Reduced Neovascularization in Aged Rats: A Study Using Lipopolysaccharide-induced Inflammation

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Abstract

Aged tissues are associated with reduced capillary density and delayed neovascularization. The aging environment is characterized by increased oxidative stress, which damages macromolecules, and thereby causes apoptosis of endothelial cells. Lipopolysaccharide (LPS) has been shown to have angiogenic property. Therefore, the objective of this study was to examine whether neovascularization after LPS-induced inflammation in aged rats is decreased when compared to adult rats. Male Wistar rats were divided into four groups: adult (aged 6-8 months), aged (aged 22-24 months), adult + LPS, and aged + LPS. Blood perfusion, capillary vascularity, plasma and tissue MDA, and tissue VEGF levels were measured. Plasma MDA in aged group was higher than in adult group ($P < 0.05$). Blood perfusion and capillary vascularity (%CV) in aged group were lower than in adult group ($P < 0.05$). Blood perfusion and %CV in aged + LPS group were significantly lower than those in adult + LPS group. Aged rats have reduced capillary vascularity when compared to adult rats. LPS could induce neovascularization in both adult and aged rats. However, aged rats have reduced neovascularization after LPS pre-treatment when compared to adult + LPS rats.

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Effective postnatal neovascularization is important for the alleviation of various pathologies such as ischemic diseases, brain diseases, and wound injuries. Various brain diseases such as stroke, Alzheimer's, and Parkinson's diseases are associated with alterations in structure and function of the cerebral vasculature.[1] Aged tissues are associated with reduced capillary density and delayed neovascularization when compared to their young counterparts.[2]

Postnatal neovascularization is a combination of two processes, namely angiogenesis and vasculogenesis. Angiogenesis is the growth of blood vessels from pre-existing endothelial cells, whereas vasculogenesis is the formation of new blood vessels from circulating stem cells.[3] The aging environment is characterized by increased oxidative stress,[4] which causes stem cells to have diminished function.[5]

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Lipopolysaccharide (LPS), a molecule found in outer membrane of Gram-negative bacteria, is known to induce strong immune response in animals. LPS has been shown to induce angiogenesis in the rat,[6] and other studies have used LPS for inflammation models in murine skin.[7,8] Furthermore, LPS has been shown to increase stem cell function in a study done by Yao *et al*.[9] They found that LPS-treated mesenchymal stem cells transplanted into infarcted rat myocardium resulted in increased vascular density, greater engrafted cell survival rate, and decreased apoptosis of the myocardium, when compared to non-treated cells.

Therefore, the objective of this study was to examine whether neovascularization after LPS-induced inflammation in aged rats is decreased when compared to adult rats.

Materials and Methods

Animal preparation

Male Wistar rats (8 weeks old; body weight 200-250 g) were obtained from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornpathom, Thailand. The experimental procedures were conducted according to the guidelines for experimental animals by the National Research Council of Thailand, and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. The animals were housed in a room with 12:12 hour light-dark cycle until they reached ages of 6 months and 22 months. All rats were allowed free access to normal chow and tap water *ad libitum*.

Skin inflammation model

Animals were divided into four groups: adult (aged 6-8 months), aged (aged 22-24 months), adult + LPS (aged 6-8 months), and aged + LPS (aged 22-24 months). Dorsal skin inflammation was induced in the LPS groups by subcutaneous injection of lipopolysaccharide (LPS; Sigma-Aldrich, USA) in the amount of 10 µg in 10 µl normal saline solution.[7] After that, the animals were individually kept in cages for 2 weeks until the day of data collection.

Determination of blood perfusion

Animals were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.). Blood flow at 8 locations circumscribing the LPS injection area was measured using a laser Doppler flowmeter. The 8 measurement locations were of equal distance apart, and at a distance of 3 mm. away from the LPS injection area. For animals not injected with LPS, an area in the non-LPS animal corresponding to the LPS injection site in the LPS animal was chosen, and blood perfusion was measured around this area in a similar manner.

