Addition of endothelial progenitor cells to renal revascularization restores medullary tubular oxygen consumption in swine renal artery stenosis

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Ebrahimi B, Li Z, Eirin A, Zhu XY, Textor SC, Lerman LO. Addition of endothelial progenitor cells to renal revascularization restores medullary tubular oxygen consumption in swine renal artery stenosis. Am J Physiol Renal Physiol 302: F1478–F1485, 2012.—Renal artery stenosis (RAS) impairs renal hemodynamics and function and affects medullary oxygenation. Although percutaneous transluminal renal angioplasty (PTRA) can improve medullary oxygenation, it may not normalize microvascular architecture. The objective of this study was to determine whether endogenous or exogenous delivery of vascular endothelial progenitor cells (EPCs) improves medullary structure and function in swine RAS. PTRA and stenting were performed in 21 pigs using implantation of irritant coils, while another group served as normal controls (n = 7 each). Two RAS groups were then treated 6 wk later with PTRA or both PTRA and EPC. Four weeks later, medullary hemodynamics, microvascular architecture, and oxygen-dependent tubular function of the stenotic kidneys were examined using multidetector computed tomography, microcomputed tomography, and oxygen-dependent tubular function. Medullary oxygenation was normalized, and fibrosis attenuated, in EPC animals, EPC were engrafted in tubular structures, oxygen-dependent tubular function was normalized, and fibrosis attenuated, despite elevated expression of hypoxia-inducible factor-1alpha and sustained downregulation of vascular endothelial growth factor. In conclusion, PTRA, with or without adjunct EPC, restores medullary oxygenation and oxygen-dependent tubular function, despite impaired medullary blood supply and oxygen uptake. These results support further development of cell-based therapy as an adjunct to revascularization of RAS.

Restoration of blood flow to the stenotic kidney by percutaneous transluminal renal angioplasty (PTRA); and stenting sometimes improves renal function and slows the progression of the injury (1, 25). Our laboratory has previously shown that PTRA can partially restore glomerular filtration rate (GFR), blood pressure, and microvascular density in the cortex, whereas renal blood flow (RBF) and cortical fibrosis were not changed (9). In clinical studies, most patients show little recovery of function or continue to lose GFR over time (20). This suggests that additional measures are required to restore microvascular integrity and function in the stenotic kidney undergoing PTRA.

Our laboratory has also previously shown that intrarenal delivery of endothelial progenitor cells (EPCs) can repair renal injury in RAS (4), as EPCs elicited increased blood flow and GFR, and reduced cortical fibrosis. Furthermore, EPCs alone did not restore medullary volume or decrease blood pressure levels and might, therefore, be useful as an adjunct to PTRA.

Most studies on reversibility of renal injury in RAS address the whole kidney. Importantly, the renal medulla can be regulated independently from the cortex and plays a central role in solute transport and concentration of urine. Due to its lower blood supply and high rates of oxygen consumption, the medulla is particularly susceptible to hypoxia and changes in renal oxygen tension (2). However, the ability of vascular interventions to restore medullary function and structure remains unclear. Therefore, this study was designed to test the hypothesis that PTRA, with or without EPC, would improve medullary structure and function in swine RAS.

MATERIALS AND METHODS

All animal procedures followed the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee. Twenty-one female domestic pigs (47.6 ± 1.3 kg) were studied for 10 wk after unilateral RAS induced by placing an irritant coil in main renal artery (15). To continuously monitor and record mean arterial pressure, a PhysioTel telemetry device (Data Science International, Arden Hills, MN) was also implanted in the femoral artery on that day.

Six weeks later, RAS animals were randomized in three groups (n = 7 each), and the degree of stenosis determined using renal angiography. One group (RAS) underwent a sham procedure, another group (RAS+PTRA) underwent PTRA and stenting, and the third group (RAS+PTRA+EPC) also received intrarenal EPC after PTRA. For stenting, under fluoroscopic guidance, a balloon carrying a mounted stent was directed proximal to the stenotic region and then inflated. Balloon expansion dilated the stenosis and implanted the stent into the wall of the renal artery, and the balloon was then deflated and removed. RAS+PTRA+EPC also received an intrarenal infusion...
of EPCs (10^6 cells/ml suspended in 10 ml of saline) over 5 min after PTRA. A fourth group of pigs underwent only sham procedures (angiography, saline infusion) and served as normal controls (n = 7).

