Akt/Protein Kinase B and Endothelial Nitric Oxide Synthase Mediate Muscular Neovascularization Induced by Tissue Kallikrein Gene Transfer

Costanza Emanueli, PhD; Maria B. Salis, PhD; Sophie Van Linthout, PhD; Marco Meloni, BS; Elisa Desortes, Pharm Chem D; Jean-Sebastien Silvestre, PhD; Michel Clergue; Carlos D. Fiqueroa, PhD; Sergio Gadau, VMD, PhD; Gianluigi Condorelli, MD, PhD; Paolo Madeddu, MD

Background—Angiogenesis gene therapy with human tissue kallikrein (hTK) has shown promise for ischemic disease. The present study was undertaken to (1) assess an optimal gene transfer modality, (2) clarify hTK angiogenic pathways, and (3) discount possible side effects.

Methods and Results—The hTK gene was transferred to murine adductors by increasing doses of an adenovirus (Ad.hTK). Heterologous protein production was evaluated by ELISA and immunohistochemistry. Structural and functional characteristics of hTK-induced neovascularization were assessed. Muscular endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF)-A mRNA and protein content were evaluated by real-time polymerase chain reaction and Western blotting. The ability of hTK to phosphorylate-activate Akt/protein kinase B (Akt-B) and VEGF receptor 2 (VEGF-R2) was also determined. Implication of the aforementioned mechanisms in Ad.hTK-induced neovascularization was challenged by blocking Akt-B with a dominant-negative Akt construct; NOS with N\(_{\text{G}}\)-nitro-L-arginine methyl ester; and VEGF-A with neutralizing antibody, VEGF-R2 antagonist, or Ad carrying soluble VEGF-R1 gene. We found that 10\(^7\) PFU Ad.hTK led to peak increases in capillary and arteriole density. Newly developed arterioles persisted for up to 8 weeks. Ad.hTK did not change microvascular permeability. Ad.hTK upregulated eNOS mRNA and protein and activated Akt-B through Ser-473 phosphorylation. Inhibitory studies documented that these biochemical events were instrumental to Ad.hTK-induced neovascularization. In contrast, Ad.hTK neither affected VEGF-A and VEGF-R2 levels nor increased VEGF-R2 phosphorylation. Consistently, Ad.hTK-induced neovascularization was not disturbed by any of the different approaches used to block VEGF-A.

Conclusions—Our findings provide new information on the pathway involved in hTK-induced neoangiogenesis and represent an advancement toward clinical applications with Ad.hTK. (Circulation. 2004;110:1638-1644.)

Key Words: gene therapy ■ nitric oxide synthase ■ angiogenesis ■ muscle ■ bradykinin

Neovascularization gene therapy has been proposed as a rescue for ischemic disease.\(^1,2\) After successful application in animal models, the strategy has been transferred from the bench to the bedside. However, clinical results have not matched the level of efficacy for which researchers had hoped.\(^3,7\) The serious side effects derived from the use of robust infecting doses of viral vectors and unregulated expression of angiogenic genes\(^7,8\) point to the necessity that clinical application must be preceded by in-depth clarification of the involved pathways and improvements in the therapeutic-risk index. Furthermore, simple initiators of angiogenesis may not represent the best option to construct a durable and well-tempered neovascularization. In this area of high medical need, substances capable of successfully completing the angiogenic process appear more suitable to the task.

The serine protease human tissue kallikrein (hTK), a recent entry in the angiogenic factor family, has shown promise for the treatment of ischemic disease. Kallikrein-kinin system components are upregulated in animal models of and in patients with peripheral vascular disease,\(^9,10\) and local hTK gene delivery enhances native neovascularization and accelerates tissue healing.\(^9\) It is noteworthy that hTK does not require an ischemic environment for its curative properties.\(^11\)

