Endogenous Adrenomedullin Protects Against Vascular Response to Injury in Mice

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Background—In our previous study, adrenomedullin (AM) overexpression could limit the arterial intimal hyperplasia induced by cuff injury in rats. However, it remains to be elucidated whether endogenous AM plays a role against vascular injury.

Methods and Results—We used the AM knockout mice to investigate the effect of endogenous AM. Compared with wild-type (AM+/+/) mice, heterozygous AM knockout (AM+/−/−) mice had the increased intimal thickening of the cuff-injured femoral artery, concomitantly with lesser AM staining. In AM+/−/− mice, cuff placement increased both the production of superoxide anions (O2−) measured by coelentarazine chemiluminescence and the immunostaining of p67 phox and gp91 phox, subunits of NAD(P)H oxidase in the adventitia, associated with the increment of CD45-positive leukocytes, suggesting that the stimulated formation of radical oxygen species accompanied chronic adventitial inflammation. Not only the AM gene transfection but also the treatment of NAD(P)H oxidase inhibitor apocynin and membrane-permeable superoxide dismutase mimetic tempol could limit cuff-induced intimal hyperplasia in AM+/−/− mice, associated with the inhibition of O2− formation in cuff-injured artery.

Conclusions—The overproduction of oxidative stress induced by the increased NAD(P)H oxidase activity might be involved in cuff-induced arterial intimal hyperplasia in AM+/−/− mice. Thus, it is suggested that endogenous AM possesses a protective action against the vascular response to injury, possibly through the inhibition of oxidative stress production.

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Key Words: antioxidant ■ inflammation ■ endothelium ■ arteries ■ peptides

Adrenomedullin (AM) is a novel vasorelaxant peptide originally isolated from the human pheochromocytoma.1,2 Cultured endothelial and vascular smooth muscle cells (VSMCs) have been shown to be not only sources but also targets of AM, suggesting its role as an autocrine/paracrine factor in the cardiovascular tissues.3,4 In addition to the vasodilator action, AM inhibits the proliferation and migration of cultured aortic VSMCs, possibly through either the elevation of intracellular cAMP or an NO-dependent pathway of endothelial NO synthase.5 Moreover, AM inhibits angiotensin II (AII)-induced proliferation and migration of VSMCs, possibly through inhibiting oxidative stress production.6,7 We have recently demonstrated that AM-deficient mice had severe cardiovascular damage and increased oxidative stress with AII and salt loading.8

Intimal thickening is an early, essential stage in the development of vascular injury. Recently it has been reported that placement of a nonconstrictive cuff around an arterial segment in rodents results in a reproducible, concentric intimal hyperplasia.9-13 Intimal thickening induced by this technique results from the excessive accumulation of VSMCs and the deposition of extracellular matrix in the intima of the vessel wall followed by adventitial inflammatory cell infiltration,14 which is intimately related to oxidative stress.15,16 Recently, we reported that local AM overexpression effectively limited the arterial intimal hyperplasia induced by cuff injury in rats, suggesting that intrinsic AM possesses the potential to protect organs from damage, possibly through the antioxidant effect.12

To additionally elucidate the plausible role of AM as an endogenous vasoprotective substance, in the present study, we studied the effect of cuff placement around the femoral artery on intimal thickening and oxidative stress production in target-gene–disrupted mice of AM alone.8 In addition, we examined the effects of either AM gene transfection or the treatments of apocynin, a specific inhibitor of NAD(P)H oxidase, and tempol, a membrane-permeable superoxide dismutase (SOD) mimetic, on cuff-induced intimal thickening and superoxide (O2−) production in AM-deficient mice.

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Methods

Construction of an Adenovirus Vector Carrying the AM Gene

We constructed a replication-defective adenovirus carrying the AM gene under CAG promoter (AxCAAM), as previously reported.12 A full-length coding region of AM was blunt-ended and cloned into a cosmid, pAXCam (Adenovirus expression kit, Takara Co). The resulting cosmid, pAXCAAM, was cotransfected into the 293 embryonic cell line with EcoT221-digested DNA-TPC (from Ad5sd) to generate AxCAAM carrying the coding region of AM. Human AM cDNA was kindly provided by Dr N. Minamino and Dr K. Kangawa (the National Cardiovascular Center, Osaka, Japan).

