Published in final edited form as:

Arch Physiol Biochem. 2011 February ; 117(1): 1–7. doi:10.3109/13813455.2010.512042.

X-ray imaging of differential vascular density in MMP-9^{−/−}, PAR-1^{−/+}, hyperhomocysteinemic (CBS^{−/+}) and diabetic (Ins2^{−/+}) mice*

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Abstract

Although protease activated receptor-1 (PAR-1) and matrix metalloproteinase-9 (MMP-9) play significant role in vascular remodelling in hyperhomocysteinemia (HHcy due to cystathionine beta synthase deficiency, CBS^{−/+}) and diabetes, mechanism remains nebulous. We hypothesized that differential vascular density and remodelling in different vascular beds in HHcy and diabetes were responsible for an adaptive metabolic homeostasis during the pathogenesis. To test this hypothesis, vascular density in mice lacking PAR-1, MMP-9, CBS and Insulin-2 gene mutant (Ins2^{−/+}, Akita) was measured and compared with wild type (WT, C57BL/6J) mice. The vascular density was detected by x-ray angiography using KODAK 4000 MM image station, using barium sulphate as contrasting agent. The % vascular density in the hearts of WT, CBS^{−/+} (HHcy), MMP-9^{−/−}, PAR-1^{−/+} and Ins2^{−/+} (type-1 diabetes) was 100 ± 2.8 , 85 ± 3.3 , 90 ± 3.3 , 95 ± 3.8 and 73 ± 1.7 , respectively. The vascular density in CBS^{−/+} and Akita hearts decreased while it was increased in lungs of CBS^{−/+} and MMP-9^{−/−}. There was decreased vascular density in liver and kidney of Akita mice. Vascular density in brain, kidney and mesentery was decreased in CBS^{−/+} mice. These findings support the notation that metabolic derangement in diabetes and HHcy causes the chronic decline and/or rarefaction in vascular density.

Keywords

remodelling; hyperglycaemia; portal circulation; heart; lung; renal; liver; mesentery; endostatin; angiotensin; capillary rarefaction; angiography

Introduction

Remodelling by its very nature implies synthesis and degradation of extracellular matrix (ECM). Matrix metalloproteinases (MMP) degrade ECM and alters the concentration and composition of ECM components. The protease activated receptor (PAR, a G-protein coupled receptor, GPCR) plays significant role in activating downstream signalling pathways Gi (inhibitory) and Gs (stimulatory) (Conant *et al.*, 2002). This leads to accumulation of focal adhesive complex and stimulates the kinases, a process involving in vasculogenesis/angiogenesis (Macfarlane *et al.*, 2001; Tsopanoglou & Maragoudakis, 2007).

* A part of this study was supported by NIH grants: HL-71010; HL-74185; HL-88012; and NS-51568.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

The elevated levels of homocysteine (Hcy) known as hyperhomocysteinemia (HHcy) cause vascular dementia, cerebral vascular complication and retardation (Nilsson *et al.*, 2008; Sala *et al.*, 2008; Davis *et al.*, 2009; Kivipelto *et al.*, 2009; Regalado Dona *et al.*, 2009).

Hyperhomocysteinemia causes constrictive vascular remodelling by increased collagen deposition in the vessel wall (Guo *et al.*, 2008). There is linear correlation between plasma homocysteine levels and aortic wall thickness and HHcy causes increase in aortic blood pressure, resistance, wall thickness by ECM remodelling (Ovechkin *et al.*, 2006). We have shown that homocysteine decreases blood flow to the brain due to vascular resistance in carotid arteries (Kumar *et al.*, 2008).

Hyperglycaemic condition was shown to induce vascular remodelling by collagen deposition and increased stiffness in aortic wall (Guo *et al.*, 2008). Increased oxidative stress during hyperglycaemia uncouples endothelial nitric oxide synthase (eNOS) and induces vascular remodelling (Sasaki *et al.*, 2008). There is differential regulation of micro and macro vascular beds due to changes in matrix scaffolding and matrix degrading MMP expression in type-2 diabetes (Song & Ergul, 2006).