At present, information about molecular effector(s) of adenovirus (Ad) hTK-induced neovascularization remains
largely incomplete, being mainly limited to the basic notion that kinins generated by hTK play a fundamental role in these vascular effects. In addition, from in vitro studies we know that bradykinin (BK) activates endothelial NO synthase (eNOS) through either the phosphoinositide 3-kinase-Akt/protein kinase B (Akt-B) pathway or calcineurin-mediated mechanisms. Furthermore, vascular endothelial growth factor (VEGF)-receptor 2 (R2) transactivation by the BK-B2 receptor seemingly induces endothelial cell (EC) tube formation on Matrigel through eNOS mediation. In general, VEGF-A and Akt-B cooperate in the control of endothelial growth and viability, but recent evidence supports the possibility that Akt-B may be activated by alternative pathways independent of VEGF-A. Therefore, we considered it worthwhile to clarify whether hTK stimulates angiogenesis in vivo through Akt-B, eNOS, and VEGF-A. In view of future clinical use, we also evaluated an optimal gene transfer modality, the composition and persistence of neovascularization, and the microvascular permeability of Ad.hTK-infected muscles.

**Methods**

**hTK Gene Delivery and Histological Assessment of Muscular Neovascularization**

Procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, 1996). A dose-response curve (10<sup>3</sup> to 10<sup>9</sup> plaque-forming units [PFU]) to Ad.hTK was established in CD1 mice (Charles River, Calco, Italy). Anesthetized (2,2,2-tribromoethanol, 880 mmol/kg IP, Sigma) mice received 3 injections (3.5 μL/injection, 10.5 μL total volume) at different sites of the left adductor muscle along the projection of the femoral artery. This procedure enabled Ad vector diffusion along the entire mouse adductor. Ad carrying the luciferase gene (Ad.Luc) and saline served as controls. Each group consisted of at least 6 mice.

Two or 8 weeks after gene transfer, limb muscles were perfusion-fixed and processed for histological analyses of capillary and arteriole density, as described. Capillary density was expressed as capillary number per area (n<sub>cap/mm<sup>2</sup></sub>). Transverse section or normalized to myofiber density (n<sub>cap/n<sub>fiber</sub></sub>). Arteriole density was expressed as arteriole number per area (n<sub>art/mm<sup>2</sup></sub>). The effect of Ad.hTK on EC proliferation in vivo was evaluated. To this aim, adductors (n=5 per group) that had received Ad.hTK or Ad.Luc (each at 10<sup>9</sup> PFU) 3 days in advance were processed for immunohistochemical identification of proliferating cell nuclear antigen (PCNA) by using a monoclonal antibody (Dako). Total capillaries and PCNA-positive ECs were counted at least 25 fields at ×1000 magnification, and the number of PCNA-positive ECs per square millimeter and for every 1000 capillaries was calculated.

**Transgene Expression After Ad.hTK Gene Transfer**

We established the relation between Ad infecting dose and transgenic protein content. An ELISA (AngioProgen) selectively recognizing hTK<sup>16</sup> was used to measure hTK content in plasma and adductors (n=4 per dose) at 5 days from gene delivery, i.e., at the peak of transgene expression. Saline-injected muscles served as controls. We also measured hTK levels in the eyes (a tissue at risk for pathological angiogenesis) of mice receiving intramuscular Ad.hTK (10<sup>9</sup> PFU) or saline. Total protein concentration was measured by the Lowry method.

The cellular localization of transgenic protein in infected muscles was determined by immunohistochemistry with a polyclonal rabbit antibody that recognizes exclusively the hTK form.<sup>17</sup> Specific binding was detected with the biotin-streptavidin LSAB<sup>®</sup> system (Dako). Controls included preabsorption of anti-hTK with purified human urinary TK (Calbiochem) and replacement of anti-hTK antibody by nonimmune serum at the same dilution. Muscles injected with Ad.Luc and sterile saline served as negative controls.

**Microvascular Permeability**

Plasma protein extravasation (PPE) provoked by angiogenesis therapy may cause local edema.<sup>18</sup> Because kinins may cause similar reactions, we tested whether this undesired effect occurs after hTK application. Muscular PPE at 3 or 14 days after 10<sup>9</sup> PFU of Ad.hTK or Ad.Luc administration or saline was determined by the Evans blue method<sup>19</sup> and normalized to PPE in the contralateral, noninjected adductors. BK (10 μg/10 μL IM, given 5 minutes before Evans blue dye) served as positive control. Each group consisted of 6 mice.