Animals

We previously described the generation of AM mutant mice.8 These mice have a genetic background of C57 BL/6 strain (Tokyo Jikken Dobutsu, Tokyo, Japan). The litter male wild-type (AM+/+) mice were used as controls. Homozygous AM knockout mice were an embryonic lethal mutation; however, heterozygous AM knockout (AM+/−) mice were both viable and fertile. Thus, we used AM+/− mice that were bred in our laboratory. In our previous study, their AM levels in organs and plasma were almost half those of AM+/+ mice.8

Adenovirus-Mediated Gene Transfer Into Cuffed Arteries

The surgical procedures of cuff placement were applied according to the method described previously with some modification.9,12 All procedures were performed under sterile conditions according to the Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo. The sterile surgical procedure also prevents inconsistently enhanced vascular injury attributable to infection. Mice were anesthetized with sodium pentobarbital, 45 mg/kg IP. The femoral artery was isolated from the surrounding tissues, and a silicone rubber tube (2 mm long; inner diameter, 0.64 mm; outer diameter, 1.20 mm; Plastics One), cut longitudinally to open the tube, was loosely placed around the artery. Then a 6-μL portion of virus fluid (1.0×10 plaque-forming units per mL) was delivered into a space between the cuff and the artery for viral infection.17 Some mice underwent isolation of the femoral artery without cuff placement (sham-operated mice). After recovery from anesthesia, the animals were given standard diet and water ad libitum.

Treatments of Apocynin and Tempol

To examine the involvement of oxidative stress in cuff-injured intimal thickening, apocynin (15 mmol/L) and tempol (10 mmol/L) dissolved in tap water were given orally after recovery from anesthesia.

Detection of Superoxide by Coelentarazine

Production of O2− was measured by coelentarazine-dependent chemiluminescence response, as described previously.19 Briefly, after the cuff-injured artery specimen (2-mm length) was isolated and cleaned of fat and loose connective tissues, the specimen was placed in a modified Krebs-Ringer solution composed of (in mmol/L) NaCl 119, HEPES 20, KCl 4.6, MgSO4 1.0, NaHPO4 0.15, KH2PO4 0.4, NaHCO3 1.2, and glucose 5.5. The specimen was then transferred into the test tubes containing coelentarazine (5 μmol/L). The tube was placed in a luminometer Luminescence Reader BRL-301 (Aloca). After 30-minute equilibration, repeated measurements were interpreted every 30 seconds and an average value was reported over a 5-minute period. We found that counts did not significantly increase with longer periods of measurements. Wet weights were obtained for each vascular segment to allow normalization of data.

The assay was done after the addition of phorbol 12-myristate 13-acetate (PMA) (5 μmol/L) in the buffer medium; unstimulated O2− production was too low to detect because of very small sample size of the cuff ed arteries. PMA has been widely used as a protein kinase C agonist and causes p47phox phosphorylation and activation of NAD(P)H oxidase, resulting in O2− production.19 In a preliminary study, the artery specimens showed time-dependent increases in O2− production until 3 days later, which subsequently decreased gradually (Figure 1). In the present study, therefore, we used the artery specimens harvested 3 days later.

Immunostaining of AM, CD45, p67phox, gp91phox, and X-gal Staining

To examine the AM expression, a portion of the femoral artery, removed 14 days after the adenoviral infection, was fixed in PBS containing 4% paraformaldehyde for 2 hours at room temperature and then paraffin embedded. According to the previous reports,12 sections (4 μm in thickness) from each block of arterial tissue were stained immunohistochemically by avidin-biotin complex method using an ABC kit (Vector Laboratories) with anti-AM at a dilution of 1:10 000. An incubation with type- and class-matched irrelevant immunoglobulin (Ig) instead of primary antibody served as negative controls. Monoclonal mouse anti-human AM amimated C-terminal peptide (aa46-52) antibody was described previously.20 Immunohistochemistry of CD45 was performed using the labeled streptavidin biotin method as described previously.21 Section were dewaxed and incubated with 3% H2O2 and blocking serum and thereafter with a goat polyclonal antibody directed against CD45 (Santa Cruz Biotechnology) at 1:100 dilution. The sections were rinsed with Tris-buffered saline with 0.1% Tween 20 (TBST) and biotinylated secondary antibody against goat IgG (Dako) with 1:400 dilution. After rinsing with TBST, the sections were incubated with HRP-conjugated streptavidin solution (Dako). HRP labeling was detected using a peroxidase substrate solution with 0.8 mmol/L DAB and 0.01% H2O2. The sections were counterstained with hematoxylin before being examined under a light microscope. The section was quantified as the number of immunostained-positive cells per total number of nuclei.