Determination of capillary vascularity

A jugular vein was cannulated for the injection of 0.1 ml of 5% fluorescein isothiocyanate-labeled dextran (FITC-dextran; Sigma-Aldrich, USA) for fluorescent visualization of the microvasculature. A square full thickness incision was made on the skin surrounding the area where LPS was injected, to create a flap. A laser-scanning confocal microscope (Nikon, Japan) was used to obtain photographs of fluorescent images, taken at 4 different locations circumscribing the LPS injection area, using 20x objective lens. These 4 locations were of equal distance apart, and correspond to four of the eight locations where blood perfusion was measured. The same procedure was done for non-LPS animals. Capillary vascularity (%CV) was determined using an image analysis software (Image-Pro Plus 6.0, Media Cybernetics, USA), in which only neocapillaries (diameter < 15 µm) are selected. Capillary vascularity was defined as the areas covered by microvessels divided by the area of the entire photomicrograph frame.

Determination of tissue VEGF levels

Skin tissue in the area encompassing blood perfusion measurement sites was harvested from each animal. Tissue VEGF levels were determined by ELISA (R&D Systems, USA) using supernatants from tissue homogenate.

Determination of malondialdehyde (MDA) levels

Plasma and tissue malondialdehyde (MDA) levels (a common oxidative stress marker) were determined using a commercial assay kit (Cayman Chemical, USA).

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Differences between groups were analyzed using one-way ANOVA. Statistical significance was set at $P < 0.05$.

Table 1. Body weight, plasma malondialdehyde (MDA) level, and mean arterial blood pressure (MAP) of rats in adult and aged groups. *$P < 0.05$ *vs* adult group.

	Body weight (g)	Plasma MDA (nmol/ml)	MAP (mmHg)
Adult	565.00 ± 43.30 (n = 3)	1.84 ± 0.46 (n = 5)	99.11 ± 6.04 (n = 5)
Aged	693.33 ± 53.33* (n = 3)	11.93 ± 2.28* (n = 6)	130.00 ± 5.00* (n = 8)

Results

Body weight, plasma MDA, and mean arterial blood pressure (MAP) of adult and aged groups are shown in Table 1. Body weight, plasma MDA, and mean arterial blood pressure of aged group were significantly greater than in adult group.

Figure 1A shows the dorsal skin blood perfusion of adult and aged groups (53.40 ± 4.58 and 36.14 ± 2.13 PU, respectively). Figure 1B shows the mean ± SEM of capillary vascularity (%CV) of adult and aged groups (8.81 ± 1.39 and 5.16 ± 0.13, respectively). Blood perfusion in aged group is significantly lower than that of adult group ($P < 0.05$). Similarly, aged group has significantly lower %CV when compared to adult group ($P < 0.05$).

Figure 1C shows the dorsal skin blood perfusion of adult + LPS and aged + LPS groups (59.08 ± 7.04 and 36.99 ± 2.58 PU, respectively). Figure 1D shows the %CV of adult + LPS and aged + LPS groups (12.37 ± 1.00 and 9.00 ± 0.76, respectively). Blood perfusion in aged + LPS group is significantly lower than that of adult + LPS group ($P < 0.01$). In the same way, aged + LPS group has significantly lower %CV when compared to adult + LPS group ($P < 0.05$).

Figure 2A shows tissue VEGF levels in adult, aged, adult + LPS, and aged + LPS groups (225.55 ± 31.30, 313.83 ± 42.46, 309.75 ± 49.43, and 307.06 ± 65.88, respectively). There was no significant difference among groups. Figure 2B shows tissue MDA levels of adult, adult + LPS, aged, and aged + LPS groups (2.12 ± 0.04, 1.63 ± 0.37, 2.78 ± 0.42, and 1.82 ± 0.21, respectively). Aged + LPS group has significantly lower tissue MDA level when compared to aged group ($P < 0.05$).