PAR-1 plays crucial role in inducing angiogenic factor vascular endothelial growth factor (VEGF) and thus contributes to angiogenesis (Maeda *et al.*, 1999). PAR-1 is also involved in the vascular remodelling of aortic wall and intimal thickening (Macfarlane *et al.*, 2001; Archiniegas *et al.*, 2004). Matrix metalloproteinases especially MMP-9 induces outward vascular remodelling (Ota *et al.*, 2009). Diabetes and elevated levels of Hcy both independently induce matrix remodelling (Rajkumar *et al.*, 1999; Uemura *et al.*, 2001; Hao & Yu, 2003; Song & Ergul, 2006). The levels of Hcy are elevated in diabetic myopathies (Rajkumar *et al.*, 1999). However, it is unclear whether HHcy and diabetes induce vascular heterogeneity and remodelling in different vascular beds. The significance of the term “vascular remodelling” is related to increase in smooth muscle mass, rearrangements of cells within the vascular wall, changes in luminal dimensions associated with relatively small changes in wall thickness (Gibbons & Dzau, 1994). Here, we measured the vascular density. The differential change in the vascular density in between the MMP-9^{-/-}, PAR-1^{-/+}, CBS^{-/+} and Ins2^{-/+} as compared to WT mice may be the consequence of vascular remodelling.

Materials and methods

Animals

Wild type (WT, C57BL/6J), CBS^{-/+}, MMP-9^{-/-} and PAR-1^{-/+} and diabetic mice (Ins2^{-/+}) were obtained from Jackson laboratories, housed in the animal care facility at university of Louisville. Mice were fed with standard chow and water. Most of the knockout/mutant mouse used in this study was on similar background (i.e., C57BL/6J). The wild type mouse used in this study was C57BL/6J, and the knockouts were derived primarily from this strain. The phenotype of MMP-9^{-/-}, PAR-1^{-/+}, CBS^{-/+} and Ins2^{-/+} mice is well established (JAX mice data base). Mice were 10 to 12 weeks old with approximate weight of 25 to 30 g and all animal procedures were performed in accordance with National Institute of Health guide lines for animal research and were reviewed and approved by the Institute Animal Care and Use Committee of the University of Louisville. All mice were anaesthetized with the same dose of sodium pentobarbital according to body weight (70 mg per kg). Right carotid artery was dissected out and cannulated with PE-10 tubing. Similarly for pulmonary angiogram and hepatic angiogram, jugular vein was cannulated. All mice were heparinized with heparin saline (10 units per ml) and euthanized by supersaturated KCl solution. The KCl solution is used for the reason that heart stops in diastole with opened coronary arteries.

Preparation of contrast agent (Barium sulphate)

Barium sulphate has been widely used by the radiologists in “Barium meal”, “Barium swallow” and “Barium enema” preparations to visualize the structural and motility abnormalities of the gastro intestinal tract mostly in the paediatric population. However, if it enters the systemic circulation, barium sulphate can be lethal (Zalev, 1997). Though iodinated contrast agents are mostly used for intravascular imaging, with small animals like mice and basic X-ray, we found barium gives better vascular imaging than iodine compounds.

The size of barium particles ranges from 1 to 100 μm , depending on the solubility of barium sulphate in aqueous solution (Myojin *et al.*, 2007). We dissolved barium sulphate in 50 mM Tris-buffer (pH 5.0) and infused slowly at a constant pressure and flow with a syringe pump at 200 $\mu\text{L}/\text{min}$ using the intravascular route; this produced the optimal visualization of vascular density. Animals were dissected open to expose various organs, and angiograms were performed.

Angiogram studies

All images were taken with Kodak 4000 MM image station. Dissected animals were placed in the X-ray chamber and angiograms were captured with high penetrative phosphorous screen by 31 KVP X-ray exposures for 3 minutes and aperture settings of approximately 4.0, f-stop-12 and variable zoom for different organs.