**Molecular Pathways Activated by Ad.hTK In Vivo**

**Akt-B Phosphorylation**

Western blot analysis of Akt-B was performed on muscles harvested 3 days after 10<sup>9</sup> PFU Ad.hTK or Ad.Luc (n=6 each group) had been administered. Twenty-six micrograms of protein for each sample was loaded. The reaction was carried out using primary antibodies raised against Ser-473-phosphorylated or total Akt (Cell Signaling Technology). Tubulin served as the loading control. Specific protein was detected by chemiluminescence reaction (Amersham), followed by analysis of immunoblot density by dedicated software (Scion Corp).

**eNOS Expression: Real-Time PCR Primers**

Real-time quantitative polymerase chain reaction (PCR; ABI PRISM 7000 sequence detection system software version 1.0, Perkin-Elmer) was used to determine eNOS mRNA content in muscles (n=6 per group) harvested 3 days after the injection of 10<sup>9</sup> PFU Ad.hTK or Ad.Luc. Total RNA was isolated with TRIzol reagent (Invitrogen) and treated with DNAse (Qiagen). RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen). eNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sequences were designed on GenBank database NM-008713 and NM-008084, respectively, and were as follows: 5'-CTTCCGGTTACCCCGAGA-3' (eNOS forward), 5'-CAGAGAAGTCTCCATTGGCTG-3' (eNOS reverse), 5'-GCTGAGGCTCAGGCGACAT-3' (GAPDH forward), and 5'-TCTTACCAGGCGACACGAC-3' (GAPDH reverse). Conventional PCR products of murine eNOS (105 bp) and GAPDH (156 bp) were obtained with the primers designed for the real-time PCR and were cloned into pGEM-T Easy vector (Promega) to be used as DNA standards. eNOS cDNA level was normalized to GAPDH cDNA level.

Muscular eNOS protein content at 5 days from gene transfer was analyzed by Western blotting (n=6 muscles per group) with a rabbit polyclonal antibody (1:1000, Santa Cruz). Blots were reprobed with anti–β-actin antibody (1:1000, Santa Cruz). Immunoblot density was analyzed by Image Gauge V3.41 (Fuji Film).

**VEGF-A and VEGF-R2**

VEGF-A mRNA content was determined on the same samples used for the eNOS experiment. VEGF-A primers (designed on GenBank M95200) generate a 111-bp fragment and were as follows: 5'-CCA GCC CAG GTA CTA CGG TCC A-3' (forward) and 5'-ACA GGC CAG CAG CAC AC-3' (reverse).

Western blots for VEGF-A (rabbit polyclonal antibody at 1:2000, Santa Cruz), VEGF-R2 (goat polyclonal antibody at 1:2500, R&D Systems), and Tyr-951-phosphorylated VEGF-R2 (rabbit polyclonal antibody at 1:1000, Cell Signaling) were performed on the same samples (n=6 per group) used for eNOS analysis. After phospho-VEGF-R2 analysis, the membrane was stripped and reprobed with antibody for total VEGF-R2. In all cases, β-actin was used for normalization.
Functional Role of Akt-B, NOS, and VEGF-A in Ad.hTK-Induced Angiogenesis

Ad.hTK and Ad.DN-Akt-B Cotransfection
To elucidate the functional role of Akt-B in Ad.hTK-induced neovascularization, a cotransfection experiment with Ad.dominant negative-Akt308/547 (Ad.DN–Akt-B) was performed. Adductors received 10⁷ PFU of Ad.hTK or Ad.Luc in combination with either Ad.DN–Akt-B or Ad.Luc (each at 5 × 10⁶ PFU). Neovascularization was evaluated at 2 weeks in 6 mice per group.

NOS Inhibition
Capillary and arteriole density was counted at 2 weeks from Ad.hTK or Ad.Luc administration (each at 10⁷ PFU) in combination with the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 1.4 mmol/kg body weight daily in drinking water) or the inactive enantiomer D-NAME. Each group consisted of 6 mice.

