Carbon monoxide rescues ischemic lungs by interrupting MAPK-driven expression of early growth response 1 gene and its downstream target genes

Snigdha Mishra*, Tomoyuki Fujita*, Vibha N. Lama, Douglas Nam, Hui Liao, Morihito Okada, Kanji Minamoto, Yasushi Yoshikawa, Hiroaki Harada, and David J. Pinsky†

Department of Internal Medicine, University of Michigan, 3119N Taubman Center, 1500 East Medical Center Drive, Ann Arbor, MI 48109

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Carbon monoxide (CO), an endogenous cytoprotective product of heme oxygenase type-1 regulates target thrombotic and inflammatory genes in ischemic stress. Regulation of the gene encoding early growth response 1 (Egr-1), a potent transcriptional activator of deleterious thrombotic and inflammatory cascades, may govern CO-mediated ischemic lung protection. The exact signaling mechanisms underlying CO-mediated cytoprotection are not well understood. In this study we tested the hypothesis that inhibition of mitogen-activated protein kinase-dependent Egr-1 expression may be pivotal in CO-mediated ischemic protection. In an in vivo isogeneic rat lung ischemic injury model, inhaled CO not only diminished fibrin accumulation and leukostasis and improved gas exchange and survival but also suppressed extracellular signal-regulated kinase (ERK) activation, Egr-1 expression, and Erg DNA-binding activity in lung tissue. Additionally, CO-mediated inhibition of Egr-1 reduced expression of target genes, such as tissue factor, serpine-1, interleukin-1, and TNF-α. However, CO failed to inhibit serpine-1 expression after unilateral lung ischemia in mice null for the Egr-1 gene. In RAW macrophages in vitro, hypoxia-induced Egr-1 mRNA expression was ERK-dependent, and CO-mediated suppression of ERK activation resulted in Egr-1 inhibition. Furthermore, CO suppression of ERK phosphorylation was reversed by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazo-

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Abbreviations: Egr-1, early growth response 1; ERK, extracellular signal-regulated kinase; ERK1/2, ERK 1 and 2; Hmox-1, heme oxygenase type-1; JNK, c-Jun N-terminal kinase; L-NAME, L-nitroarginine methyl ester; MAPK, mitogen-activated protein kinase; ODQ, 1H-[1,2,4]oxadiazo[4,3-a]quinazolin-1-one; PKA, protein kinase A; RA, room air; sGC, soluble guanylate cyclase; TF, tissue factor; ZnP 1 IX, zinc protoporphyrin IX.

*SM and T.F. contributed equally to this work.

1To whom correspondence should be addressed at: Department of Internal Medicine, University of Michigan, MSRBIII, 7th Floor, 1500 East Medical Center Drive, Ann Arbor, MI 48109. E-mail: dpinsky@umich.edu.

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CO increases cGMP and activates MAPK in transplanted lungs. (A) cGMP levels of lungs before ("Preservation (-)") and after ("Preservation (+)") 6-h hypothermic ischemic preservation. Lungs were excised from rats inhaling either RA (RA/normoxia), CO (for 16 h), or treated for 16 h with CO and then flushed/preserved with ODQ (2 mg/kg). n = 6 for each group. **, P < 0.05. (B) ERK1/2 phosphorylation by hypoxia and its suppression by CO were examined 45 min after lung transplantation. UnTx, nontransplanted lungs excised from naive rats breathing RA; RA, transplanted lungs from donor rats breathing RA; CO, transplanted lungs from rats given 0.1% CO 16 h before donor harvest; CO/ODQ, transplanted lungs from rats treated with 0.1% CO for 16 h and given ODQ (2 mg/kg) just before donor lung harvest. n = 4 for each group. **, P < 0.05, compared with RA/normoxia; ***, P < 0.05, CO compared with CO/ODQ.

CO-Mediated Regulation of ERK in Transplanted Rat Lung and RAW Cells. Because NO activates several MAPKs through a cGMP-dependent mechanism (16), we investigated whether CO could activate the three major mammalian MAPK families, namely, ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK. Four groups of rat lungs were studied: (i) untreated lungs rapidly excised from anesthetized/untreated rats, (ii) transplanted lungs from donor rats breathing room air (RA) (RA/normoxia), (iii) transplanted lungs from donors treated with CO (0.1%/6 h), or (iv) transplanted lungs from donors who inhaled CO but were injected with ODQ (2 mg/kg). Although total ERK1/2 protein was un-changed, phosphorylated ERK1/2 was remarkably increased after transplantation (~11-fold for ERK1/2). Inhaled CO significantly suppressed ERK1/2 phosphorylation and activation (Fig. 1B). ODQ completely reversed CO-mediated inhibition of ERK1/2 phosphorylation (Fig. 1B). JNK and p38 MAPK were also activated in transplanted lungs (~4-fold increase of phospho-JNK and 6-fold increase of phospho-p38 MAPK) but were not significantly regulated by CO (Fig. 7, which is published as supporting information on the PNAS web site).