Data collection and statistics

The angiograms of ex-vivo organs were recorded. Vascular density was determined using Image-Pro Plus software. The images were analysed by depicting the pixels in arbitrary units (AU). The value from wild type (WT) mouse was considered 100%. The values for CBS^{-/+}, MMP-9^{-/-}, PAR-1^{-/+} and Ins2^{-/+} were compared with WT. Each bar was the mean $\pm$ SD from $n = 6$ in each group. Student *t*-test was used to calculate the statistical significance. A $p < 0.05$, compared with WT was considered significant. The goal of this study was to determine the differential vascular density in different vascular beds. Therefore, to compare the same organ in different strains of mice, we used WT, CBS^{-/+}, PAR^{-/+}, MMP-9^{-/-} and Ins2^{-/+} mice. We can only compare one organ in different animals and not the different organs.

Results

Gravimetric data

There was robust cardiac hypertrophy in CBS^{-/+} mouse compared with WT (Table 1). The liver weight was significantly decreased in MMP-9^{-/-} mouse compared with WT. This may suggest a role of MMP-9 in liver atrophy (Table 1).

Coronary vasculature in hyperhomocysteinemia and hyperglycaemia

To demonstrate whether the coronary vascular density in wild type (WT, C57BL/6J), CBS^{-/+}, MMP-9^{-/-}, PAR-1^{-/+} and Ins2^{-/+} (Akita diabetic) mice was altered, whole body soft tissue X-ray analysis was performed. Aorta and branching of various arteries and coronaries were identified (Figure 1A). Although vascular density in MMP-9^{-/-} and PAR-1^{-/+} was similar to WT, the quantitative by image-pro software (Figure 1B, Image-Pro panel) data revealed a decrease in vasculature in CBS^{-/+} and ins2^{-/+} mice as compared to WT mice (Figure 1B).

Cerebral vasculature in hyperhomocysteinemia and hyperglycaemia

Similar to coronaries, we observed a decrease in cerebral vascular density in CBS-/+ and ins2-/+ as compared to other strains of mice (Figure 2).

Differential vascular density in pulmonary, liver, mesentery, and renal tissues

The whole body soft-tissue barium contrast X-ray angiography visualized lung, liver, mesentery and kidney (Figure 3). Contrary to heart and brain, the quantitative analysis of vasculature in the lung demonstrated increase in vascular density in CBS-/+ and MMP-9-/- compared with WT mice. Interestingly, the pulmonary vasculature was decreased in diabetic mice when compared with WT mice (Figure 4). The vascular density in hepatic tissue was lower in CBS-/+ and Ins2-/+ compared with WT mice (Figure 5). In the mesentery, the vascular density was decreased in HHcy-CBS-/+ and PAR-1-/+ mice compared with WT controls (Figure 6). However, in MMP-9-/- mice the mesenteric vascular density was increased when compared with WT mice (Figure 6). In the kidney, the vascular density in CBS-/+ and diabetic Akita mice was decreased compared with WT controls (Figure 7A). Similar results were obtained for renal vein, when we infused barium contrast into the jugular vein (Figure 7B). The excess contrast from the infusion of right jugular vein entered the inferior venacava, and filled the renal veins retrogradely and the images were captured. The results showed that there was a decrease in venous vasculature in both CBS-/+ and Ins2-/+ mice suggesting the differential role of hyperhomocysteinaemia and hyperglycaemia in vascular remodelling in different vascular beds.

Discussion

Although numerous studies have been carried out with MMP-9-/-, PAR-1-/-, CBS-/+ and Ins2-/+ mice, there is no known vasculature-associated modification studied in MMP-9-/-, PAR-1-/-, CBS-/+ and Ins2-/+ mice. Our study demonstrated for the first time a correlation between the increase/decrease of vascular density and the type of knockouts in mice.