Inhibition of VEGF-A Action
The role of VEGF-A was addressed by 3 approaches: (1) A VEGF-A neutralizing antibody (2.5 μg ip twice a week, R&D Systems) or nonimmune IgG was given in combination with Ad.hTK or Ad.Luc (10⁶ PFU IM). (2) The VEGF-R2 antagonist PTK 787 (kindly provided by Dr J. Wood, Novartis Pharma AG, Basel, Switzerland), that was previously shown to block VEGF-A–induced angiogenesis, was given in drinking water (25 mg/kg body weight per day for 15 days) starting 1 day before Ad.hTK or Ad.Luc (10⁶ PFU IM). Control mice drank regular water. (3) An Ad carrying soluble VEGF-R1 gene (Ad.s-flt-1, 10⁹ PFU, kindly provided by Drs S.A. Karumanchi, Beth Israel Deaconess Hospital and Harvard Medical School, Boston, Mass, and R. Mulligan, Harvard Medical School and Children’s Hospital, Boston, Mass) was cotransfected with Ad.hTK or Ad.Luc (each at 10⁸ PFU). Soluble VEGF-R1 is able to entrap several VEGFs, including VEGF-A. Therefore, it inhibits the biological effects of VEGF-A.

The capacity of VEGF-A antibody, PTK 787, or Ad.s-flt-1 to block VEGF-A–induced neovascularization was confirmed by using them or their respective controls (nonimmune IgG, normal drinking water, or Ad.Luc) in mice whose muscles were infected with Ad.VEGF-A (10⁹ PFU). Mice (n=6 per group) were humanely killed at 14 days from gene transfer for evaluation of neovascularization.

Akt-B Involvement in BK-Induced EC Proliferation In Vitro
We evaluated whether Akt-B blockade by wortmannin inhibits the BK-induced proliferation of human umbilical vein ECs (HUVECs). Proliferation was evaluated by MTS assay (Promega). HUVECs were seeded on 96-well plates. After 4 hours, the medium was changed to medium 199 containing reduced fetal bovine serum (2%) plus wortmannin (10 nmol/L) or vehicle (phosphate-buffered saline). To avoid BK degradation, the angiotensin-converting enzyme inhibitor captopril (10⁻⁵ mol/L) was added 2 hours before BK (10⁻⁵ to 10⁻⁶ mol/L) or its vehicle. Proliferation was assessed after 48 hours. Each experiment was repeated 8 times.

Statistical Analysis
Results are expressed as mean±SEM. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons in which ANOVA indicated significant differences, the statistical value was determined according to the Bonferroni method. Differences within and between groups were determined by a paired or unpaired Student t test, respectively. A probability value <0.05 was interpreted to denote statistical significance.

Results
hTK Protein Expression
As shown in Figure 1A, muscular hTK protein increased dose-dependently after Ad.hTK administration. hTK was not detected in plasma, contralateral noninjected muscles, or eyes. Skeletal myocytes expressed transgenic protein in variable amounts that in some cells appeared as small dots, whereas in others it was associated with the cell membrane (Figure 1B, i and ii). No immunoreactivity was observed in muscles injected with saline or Ad.Luc or when anti-hTK antibody was preabsorbed with purified hTK (iii) or Ad.Luc-injected muscle (iv) did not display any immunoreactive signal. Bar corresponds to 50 μm. Abbreviations are as defined in text.
Ad.hTK Titration and Microvascular Effects
At 14 days from gene transfer, Ad.hTK increased capillary and arteriole density in a dose-related manner (see Figure 2A and 2B). The plateau was reached with $10^7$ PFU, indicating that moderate infecting doses are sufficient to elicit maximal biological effect. Results were confirmed after normalization by myofiber density (data not shown).

Previously, we reported that Ad.hTK-promoted capillarization is stable for up to 4 weeks, a time by which transgene expression has expired. Here, we examined the destiny of newly formed vessels over a longer period. At 8 weeks, capillary density of Ad.hTK-injected tissue had returned to the levels of Ad.Luc- or saline-injected muscles (Figure 2C). In contrast, arteriole density remained elevated (Figure 2D), with the increase encompassing vessels of any size of luminal diameter (data not shown).

As expected, Ad.hTK increased EC proliferation (0.05 versus 0.012 PCNA-positive ECs/mm² in Ad.Luc and 0.12 versus 0.051 PCNA-positive ECs in every 1000 capillaries at 3 days after injection; $P<0.05$ for both comparisons). Data Supplement Figure I (available online only at http://www.circulationaha.org) shows representative images of PCNA-stained sections from muscles injected with Ad.hTK (B) or Ad.Luc (A).