Discussion

The results of this study indicated that plasma MDA of aged group was significantly higher than that of adult group (Table 1). Additionally, %CV and skin blood perfusion values of aged group were significantly less than that of adult group (Figure 1A-1B). This implies that there is a loss of microvessels in association with aging. The oxidative stress theory of aging states that organisms age as a result of molecular damage due to increased reactive oxygen

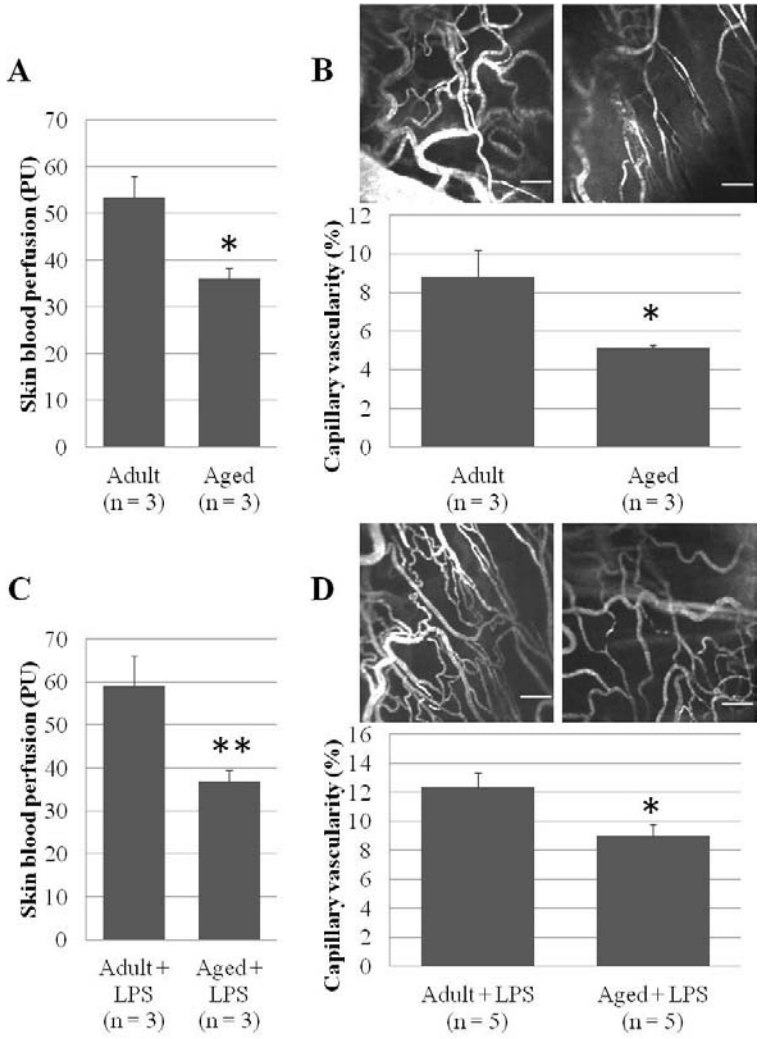

Figure 1. (**A**) Dorsal skin blood perfusion in perfusion units (PU) of adult and aged groups. **P* < 0.05 *vs* adult group. (**B**) Percent of capillary vascularity in adult and aged groups, with representative fluorescent images of microvessels at top, taken by confocal microscope at 20x objective lens (scale bar: 100 μm). **P* < 0.05 *vs* adult group. (**C**) Dorsal skin blood perfusion of adult + LPS and aged + LPS groups. ***P* < 0.01 *vs* adult + LPS group. (**D**) Percent of capillary vascularity in adult + LPS and aged + LPS groups, with representative fluorescent images of microvessels at top. **P* < 0.05 *vs* adult + LPS group.

species.[10] Furthermore, oxidative stress has been shown to promote apoptosis of endothelial cells, thereby causing loss of microvessels.[11-13] Our results are consistent with these findings.