For immunofluorostaining of p67phox and gp91phox, components of NAD(P)H oxidases, we applied the following method.12,22 Arterial specimens were cryoprotected in 20% sucrose in PBS and embedded in Tissue-Tek OCT compound (Sakura Fintek). Frozen sections were cut 4 μm thick, fixed in cold acetone, air dried, washed, and blocked with 1% goat serum for 30 minutes at room temperature. The sections were incubated with Tris-buffered saline with 0.1% Tween 20 (TBST) and biotinylated secondary antibody against goat IgG (Dako) with 1:400 dilution. After rinsing with TBST, the sections were incubated with HRP-conjugated streptavidin solution (Dako). HRP labeling was detected using a peroxidase substrate solution with 0.8 mmol/L DAB and 0.01% H2O2. The sections were counterstained with hematoxylin before being examined under a light microscope. The section was quantified as the number of immunostained-positive cells per total number of nuclei.

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540-nm laser lines of the krypton/argon ion laser (model 5470K; Ion Laser Technology), respectively. Three different samples were examined for each mouse euthanized 3 days after the cuff placement. The X-gal staining of the section from the artery infected with AxCALacZ, removed 5 days after the adenoviral infection, was performed as described. A replication-defective adenovirus carrying the Escherichia coli β-galactosidase gene, AxCALacZ, was kindly provided by Dr I. Saito (University of Tokyo, Tokyo, Japan).

Measurement of the Intimal to Medial Cross-Sectional Areas of Cuff-Injured Arteries
A portion of the femoral artery was harvested at 14 days after the cuff placement. The artery was fixed in PBS containing 4% paraformaldehyde for 4 to 6 hours at room temperature and then paraffin embedded. Two round cross-sections per 2-mm length of artery specimens, stained with H&E, were photographed, and the cross-sectional areas of the intimal and medial regions of the sections were measured using an image-analyzing software package (Image-Pro PLUS). To confirm the accuracy of the measurement of the intimal and medial regions, sections of sham-operated and cuff-injured artery from AM mice and AM mice were stained using elastic Verhoeffen von Gieson method. The intimal and medial cross-sectional areas and intimal-to-medial (I/M) area ratio of each artery were determined by averaging the values for 2 sections to evaluate the intimal mass in each artery.

Statistical Analysis
All values are expressed as mean±SEM. Intimal and medial thickness and I/M ratios were analyzed by 1-way ANOVA followed by the post hoc test. O2 production data were analyzed by 2-way ANOVA followed by the Tukey’s test. Differences at P<0.05 were considered to be statistically significant.

Results
Intimal Hyperplasia in Cuff-Injured Arteries in AM and AM Mice
The intimal hyperplasia developed in the femoral artery 14 days after a silicone cuff was placed around the artery in AM mice (Figures 2A and 2B). There was no significant difference in medial thickening among the experimental groups. The intimal area of the section from cuff-injured artery was greater in AM mice compared with AM mice (Figures 2C and 2D). There was no significant difference in medial thickening among the experimental groups. The intimal area of the section from cuff-injured artery was greater in AM mice compared with AM mice (P<0.01; Table); the I/M ratio of the cuff-injured artery was 24±4% and 45±8% (P<0.01) at 14 days after injury in AM and AM mice, respectively (Figure 3).

AM Staining in the Cuff-Injured Arteries
A moderately increased AM staining was detected in perivascular cells in the cuff-injured artery of AM mice (Figure 4A). However, AM mice had less AM staining in cuff-injured artery (Figure 4B).