Four weeks after treatment, or sham, animals underwent blood oxygenation level-dependent (BOLD) magnetic resonance imaging (MRI). The BOLD response, \( \Delta R2^* \), was used as a measure of oxygen-dependent tubular function (8, 27). Before each in vivo study, animals were anesthetized (Telazol 5 mg/kg and xylazine 2 mg/kg in saline), and anesthesia maintained with intravenous ketamine (0.2 mg/kg·min^-1) and xylazine (0.03 mg/kg·min^-1) (for computed tomography (CT)), or inhaled 1–2% isoflurane (for MRI) throughout the course of imaging. BOLD images were collected before and 15 min after furosemide (0.5 mg/kg) injection through an ear vein catheter, to inhibit Na/KCl reabsorption in the thick ascending limb of Henle’s Loop.

Renal function was evaluated 3–4 days later in anesthetized pigs using contrast-aided multidetector computed tomography (MDCT), to calculate cortical and medullary volumes, regional renal perfusion, GFR, and RBF (7, 14).

Pigs were eventually euthanized with a lethal intravenous dose of pentobarbital sodium (100 mg/kg) several days after completion of in vivo studies, to allow recovery and contrast agent washout. Then the kidneys were removed and immersed in saline solution containing heparin. A lobe of tissue was perfused and prepared for micro-CT, and the other lobe was shock-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) or preserved in formalin for histology (4).

**EPC preparation.** Late and early outgrowth EPCs were collected as previously described (4, 5). Late cells were cultured from mononuclear cells collected 3 wk before the injection and early EPCs 1 wk before injection. Before delivery, cells were labeled with fluorescent beads and membrane dye (CM-DiI, 5 μl/ml). The final blend of a similar number of early and late cells (total 10^7) was delivered into the renal artery of the stenotic kidney.

**BOLD imaging.** BOLD scans were performed on a 3T (GE Medical Systems, Milwaukee, WI) scanner using fast gradient echo with 16 echoes. Imaging parameters were set to repetition 100 ms, 2.1–27 ms, 40°, 32 cm, 7 mm, and 256 planes using 16 echoes. Systems, Milwaukee, WI) scanner using fast gradient echo with renal artery of the stenotic kidney.

**EPC localization.** EPCs were localized by tracking (CM-DiI) labeled EPCs in frozen 5-μm-thick cross-sectional slices of the tissue, with 4,6-diamidino-2-phenylindole (nuclear stain) and cytookeratin (tubular marker) stains used to confirm location. EPCs were counted in both the cortex and medulla in low-power fields, and the engraftment relative to the number of injected cells was calculated.

**MDCT imaging.** A pigtail catheter was advanced through the left jugular vein sheath and placed in the superior vena cava for contrast injections, and the pigs were then transferred to the MDCT gantry (Somatom Sensation 64; Siemens Medical Solutions, Forchheim, Germany). All tomographic levels containing both kidneys were cannulated a segmental renal artery perfusing a kidney lobe was cannulated and tubular oxygen consumption.

Renal fibrosis and inflammation were quantified in 5-μm trichrome and inducible NOS (iNOS)-stained slides, respectively. Because capillaries are below the resolution of our micro-CT technique, medullary capillary density was determined at \( \times 1,000 \) magnification in H&E-stained slides using an ApoTome microscope (Carl ZEISS SMT, Oberkochen, Germany). Capillaries were identified by the presence of lumen, red blood cells, and/or an endothelial cell lining (26).

**Statistical analysis.** Results are presented in mean ± SE format. Paired Student t-test was used for comparisons within groups, and ANOVA for comparison among groups, followed by an unpaired Student t-test with Bonferroni correction. For P values < 0.05, differences were considered significant.

**RESULTS**

An average of 16.3 ± 2.0% of the total injected EPCs were detected in the stenotic kidney. In each slice, \( \sim 76.1\% \) of the cells were located in the cortex and observed in the vascular and tubular structures, whereas, in the medulla, the remaining cells were observed in the interstitium or engrafted in tubules (Fig. 1A).