Based on the results of LPS pre-treatment, there was no LPS toxicity, and the body weight of aged and aged + LPS groups did not show any significant difference (693.33 ± 53.33, n = 3; 643.33 ± 20.28, n=3; respectively). Weight loss can be used as an indicator of LPS toxicity, as shown by several studies.[14,15]

Interestingly, two weeks after injection of LPS, %CV in aged + LPS group was still lower than that of adult + LPS group. Consistent with this finding, the skin blood perfusion in aged + LPS group was also less than adult + LPS group. These results indicate that LPS-induced neovascularization in aged rats are impaired when compared with adult rats (Figure 1C-1D). However, a previous study done by Fernandes *et al.* showed that hypertension caused a reduction in the number and function of endothelial progenitor cells that contribute to neovascularization.[16] Thus, there is a possibility that the reduction in neovascularization in aged rats may be involved with this aged-induced hypertension, since the aged rats

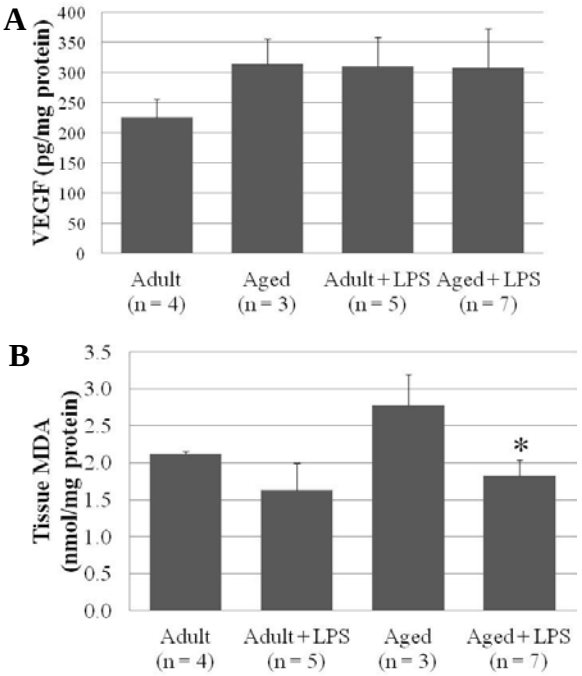

Figure 2. (**A**) Tissue VEGF levels in adult, aged, adult + LPS, and aged + LPS groups. (**B**) Tissue malondialdehyde (MDA) levels of adult, adult + LPS, aged, and aged + LPS groups. * *P* < 0.05 *vs* aged group.

had significantly higher mean arterial blood pressure than the adult rats.

The percent changes in %CV after LPS treatment in adult and aged groups have been compared. In adult rats, after LPS treatment, there is 40.41% increase in %CV when compared with their respective, non-treated control. In aged rats, there is 74.42% increase in %CV when compared with the non-treated aged group. Interestingly, there is a greater increase in %CV of aged rats after LPS treatment.

VEGF, or vascular endothelial growth factor, is known as an important angiogenic factor for inducing angiogenesis. It was expected that VEGF should increase in tissue in which greater neovascularization occurred. However, our tissue VEGF results do not reach statistical significance. This may be due to the late measurement timeframe at two weeks, as it was found in the previous study done in corneal neovascularization that VEGF protein seem to decline to control level after one week post-induction.[17]

The interesting finding of tissue MDA in aged + LPS group that was lower than in aged group supports the idea that LPS-induced inflammation could increase capillary vascularity, which enhanced more blood perfusion and then decreased oxidative stress. Moreover, this increased neovascularization after LPS-induced inflammation may be useful as a study model of inflammation-induced stem cell function in the future.

Conclusion

The present study has demonstrated that aged rats have reduced capillary vascularity when compared to adult rats, possibly due to the increased oxidative stress which damages the endothelial cells as well as microvasculature. Furthermore, aged rats have reduced neovascularization after LPS-induced inflammation when compared to adult rats.

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Conflict of Interest

None to declare.

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