CD45 Staining of the Cuff-Injured Arteries
In AM mice, CD45-positive leukocytes were increased in adventitia of cuff-injured arteries compared with sham-operated arteries (Figures 5A and 5B; adventitia, 1.1±0.7% and 15.6±4.8%). Although CD45-positive cells were almost the same in sham-operated arteries between AM mice (Figure 5D; 5.9±3.8%), they were much more increased in cuff-injured arteries from AM mice; the CD45-positive leukocytes were observed in intima (25.0±8.3%) and media (44.2±10.5%) as well as adventitia (57.6±15.4%) (Figure 5E).

AxCAAM Limits Cuff-Induced Intimal Formation Associated With Reduction of CD45-Positive Cell Infiltration
The AxCAAM-infected arteries showed a prominent reactivity for AM (data not shown). Moreover, perivascular cells of artery infected with AxCALacZ expressed intense β-galactosidase activity (data not shown), as in a previous report, and a weak staining with AM to the cuff-injured artery with AxCALacZ infection (data not shown). The infection of AxCAAM significantly decreased intimal formation in cuff-injured artery harvested 14 days later; I/M ratio was 9.8±1.1% and 9.0±1.1% (NS) in AM mice and
AM<sup>+/−</sup> mice, respectively (Table and Figures 2E and 3); I/M ratio of AxCAAM-infected artery of AM<sup>+/−</sup> mice was comparable to that of AxCAAM-infected control of AM<sup>+/−</sup> mice. In contrast, intimal formation in cuff-injured artery infected with AxCALacZ was comparable to that of arteries not infected with the virus (Figure 2F). Concomitantly, adventitial CD45-positive cell infiltration was inhibited by AxCAAM infection (Figures 5C and 5F; 7.6 ± 2.4% and 4.2 ± 1.9%, AM<sup>+/−</sup> and AM<sup>+/−</sup> mice, respectively).

**Treatments of Apocynin and Tempol Limit Cuff-Intimal Formation**

The treatments of both apocynin and tempol significantly decreased intimal formation in cuff-injured artery (Table and Figures 2G, 2H, and 3); I/M ratio of cuff-injured artery in both apocynin- and tempol-treated AM<sup>+/−</sup> mice was comparable to that of AM<sup>+/+</sup> mice. Therefore, the inhibition of O<sub>2</sub><sup>−</sup> production may effectively limit intimal hyperplasia after cuff injury in AM<sup>+/−</sup> mice.

**Superoxide Production and Immunofluorostaining of p67<sup>phox</sup> and gp91<sup>phox</sup> in Cuff-Injured Arteries**

There was no significant difference in O<sub>2</sub><sup>−</sup> production of sham-operated artery specimens between AM<sup>+/−</sup> and AM<sup>+/−</sup> mice (Figure 6A). Cuff injury could increase O<sub>2</sub><sup>−</sup> production in both mice. However, O<sub>2</sub><sup>−</sup> production in the cuff-injured artery specimens of AM<sup>+/−</sup> mice was significantly greater than that of AM<sup>+/−</sup> mice (4.7 ± 0.7 × 10<sup>3</sup> versus 2.8 ± 0.4 × 10<sup>1</sup> cpm/mg, P < 0.05). Moreover, the in vitro administration of AM in the cuff-injured artery specimens could decrease O<sub>2</sub><sup>−</sup> production in AM<sup>+/−</sup> mice (Figure 6B, left); AM inhibited O<sub>2</sub><sup>−</sup> production in cuff-injured artery specimens of AM<sup>+/−</sup> mice more greatly than in those of AM<sup>+/−</sup> mice (37.1 ± 9.7% versus 22.1 ± 9.2%, P < 0.05). By the in vivo study, in addition, the infection of AxCAAM in AM<sup>+/−</sup> mice could significantly decrease O<sub>2</sub><sup>−</sup> production in cuff-injured artery specimens (Figure 6B, right).