**Hemodynamics.** The RAS, RAS+PTRA, and RAS+PTRA+ EPC groups all developed significant and similar RAS by 6 wk...
after implantation of the irritant coil (76 ± 6, 73 ± 6, and 71 ±
3%, respectively). Subsequent PTRA in 14 pigs was techni-
cally successful (residual stenosis <10%) and induced reduc-
tion in mean arterial pressure (−11 ± 10 and −16 ± 4 mmHg
in RAS+PTRA and RAS+PTRA+EPC, respectively), which
remained unchanged in RAS (−2 ± 7 mmHg).

Four weeks after intervention or sham, cortical volume
remained smaller in RAS and RAS+PTRA compared with
normal, but was restored to normal size in RAS+PTRA+EPC
(Table 1). Medullary volumes in RAS and RAS+PTRA+EPC
tended to be lower (P = 0.09 and P = 0.06, respectively), and
in RAS+PTRA were significantly smaller, than in the control
group. RBF, cortical blood flow, and GFR were lower in RAS
animals compared with the control. In RAS+PTRA, cortical
blood flow and GFR were not different than normal shams.
Furthermore, in RAS+PTRA+EPC, both were fully restored.
Medullary blood flow in RAS, RAS+PTRA, and RAS+PTRA+
EPC all tended to remain reduced (P = 0.06, P = 0.06, and
P = 0.09 vs. normal, respectively).

Medullary tubular oxygen-dependent function. Basal me-

ulillary R2* values were slightly (but not significantly) elevated
in RAS and RAS+PTRA+EPC compared with shams (Table
1). The change in R2* after furosemide primarily reflects
the change in deoxyhemoglobin related to inhibition of active
tubular chloride transport and is, therefore, considered a sur-
rogate marker for this aspect of tubular function (27). After
furosemide, medullary R2* declined in all groups, except for
RAS. The degree of change in the BOLD index in response
to furosemide, %ΔR2*, was attenuated in RAS and RAS+PTRA
(P < 0.05 vs. normal), but was preserved in RAS+PTRA+EPC
(Fig. 1B).

A weak but significant correlation was observed between
GFR and medullary BOLD response (R2 = 0.2, P < 0.05) and
between cortical blood flow and the medullary perfusion (R2 =
0.2, P < 0.05).

Microvasculature. Significant loss of medullary microves-
sels with diameter smaller than 100 μm was observed in RAS,
RAS+PTRA, and RAS+PTRA+EPC kidneys (Figs. 2, A and
B). Histology revealed that the medullary capillary densities
were significantly lower in all three groups compared with
the shams (Fig. 2C).

Tissue studies. Expression of VEGF in the cortex in
RAS+PTRA+EPC restored close to the normal, but remained
impaired in the other two RAS groups, whereas eNOS expres-
sion was significantly reduced in all RAS groups. The expres-
sion of NAD(P)H oxidase p47 tended to be higher than normal
in RAS (P = 0.09) and RAS+PTRA (P = 0.07), but did not
reach statistical significance levels due to variability (Fig. 3A).

In the medulla, VEGF in all three RAS groups was reduced
compared with normal (P < 0.05, Fig. 3B), and the expression
eNOS was significantly elevated in RAS+PTRA and
RAS+PTRA+EPC (P < 0.05), but not in untreated RAS.
HIF-1α expression, as an index of hypoxia, significantly in-
creased in RAS+PTRA+EPC, strongly tended to increase in
RAS+PTRA (P = 0.06), but remained unchanged in RAS
compared with the control group. The expression of NADPH
was increased in all three RAS groups (P < 0.05 vs. normal).
On the other hand, the expression of TNF-α in the medulla was
significantly elevated in RAS and RAS+PTRA, but was re-
duced in RAS+PTRA+EPC (P = 0.09 vs. normal).

Medullary tubulointerstitial fibrosis (trichrome staining) was
significantly greater than normal in all experimental groups,
but was significantly blunted in RAS+PTRA+EPC compared
with RAS and RAS+PTRA (Fig. 4). Furthermore, immuno-

activity of the inflammatory marker iNOS showed a similar
pattern and was also significantly reduced compared with that
of the other two RAS groups (Fig. 4).

