. Jopek, B. Kapelak, P. Przybylowski, K. Wierzbiicki, R. Korbut, D. G. Harrison, K. M. Channon, *Arterioscler. Thromb. Vasc. Biol.*, 2006, 26, 333.

[10] Y. Steffen, G. Vuillaume, K. Stolle, K. Roewer, M. Lietz, J. Schueller, S. Lebrun, T. Wallerath, *Nitric Oxide*, 2012, 27, 176.

[11] F. Giacco, M. Brownlee, *Circ. Res.*, 2010, 107, 1058.

[12] C. G. Pearce, S. F. Najjar, M. R. Kapadia, J. Murar, J. Eng, B. Lyle, O. O. Aalami, Q. Jiang, J. A. Hrabie, J. E. Saavedra, L. K. Keefer, M. R. Kibbe, *Free Radic. Biol. Med.*, 2008, 44, 73.

[13] M. R. Kapadia, L. W. Chow, N. D. Tsihlis, S. S. Ahanchi, J. W. Eng, J. Murar, J. Martinez, D. A. Popowich, Q. Jiang, J. A. Hrabie, J. E. Saavedra, L. K. Keefer, J. F. Hulvat, S. I. Stupp, M. R. Kibbe, *J. Vasc. Surg.*, 2008, 47, 173.

[14] R. Zhang, R. Liu, C. Liu, L. Pan, Y. Qi, J. Cheng, J. Guo, Y. Jia, J. Ding, J. Zhang, H. Hu, *Biomaterials*, 2020, 230, 119605.

[15] C. L. Liu, X. Zhang, J. Liu, Y. Wang, G. K. Sukhova, G. R. Wojtikewicz, T. Liu, R. Tang, S. Achilefu, M. Nahrendorf, P. Libby, J. Guo, J. Y. Zhang, G. P. Shi, *Nat. Commun.*, 2019, 10, 3978.
[16] J. F. Bentzon, F. Otsuka, R. Virmani, E. Falk, Circ. Res. 2014, 114, 1852.
[17] T. Hoshino, L. Sissani, J. Labreuche, G. Ducrocq, P. C. Lavalle, E. Mesequier, C. Guidoux, L. Cabrejo, C. Hobeau, F. Gongora-Rivera, P. J. Touboul, P. G. Steg, P. Amarenco, A. Investigators, JAMA Neurol. 2018, 75, 203.
[18] H. Li, S. Horke, U. Forstermann, Atherosclerosis 2014, 237, 208.
[19] S. Basu, Scand. J. Food Nutr. 2007, 51, 48.
[20] F. Ito, Y. Sono, T. Ito, Antioxidants 2019, 8.
[21] Y. Zhao, Y. Yang, R. Xing, X. Cui, Y. Xiao, L. Xie, P. You, T. Wang, L. Zeng, W. Peng, D. Li, H. Chen, M. Liu, Atherosclerosis 2018, 271, 26.
[22] S. E. Heinonen, G. Genove, E. Bengtsson, T. Hubschle, L. Akesson, K. Hiss, A. Barneau, S. Yla-Herttuala, A. C. Jonsson-Rylander, M. F. Gomez, J. Diabetes Res. 2015, 2015, 404085.
[23] M. E. Hogg, V. N. Varu, A. K. Vavra, D. A. Popowich, M. N. Banerjee, J. Martinez, Q. Jiang, J. E. Saavedra, L. K. Keefer, M. R. Kibbe, Free Radic. Biol. Med. 2011, 50, 1065.
[24] R. C. Morales, E. S. Bahnson, G. E. Havelka, N. Cantu-Medellin, E. E. Kelley, M. R. Kibbe, Redox Biol. 2015, 4, 226.
[25] D. J. Toft, T. J. Moyer, S. M. Standley, Y. Ruff, A. Ugolkov, S. I. Stupp, V. L. Cryns, ACS Nano 2012, 6, 7956.
[26] J. M. Chan, L. Zhang, R. Tong, D. Ghosh, W. Gao, G. Liao, K. P. Yuet, D. Gray, J. W. Rhee, J. Cheng, G. Golomb, P. Libby, R. Langer, O. C. Farokhzad, Proc. Natl. Acad. Sci. 2010, 107, 2213.
[27] A. K. Vavra, G. E. Havelka, J. Martinez, V. R. Lee, B. Fu, Q. Jiang, L. K. Keefer, M. R. Kibbe, Nitric Oxide 2011, 25, 22.
[28] T. J. Moyer, H. A. Kassam, E. S. Bahnson, C. E. Morgan, F. Tantakitti, T. L. Chew, M. R. Kibbe, S. I. Stupp, Small 2015, 11, 2750.
[29] M. W. Meyers, J. S. Rink, Q. Jiang, M. E. Kelly, J. M. Vercammen, C. S. Thaxton, M. R. Kibbe, Physiol. Rep. 2017, 5, e13128.
[30] H. A. Kassam, D. C. Gillis, B. R. Dandurand, M. R. Karver, N. D. Tsiridis, S. I. Stupp, M. R. Kibbe, Nanomaterials 2020, 10, 420.