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Bar graphs show the I/M area ratio at 14 days after cuff placement. Cuff injury increased the I/M area ratio in both AM<sup>+/+</sup> and AM<sup>+/−</sup> mice; however, AM<sup>+/−</sup> mice had greater increases in I/M area ratio induced by cuff injury than AM<sup>+/+</sup> mice. The transfection of AxCAAM significantly decreased the I/M area ratio 14 days after cuff placement, but AxCALacZ did not suppress the neointimal formation of cutferd arteries. The treatments of apocynin and tempol also significantly decreased the I/M area ratio 14 days after cuff placement. Data are mean ± SEM. Open column, AM<sup>+/+</sup> mice; shaded column, AM<sup>+/−</sup> mice. Sham indicates sham-operated artery; Cuff, cuff-injured artery; AxCAAM, cuff-injured artery transfected with AxCAAM; AxCALacZ, cuff-injured artery transfected with AxCALacZ; and Apocynin and Tempol, cuff-injured artery in the apocynin-treated and tempol-treated mice, respectively. n = 5 to 7 in each group. *P < 0.05 vs Sham; †P < 0.001 vs Sham; ‡P < 0.001 vs Cuff; §§P < 0.001 vs AM<sup>+/+</sup>.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Representative histological and immunohistochemical photomicrographs of cross-sections of femoral arteries with AM. Sections of the arteries were obtained 14 days after the cuff placement. Cuff-injured artery of AM<sup>+/+</sup> mice showed moderately increased staining with AM in the adventitia (A). In contrast, AM<sup>+/−</sup> mice showed less staining with AM in cuff-injured artery (B). Original magnification ×200. Bar = 100 μm.
was also upregulated at the cuff-injured artery of AM 13/11001 in cuff injury models of femoral 13 arteries. 9,10 In our hands, cuff injury results in predictable growth and positive vascular remodeling have been reported formation of neointima without affecting external elastic mal thickening, possibly through the failure to inhibit oxida-
plasia and that AM deficiency aggravates cuff-induced inti-

O2 (data not shown). Thus, it is suggested that overproduction of increased O2 13/11002 intimal thickening, associated with the inhibition of the SOD mimetic tempol could significantly limit cuff-induced production in the cuff-injured artery specimens (Figure 6C). Moreover, p67 phox , a cytosolic component of NAD(P)H oxidase, was decreased O2 13/11002/13/11001/13/11002 0.05) decrease O2 13/11021 P mice could significantly ( 13/11001/13/11001 13/11001/13/11021 P mice, compared with that of AM 13/11001 mice, particularly in the adventitia (Figure 7). Another membrane-spanning polypeptide subunit of NAD(P)H oxidase, gp91 phox , was also upregulated at the cuff-injured artery of AM 13/11001 mice (data not shown). Thus, it is suggested that overproduction of O2 induced by cuff injury might be attributable at least partly to the increased NAD(P)H oxidase activity.

**Discussion**

It was clearly shown in this study that AM 13/11001 mice had the severe intimal thickening induced by cuff placement around the femoral artery associated with enhanced adventitial CD45-positive leukocytes accumulation and increased O2 production. Moreover, not only local AM overexpression but also a specific NAD(P)H oxidase inhibitor apocynin and a SOD mimetic tempol could significantly limit cuff-induced intimal thickening, associated with the inhibition of the increased O2 production. Evidence presented suggests that oxidative stress is implicated in cuff-induced intimal hyperplasia and that AM deficiency aggravates cuff-induced intimal thickening, possibly through the failure to inhibit oxidative stress production.

Cuff model has been previously described in rabbits and rats. 9,10 In our hands, cuff injury results in predictable formation of neointima without affecting external elastic lamina and media (Figure 2) in mice. However, medial growth and positive vascular remodeling have been reported in cuff injury models of femoral 13/11 as well as carotid 24 arteries. The discrepant responses might be attributable to different experimental conditions; silicone cuff was placed around the femoral artery for 14 days in the present experiment, whereas polyethylene cuff was placed for 28 days in the above-mentioned experiment. 13,24 In fact, some investigators demonstrated that the media was not affected in polyethylene cuff–placed femoral arteries of mice for 14 days. 11,14,25 Moreover, responses of femoral arteries to cuff placement might be different among various model animals. Thus, neointima formation is a common and essential finding of the cuff placement model that should be a focus of research.