Vascular imaging is a wide area of interest in cancer research and other vascular diseases. Differences in cerebrovascular anatomy in different strains of mice were previously reported and suggested that there was altered vascular anatomy and density in genetically engineered transgenic mice (Maeda *et al.*, 1999; Beckmann, 2000; Chalothorn *et al.*, 2007). The same information was also shown with high resolution magnetic resonance angiogram (MRA) in *in-vivo* mouse brain (Beckmann, 2000). Here we elucidated the role of MMP-9 and PAR-1 in differential expression of vascular heterogeneity in hyperhomocysteinemia and hyperglycaemia using transgenic mice-lacking CBS, MMP-9, PAR-1, and diabetic insulin-2 (Ins2-/+) mice and compared with the regular wild type (WT, C57BL/6J) mice. We have demonstrated further beneficial use of barium contrast X-ray angiography which is highly cost effective and uses the very basic X-ray machine, unlike high resolution CT and MRI. Overall, here we discovered the use of basic X-ray machine for producing high resolution images using the cost effective barium sulphate contrast.

The apparent aortic diameter in diabetic Ins2-/+ Akita mice was higher than other strains. This may suggest that in hyperglycaemia there was intravascular remodelling. Interestingly, the MMPs were activated in aorta of diabetics (Song & Ergul, 2006; Gorgone *et al.*, 2009). Although MMP-9-/-, PAR-1-/+ showed similar vascular density as wild type, the decrease in coronary vasculature in HHcy-CBS-/+ and diabetic hearts may reveal a role of Hcy and glucose in decrease in vascular density (Figure 1). Previously, we have demonstrated that Hcy decreased the angiogenesis (Shastri and Tyagi, 2004). Here we also suggest that hyperglycaemia decreases vascular density in heart.

Although it is known that Hcy caused cerebral vascular dementia (Nilsson *et al.*, 2008; Davis *et al.*, 2009; Kivipelto *et al.*, 2009; Regalado Dona *et al.*, 2009), it was unclear whether it led to decrease in vascular density and vasospasm. We demonstrated the HHcy caused decrease in vascular density in the brain (Figure 2).

Contrary to heart and brain the pulmonary vasculature was increased in HHcy as well as in MMP-9^{-/-} mice. These results may reveal that during compensatory remodelling, CBS^{-/+} mice may be increasing their pulmonary circulation. Similarly, in MMP-9^{-/-} mice, we demonstrated increased vascular density and decreased generation of anti-angiogenic factors (angiostatin and endostatin) (Givvimani *et al.*, 2010). Here, we show increase in vascular density in these mouse lungs (Figure 4).

The portal hypertension due to hyperhomocysteinemia has been reported (Distrutti *et al.*, 2008; Gorgone *et al.*, 2009). We show here that in HHcy (CBS^{-/+} mice) there was decrease in vascular density (Figure 5). Similarly, in diabetes, hyperglycaemia may also decrease the hepatic circulation.

Barium contrast infused through carotid arteries pass through the aorta to the entire systemic circulation perfusing all the patent vasculature. The mesenteric vasculature is dissected out carefully and exposed to X-ray. The results showed that there is increased vascular density in MMP-9^{-/-} mice and contrary to this, the mesenteric vascular density is decreased in CBS^{-/+} and PAR-1^{-/+}, stating the role of MMP-9, PAR-1 along with hyperhomocysteinemia in mesenteric vascular remodelling (Figure 6). These data once again showed the differential vascular heterogeneity in different vascular beds of different strains of mice.

The kidneys were also well exposed and radio graphed, to get the X-ray angiogram. The results from this study show that there was decreased renal arterial density both in CBS^{-/+} and Ins2^{-/+} mice in comparison to wild type control C57BL/6J mice (Figure 7). This again revealed the role of hyperhomocysteinemia and hyperglycaemia in the vascular remodelling in renal arteries. Similar pattern was observed in the renal venous circulation with the infusion through right jugular vein.

Conclusion

Although PAR-1, MMP-9, CBS, and insulin gene products are associated with vasculogenesis/angiogenesis, there is no study that compares the difference in vascular density with respect to PAR-1^{-/+}, MMP-9^{-/-}, CBS^{-/+} and Ins2^{-/+} genes. Our study is the first to compare the role of PAR-1, MMP-9, CBS and insulin mutant gene products in differential vascular density in different vascular beds.