Ad.hTK Gene Transfer Does Not Alter Microvascular Permeability
Ad.hTK did not increase PPE (data not shown), whereas the positive control BK increased PPE by 4.96-fold ($P<0.01$).

Ad.hTK Stimulates Akt-B Phosphorylation
As shown in Figure 3, Ad.hTK augmented Akt-B phosphorylation in Ser-473-treated preparations ($P<0.01$ versus Ad.Luc treated or untreated). Ad.hTK did not change the ratio between total Akt-B and tubulin (data not shown).

Ad.hTK Upregulates eNOS Expression
Ad.hTK increased eNOS mRNA by 2.84 times ($P<0.05$ versus Ad.Luc, Figure 4A). As shown in Figure 4B, Ad.hTK also augmented eNOS protein content (0.15 ± 0.02 versus 0.09 ± 0.01 eNOS/β-actin ratio in Ad.Luc, $P<0.05$).

Ad.hTK Does Not Affect VEGF-A Expression
Ad.hTK did not change VEGF-A expression at the mRNA ($11±1$ versus $9±2$ [VEGF-A/GAPDH] × 10⁴ in Ad.Luc, $P=NS$) or protein (0.17±0.02 versus 0.16±0.02 VEGF-A/β-actin in Ad.Luc, $P=NS$) level.

Figure 2. Bar graphs show effect of Ad.hTK on muscular microvessel density. Increasing infecting doses of Ad.hTK (filled columns) or Ad.Luc (dotted columns) or no agent (0, open column) were injected into adductors. Capillary and arteriole density was assessed after 2 (A and B) or 8 (C and D) weeks. Values are mean±SEM and number within each column represents sample size. *$P<0.05$, **$P<0.01$ vs Ad.Luc. Abbreviations are as defined in text.

Figure 3. Western blot bands show increased Ser-473-phospho-Akt-B content and unchanged total Akt-B levels in muscles infected with Ad.hTK. Control muscles received Ad.Luc. Abbreviations are as defined in text.
Ad.hTK Does Not Change VEGF-R2 Expression or Phosphorylation State

Ad.hTK did not change VEGF-R2 content (0.11 ± 0.01 versus 0.13 ± 0.04 VEGF-R2/actin in Ad.Luc, P = NS) or phosphorylation state (4.00 ± 1.51 versus 5.63 ± 0.71 phosphorylated/total VEGF-R2 in Ad.Luc, P = NS).

Functional Implication of Akt-B and eNOS in hTK-Induced Neovascularization

Ad.hTK-induced neovascularization involves both Akt-B and eNOS. In fact, hTK-induced angiogenesis and arteriogenesis were significantly inhibited by either Ad.DN-Akt-B (Figure 5A and 5B) or L-NAME (Figure 6A and 6B).

hTK-Induced Angiogenesis Is Independent of VEGF-A

As shown in Data Supplement Figure II (available online at http://www.circulationaha.org), Ad.hTK-induced neovascularization was not affected by VEGF-A neutralizing antibody (A and B), PTK 787 (C and D), or Ad.s-flt-1 (E and F). By contrast, each of the 3 compounds blocked angiogenesis and arteriogenesis caused by Ad.VEGF-A (data not shown). These results suggest that the angiogenic action of hTK is not mediated by either VEGF-A–dependent or -independent activation of VEGF-R1 or VEGF-R2.

Akt-B Mediates BK-Induced HUVEC Proliferation In Vitro

The Akt-B inhibitor wortmannin reduced BK-induced HUVEC proliferation (data not shown).

Discussion

Our study documents that a low infecting dose (10^6 PFU) of Ad.hTK was sufficient to promote capillary and arteriolar growth in limb muscle. With 10^7 PFU, microvascular density was further augmented. No additional effect occurred with escalating doses despite incrementally increased immunoreactive hTK. Our findings introduce the novel concept that low-dosage gene transfer could be effectively applied in vascular medicine with obvious reduction of side effects. This advantageous property could be conferred by the presence of a secretory signal in the hTK encoding sequence, allowing release of recombinant protein from infected cells into the extracellular environment. Thus, a few transduced cells are enough to release an optimal amount of hTK for the desired biological effect to be achieved. Moreover, the enzymatic nature of hTK allows a reduction in infection levels, as inasmuch as single molecule of enzyme generates angiogenic kinin effectors continuously. It should be cautiously noted that gene transfer efficiency could be reduced in aged or diseased animals. However, low-dosage Ad.hTK showed the capacity of preventing muscular microangiopathy in type I diabetic mice.