DISCUSSION

This study shows that, in contrast to the cortex, PTRA, with
or without adjunct EPC delivery, conferred no recovery of
medullary hemodynamics and microvascular architecture. In
this experimental study, restoration of main vessel blood flow
by PTRA was associated with partial recovery of cortical
volume and GFR. However, medullary capillary and micro-
vascular density in treated swine remained below normal and
similar to those in untreated RAS animals, and medullary
blood flow tended to be reduced in all experimental groups.
In contrast, changes in deoxyhemoglobin after furosemide, taken
as a measure of oxygen consumption related to tubular solute
transport, indicated greater recovery of tubular function in

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RAS+PTRA+EPC compared with RAS+PTRA alone. Furthermore, inflammation and tubulointerstitial fibrosis in EPC-treated kidney medullas were reduced, although they remained above that in sham animals, and markers of hypoxia (HIF-1α) and oxidative stress (p47) were unchanged compared with RAS+PTRA alone. This study, therefore, implies that the combination of PTRA and EPC confers selective improvement in medullary oxygen-dependent tubular function at the expense of lingering hypoxia, yet a complementary decrease in inflammation likely prevents enduring fibrosis.

A hemodynamically significant RAS leads to a decrease in RBF and, when severe, can decrease renal oxygen supply (11), which can trigger a vicious cycle of progressive kidney injury. In fact, chronic hypoxia in the tubulointerstitium has been proposed to represent a “common pathway” to end-stage renal failure (19, 22). Interstitial fibrosis resulting from hypoxia in turn aggravates peritubular capillary rarefaction and reduces oxygen delivery to the microcirculation, thereby perpetuating a chain of events of irreversible injury.

In this study, RAS animals developed hypertension and showed decreases in RBF and GFR. BOLD MRI showed a blunted reduction of R2* after furosemide in RAS, implying attenuated oxygen consumption related to tubular transport, and ex vivo studies revealed an increase in medullary tubulointerstitial fibrosis. Furthermore, micro-CT imaging revealed measurable loss of small medullary vessels, and histology confirmed regression of medullary capillaries.

Lack of sufficient blood supply to the medulla may result in impaired oxygen delivery and potentially hypoxia. Interestingly, despite impaired blood flow, vascular rarefaction, oxi-

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<th>Table 1. Single kidney function and volume after 10 wk of renal artery stenosis, 4 wk after intervention, or sham</th>
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<td><strong>Shame</strong></td>
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Values are means ± SE. RAS, renal artery stenosis; PTRA, percutaneous transluminal renal angioplasty; EPC, endothelial progenitor cells; R2*, blood oxygenation index. *P < 0.05 vs. sham. †P < 0.05 vs. RAS.

Fig. 2. A: microcomputed tomography images of renal microvessels in sham, RAS, RAS+PTRA, and RAS+PTRA+EPC. The vascular density was estimated in outer strip of medulla (highlighted) in all groups. Medullary densities of vessels with diameters smaller than 100 µm were decreased in all groups compared with sham (B), as was capillary density estimated from histology (C). Values are means ± SE. *P < 0.05 vs. sham.
dative stress, and fibrosis, medullary HIF-1α expression and basal R2* (a measure of deoxyhemoglobin levels) remained unaltered in RAS, possibly due to a parallel decrease in oxygen-dependent tubular transport and thereby oxygen consumption. Our previous studies have shown that basal R2* in poststenotic human kidneys only increases during very severe ischemia (11). In fact, this disruption in tubular function might represent a primary protective mechanism to minimize hypoxia.

Fig. 3. Representative (two bands per group) immunoblots demonstrating protein expression of VEGF, endothelial nitric oxide synthase (eNOS), and P47 in the cortex (A) and VEGF, eNOS, hypoxia-inducible factor-1α (HIF-1α), P47, and TNF-α in medulla (B). Values are means ± SE. *P < 0.05 vs. sham.
and preserve tubular cells, secondary to a decrease in filtrate input (GFR) or both.

Our study shows that PTRA alone partially restored medullary oxygenation response to furosemide in RAS, although it remained lower than that in the control group. Interestingly, this enhancement was achieved without change in tubulointerstitial fibrosis or capillary density. Since PTRA restored GFR, it is possible that an increase in tubular solute delivery accounted for the improved tubular transport function, which then became more responsive to furosemide. This postulation is supported by the modest but significant correlation between BOLD and GFR in the RAS groups. On the other hand, this increase in oxygen-dependent tubular transport and oxygen consumption might have, in turn, induced hypoxia, as reflected in the trend for medullary expression of HIF-1α to increase.