Cuff would work as a foreign body, inducing immune response and inflammation. In the present study, the adventitia of cuff-injured artery showed infiltration of inflammatory cells, similar to the previous studies. 11,26 In fact, vascular injuries by cuff placement 14 and balloon angioplasty 27 are
-associated adventitial inflammation characterized by leukocyte accumulation, which was proposed as one of major determinants of subsequent intimal hyperplasia. It should be noted in the present study that immunostaining of p67phox and gp91phox was increased predominantly in the adventitia of cuff-injured artery, because NAD(P)H oxidase proteins have been demonstrated to be localized exclusively in the adventitia with no substantial expression in the media. In addition, Shi et al recently showed high levels of ROS produced by NAD(P)H oxidase in activated adventitial fibroblasts from balloon-injured porcine coronary arteries, providing pivotal growth signals for adventitial fibroblasts. Moreover, it is conceivable that an adequate anti-ROS therapy in the adventitial fibroblasts and VSMCs is potentially able to counteract neointimal formation. Also, there is a growing body of evidence suggesting that proliferating adventitial cells might migrate to the intima. Thus, it strongly supports the plausible hypothesis on the possible involvement of adventitial ROS, which was produced in inflammatory cells, in vascular response to injury.

In addition to adventitial immunostaining of the components of NADPH oxidase, the present study consistently suggests the critical role of the oxidative stress in cuff-induced intimal hyperplasia. First, $\text{O}_2^-$ production in cuff-injured artery was augmented. Second, the treatments of apocynin and tempol could limit cuff-induced intimal hyperplasia associated with decreased $\text{O}_2^-$ production. This has been supported by several lines of evidence indicating that NAD(P)H oxidase plays a crucial role in the formation of cuff-induced intimal thickening. In the present study, moreover, local AM overexpression could not only limit cuff-induced intimal thickening but also decrease $\text{O}_2^-$ production associated with ameliorated cuff-induced inflammatory inflammation in AM$^{-/}$ mice. The anti-inflammatory action of AM might be attributed to the inhibition of ROS generation. Alternatively, the inhibitory effect of AM on cuff-induced intimal hyperplasia may be at least partly attributable to its inhibition of ROS production; the in vitro administration of AM reduced the increased $\text{O}_2^-$ production in the cuffed-artery specimens of AM$^{-/}$ mice (Figure 6B, left). Supporting this finding, AM was reported to inhibit the generation of ROS in cultured mesangial cells and macrophages. Moreover, AM inhibits AII-induced proliferation and migration of cultured aortic VSMCs possibly through inhibiting AII-induced $\text{O}_2^-$ production via NAD(P)H oxidase activation. A more recent report demonstrated that AM inhibited $\text{O}_2^-$ production in cardiac ischemia/reperfusion model of rats associated with suppression of NAD(P)H oxidase but not xanthine oxidase activity, supporting our hypothesis. The antioxidant effect of AM may be intimately related to the increased intracellular cAMP and NO-cGMP pathway. AM-induced inhibition of NAD(P)H oxidase activity has been proposed to be attributable to NO-cGMP pathway, which is compatible with our previous report that AM overexpression limited intimal hyperplasia associated with endothelial NO synthase overexpression. However, additional studies are required to clarify its precise mechanisms.

In the present study, cuff placement could upregulate AM expression in the injured artery of AM$^{-/}$ mice but not in that of AM$^{-/-}$ mice (Figure 4). It is consistent with the results of the previous study showing that AII and salt loading could increase cardiac AM expression in AM$^{-/}$ mice but not in AM$^{-/-}$ mice. Thus, AM deficiency could be critical to aggravate intimal hyperplasia induced by cuff injury. It is supported by the present findings that the in vivo treatment of AxCAAM could not only normalize the increased $\text{O}_2^-$ production in cuff-injured artery of AM$^{-/}$ mice but also that the in vitro administration of AM in cuff-injured artery specimens reduced it more greatly in AM$^{-/}$ mice than in AM$^{-/-}$ mice. Moreover, several lines of evidence suggest that oxidative stress could increase AM production in vascular endothelial cells and VSMCs. In turn, locally generated AM might counteract the proliferation and migration of VSMCs induced by cuff injury, possibly through inhibiting oxidative stress production; AM might be an endogenous vasoprotective substance.

In summary, AM$^{-/-}$ mice showed marked cuff-injured intimal thickening concomitant with increased oxidative stress. Both local AM overexpression and the antioxidants could inhibit cuff-induced intimal hyperplasia, associated with reducing oxidative stress production. Endogenous AM may counteract the generation of ROS induced by cuff injury and thus result in the protection of the vascular response to injury.

References


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