Ad.hTK encodes for a pre-pro form of tissue kallikrein. Activation to the mature enzyme was demonstrated in a previous study, where we found increased kinin levels in Ad.hTK-infected, normoperfused mouse adductors. Moreover, the ELISA used in the present study is able to recognize only active kallikrein.

**Figure 4.** A, Bar graph shows increased eNOS mRNA levels in Ad.hTK-infected muscles. *P < 0.05 vs Ad.Luc. B, Western blot bands show increased eNOS protein content in Ad.hTK- vs Ad.Luc-infected muscles. β-Actin levels were similar between groups. Abbreviations are as defined in text.

**Figure 5.** Bar graphs show effect of dominant-negative Akt-B (Ad.DN-Akt) on Ad.hTK-induced increases in capillary (A) and arteriole (B) density. Mice were injected with Ad.hTK or Ad.Luc (as control vector of Ad.hTK) in combination with Ad.DN-Akt or Ad.Luc (as control vector of Ad.DN-Akt). Neovascularization was assessed after 2 weeks. Values are mean ± SEM, and number within each column represents sample size. **P < 0.01 vs controls given Ad.Luc instead of Ad.hTK; *P < 0.05 vs controls given Ad.Luc instead of Ad.DN-Akt. Abbreviations are as defined in text.
A novel finding of the present study consists of the demonstration that hTK generates a stable and well-
tempered vascularization. Muscularization of nascent cap-
illaries and stimulation of growth and remodeling of
preexisting collateral vessels may account for arteriogen-
esis promoted by hTK. Support for the latter possibility
comes from clinical studies showing that hTK is involved
in adaptive growth of collateral circulation. This result
could have clinical implications, inasmuch as arterioles are
principaly responsible for providing tissue relief after
the occurrence of vascular occlusion.

The present study provides novel insight into the mech-
anism mediating Ad.hTK-induced neovascularization. Ki-
nins activate the phosphoinositide 3-kinase–Akt-B path-
way in cultured ECs, and here we report for the first time
that Akt-B blockade inhibits the proliferative effect of BK
on ECs. Moreover, our in vivo studies documented that
Akt-B and eNOS are functionally involved in the neovas-
cularization pathway of Ad.hTK, whereas VEGF-A or its
receptors play no role. Ad.hTK-produced kinins share
important features with VEGF-A, because they induce
vasodilation, angiogenesis, and NO production. However,
we found that Ad.hTK did not affect VEGF-A expression.
Most important, VEGF-A blockade failed to inhibit
Ad.hTK-induced neovascularization. Therefore, we con-
clude that VEGF-A does not participate in Ad.hTK-
duced microvessel growth. Furthermore, relevant to a
clinical perspective, Ad.hTK does not cause the increase in
microvascular permeability observed with VEGF-based
gene transfer. Safety of Ad.hTK gene therapy is addi-
tionally supported by the observation that transgene ex-
pression was confined to the injection site, thereby not
dangering distant organs, including the retina.

In conclusion, the present discoveries unravel novel mech-
anism responsible for Ad.hTK-induced neovascularization
and represent further progress toward its clinical application.
The discovery that hTK induces a VEGF-A-independent
activation of the Akt–eNOS pathway may have relevance for
therapeutic strategies alternative to or combined with
VEGF-A.

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Figure 6. Bar graphs show effect of NOS blockade by L-NAME on Ad.hTK-induced increases in capillary (A) and (B) density. Mice were injected with Ad.hTK or Ad.Luc in combination with L-NAME or inactive D-NAME. Neovascularization was assessed after 2
weeks. Values are mean±SEM, and number within each column represents sample size.
**P<0.01 vs Ad.Luc; #P<0.05, ##P<0.01 vs D-NAME. Abbreviations are as defined in text.


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