The sustained increase in oxidative stress (represented by p47 expression) was also consistent with hypoxic conditions. The concurrent upregulation of eNOS expression may have aimed to compensate for the increased oxidative stress and diminished oxygen supply, or to facilitate nitric oxide-dependent tubular transport in the face of persistently impaired medullary blood flow (6).

Remarkably, in RAS+PTRA+EPC, the response to furosemide was fully restored to control values, yet, similar to the RAS+PTRA group, the avid tubular transport might have exacerbated hypoxia, as suggested by upregulation of medullary HIF-1α expression that has reached statistical significance. Speculatively, oxygen-related tubular function in this group might have been bolstered partially as a result of restored GFR and EPC engraftment into medullary tubular structures, as well as their autocrine and paracrine activities. For example, the anti-inflammatory effect of EPC (decreased TNF-α and iNOS expression) and, consequently, reduced fibrosis might have facilitated oxygen extraction by tubular cells. Similar to RAS+PTRA, in RAS+PTRA+EPC we observed upregulation of eNOS expression, possibly to increase the medullary blood flow and tubular function and offset oxidative stress and hypoxia. Contrarily, as opposed to the cortex after intrarenal EPC delivery (4), no significant improvement in medullary microvascular density or VEGF expression was observed in RAS+PTRA+EPC, perhaps because no EPC engrafted in medullary vessels, or the vessels were replaced by interstitial fibrosis. Reduced expression of VEGF, despite elevated expression of HIF-1α, may reflect an intrinsic protective anti-inflammatory mechanism that is activated even at the cost of hypoxia (16, 17, 21). Nevertheless, despite evidence of lingering hypoxia, medullary tubulointerstitial fibrosis was blunted in RAS+PTRA+EPC compared with RAS and RAS+PTRA. Possibly, EPC inhibited fibrosis directly or through their anti-inflammatory effect, thereby interrupting the vicious cycle of renal disease progression.

The weak correlation of cortical blood flow with medullary perfusion supports the notion that the influence of hemodynamic improvements in cortex on BOLD response is modest. The differential expression level of VEGF, eNOS, and NAD(P)H in the cortex compared with the medulla

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**Fig. 4.** Medullary staining of inducible nitric oxide synthase iNOS (A; top) as an index of inflammation and trichrome (A; bottom) of tubulointerstitial fibrosis (×40), and the quantifications (B). Values are means ± SE. *P < 0.05 vs. sham. †P < 0.05 vs. RAS+PTRA+EPC.
argue that those regions are regulated differently in RAS. Indeed, EPC seem to be less effective in improving medullary hemodynamics and vascular structure than those of the cortex.

Limitations. Human RAS is a multifactorial disease, which is difficult to fully simulate in animal models. In addition, RAS in humans develops over longer periods of time than in our model, which might affect the interplay between RAS and other factors involved in progression of the disease. Nevertheless, the chain of events and the mechanism of tissue injury in our RAS model closely resemble those in humans (19, 23, 24). We used the arcuate arteries as the accepted anatomic boundary between the cortex and outer medulla in our micro-CT analysis, yet we cannot rule out the possibility that some cortex or inner medulla was inadvertently included. The reason for preferential engraftment of EPC in medullary tubules rather than blood vessels needs to be elucidated in future studies. Speculatively, strategies can be developed to increase their vascular engraftment, such as enrichment with VEGF receptors, or adjunct therapies to simultaneously increase vascular density and decrease remodeling. Finally, a renal protective effect of isoflurane in ischemia-reperfusion has been reported in the past (28). Although a short-term exposure to the anti-inflammatory properties of isoflurane anesthesia is more likely to affect acute rather than the chronic renal inflammation and injury observed in our model, its potential influence on renal hypoxia assessed by BOLD MRI cannot be completely ruled out and warrants further investigation. In conclusion, PTRA improved GFR and solute delivery to the medulla, which might somewhat aggravate medullary hypoxia, in the absence of small-vessel neovascularization and an increase in medullary blood flow. Adjunct delivery of EPC further augmented RBF and tubular transport function, but, although this functional burden led to a slight further rise in medullary oxygen consumption and thereby hypoxia, this intervention decreased medullary fibrosis, possibly due to increased availability of nitric oxide or direct anti-fibrotic and anti-inflammatory effects of EPC. This study, therefore, supports further development of cell-based approaches as an adjunct therapy to preserve medullary outcomes in RAS after PTRA.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