Limitations

One of the limitations of this study is that it is post mortem angiography and the animal will not be alive after the procedure as barium is toxic in the blood stream and more over use of KCl to stop the heart in diastole will kill the animal otherwise.

There is no explanation for the biological mechanisms beyond the organ-specific increase or decrease in vascular density; when we envisage a reduction of vascular mass (or a rarefaction); a complementary measurement of the blood flow may complete the data.

Several types and levels of “vascular heterogeneity” are known, the significance of this term in this particular context is questionable. Although “vasculogenesis” occurs during the very

early developmental stages of an organism when the blood vessel pathways are created, we actually measured the existing vascular density and compared between the knockouts.

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Figure 1.

A. Barium contrast arterial angiograms of the different strains of mice with heart intact along with aorta and other major vessels. B. Coronary angiogram of ex-vivo heart. Wild type (WT) mouse heart was compared with CBS-+/-, MMP-9-/-, PAR-1-/+ and Ins2-+/- Akita. The panel, Image-Pro demonstrates quantitative analysis and visualisation of vasculature using Image-Pro software. The bar graph presents image analysed with Image-Pro Plus, depicted in pixels and converted to per cent change with respect to WT. Each bar is the mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT.

Mouse Cerebral Angiograms

Figure 2.

Cerebral angiogram with barium contrast in different strains of mice. CBS^{-/-} and Ins2^{-/-} showed decrease in vascular density compared to wild type mice. Images were analysed with Image-Pro Plus and represented in a bar diagram. Each bar is mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT.

Figure 3.

Angiogram of gastro intestinal tract viscera long with liver shown in all different strains of mice CBS-/-, MMP-9-/-, PAR-1-/+ and Ins2-/+ along with C57BL/6J wild type mice. IVC (inferior vena cava) is seen along with hepatic vasculature and other viscera.

Figure 4.

Pulmonary angiogram of different strain of mice: C57BL/6J, CBS^{-/-}, MMP-9^{-/-}, PAR-1^{-/-} and Ins2^{-/-}. Images were quantified with Image-Pro analysis software and represented in bar diagrams. Each bar is the mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT.

Figure 5.

Angiogram of hepatic venous circulation in C57BL/6J, CBS^{-/-}, MMP-9^{-/-}, PAR-1^{-/-} and Ins2^{-/-} mice and the quantified images were shown in bar diagrams. Each bar is the mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT.

Mesenteric Vasculatric Angiograms

Figure 6.

The angiogram of the intestinal mesentery of the different strains of mice: C57BL/6J, CBS^{-/+}, MMP-9^{-/-}, PAR-1^{-/+} and Ins2^{-/+}. In MMP-9^{-/-} strain, the arrow indicates the higher vascular density as compared to other strains. Vascular density was assessed by Image-Pro Plus software and depicted in bar diagrams. Each bar is the mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT.

Figure 7.

A. Renal arterial angiogram in different strains of mice: C57BL/6J, CBS^{-/-}, MMP-9^{-/-}, PAR-1^{-/-} and Ins2^{-/-}. Images were quantified with Image-Pro Plus software and the data is represented in bar diagrams. Each bar is the mean $\pm$ SD from $n = 6$ in each group. * $p < 0.05$ compared with WT. B. Renal venous angiogram, following the similar pattern of vascular density as in renal artery angiogram.

Table 1

The gravimetric data of wilt type (WT), CBS^{-/+} (HHcy), MMP-9^{-/-}, PAR-1^{-/+}, and diabetic Akita (Ins2^{-/+}) mice. Values are mean from $n = 6$ in each group.

	Body (g)	Heart (mg)	Lung (mg)	Liver (mg)	Kidney (mg)	Brain (mg)
WT	28	138	211	1721	202	510
CBS ^{-/+}	30	159*	204	1662	198	484
MMP-9 ^{-/-}	26	127	251	1314*	205	484
PAR-1 ^{-/+}	31	152*	204	1738	220*	505
Ins2 ^{-/+}	24	123	145*	1692	198	457*

* $p < 0.5$ compared with WT.