Macrophages are critical regulators of inflammatory and coagulant responses in ischemic (5, 12) and transplanted (15) lungs. Therefore, we examined ERK suppression by CO in a mononuclear phagocyte (RAW) cell culture model in which hypoxia serves as a paradigm for ischemic stress. Optimal temporal profiles to study CO effects on MAPK phosphorylation and activation in vitro were performed as described in Methods and based on experimental protocols described in ref. 16. CO significantly suppressed hypoxia-induced ERK1/2-phospho-activation (~10 fold) in RAW cells (Fig. 2); suppression was abrogated by ODQ, suggesting cGMP dependence (Fig. 2), as noted in vivo. Furthermore, a cell-permeant analogue of cGMP, 8-bromo-cGMP, suppressed ERK activation (Fig. 2). Likewise, the sGC activator YC-1 also significantly suppressed ERK phosphorylation (Fig. 2). YC-1-mediated ERK inhibition was reversed by ODQ but was insensitive to protein kinase A (PKA) inhibition with H89 (data not shown). Parallel to the in vivo results, JNK and p38 MAPK were activated in hypoxic RAW cells but not significantly regulated by CO (Fig. 8, which is published as supporting information on the PNAS web site). Together, these results indicate that ERK activation in hypoxia represents the dominant MAPK regulated by CO in a cGMP-dependent manner.

CO Regulates ERK Independent of CAMP/PKA and NO. It is plausible that CO exposure could result in elevation of intracellular CAMP levels partly by reducing CAMP breakdown through inhibition of phosphodiesterase III (17). We therefore tested the effects of stimulating the CAMP–PKA pathway on ERK phosphorylation in RAW cells. Increasing intracellular CAMP by adenylyl cyclase stimulation (by using forskolin) activated ERK under normoxic...
CO-mediated regulation of Egr-1 expression in RAW cells and transplanted rat lung is cGMP-dependent. To further delineate the signal transduction cascade linking CO inhalation to repression of thrombosis and inflammation, we examined the regulation of the transcription factor Egr-1 and its downstream target gene COX-2 by hypoxia. Egr-1 is a logical candidate because it is induced by phospho-ERK1 and is a major cause of postischemic lung injury (12, 15). Hypoxic exposure caused a 7.6-fold increase of Egr-1 mRNA in RAW cells (Fig. 3A). CO treatment before hypoxia decreased Egr-1 expression by 62% (Fig. 3A). Only the specific ERK1/2 inhibitor PD98059 completely suppressed hypoxia-induced Egr-1 induction, whereas SB202190, a specific p38 MAPK inhibitor, had no effect (Fig. 3A). These results indicate that hypoxic induction of Egr-1 is primarily ERK1/2—(but not p38 MAPK-) dependent. These data strongly suggest that CO suppresses hypoxic induction of Egr-1 by interrupting ERK1/2 activation.

To ascertain the contribution of endogenous CO in Egr-1 suppression under hypoxic conditions, we examined Egr-1 expression in RAW cells. Egr-1 mRNA was strongly induced in hypoxic RAW cells, as reported in ref. 19 (Fig. 3B). Egr-1 mRNA levels were calculated by densitometric scanning of blots, with the intensity of each Egr-1 band normalized to the corresponding β-actin band. Four experiments were performed with similar results, one of which is shown. * P < 0.05. (C) Effect of inhaled CO in the presence or absence of ODQ on graft Egr-1 mRNA levels. Although a different cohort of animals was used, experimental conditions/groups were the same as described in the legend to Fig. 1B. n = 6 for each group. * P < 0.05. (D) Effect of CO on Egr-1 protein expression in nuclei from lung grafts, analyzed by Western blotting and normalized to untreated samples. n = 6 for each group. * P < 0.05. (E) Electrophoretic mobility gel shift assay performed on nuclear extracts with a 32P-labeled consensus probe for Egr-1. Lane 1 was loaded solely with buffer containing free 32P-labeled Egr-1 probe. Lane 2, nuclear extract from naive (untreated) lung; lane 3, nuclear extract from CO-treated/ transplanted lung; lane 4, nuclear extract from normoxic/ transplanted lung; lane 5, same sample as lane 4 in the presence of a 100-fold molar excess of unlabeled consensus Egr-1. The arrow indicates migration of the band corresponding to the Egr-1–DNA complex. Four experiments were performed with similar results, one of which is shown.
transplantation. As shown in ref. 5, ischemia up-regulates serpine-1 in wild-type (5194). Transplantation caused a 5.1-fold increase in fibrin accumulation lung extracts from rats heparinized immediately ante mortem. DNA binding (Fig. 3E, lane 3). Specificity of Egr-1–DNA interaction is evidenced by competition experiments in which a 100-fold molar excess of unlabeled Egr-1 probe added to the same sample as lane 4 abolished the appearance of the gel shift band. These data clearly demonstrate that CO suppresses Egr-1 induction during lung ischemia.

Functional Effects of CO on Coagulation, Fibrinolysis, and Inflammation in Lung Ischemia. To determine whether Egr-1 induction underlies protective effects of CO, we examined expression of Egr-1 target genes (TF and serpine-1) after lung transplantation. Serpine-1 suppression by CO has been shown to be a key survival mechanism in pulmonary ischemia (5). CO suppressed serpine-1 mRNA (Fig. 4A) and TF mRNA (Fig. 4B) increased by orthotopic transplantation. As shown in ref. 5, ischemia/reperfusion strongly up-regulates serpine-1 in wild-type (Egr-1+/+) mice (Fig. 4C). Interestingly, mice null for the Egr-1 gene (Egr-1−/−) exhibited a modest induction of serpine-1 after ischemia/reperfusion, indicating that Egr-1 is an important regulator of ischemia-driven serpine-1 induction. In this identical model, we showed that CO suppressed serpine-1 induction by 60% (5). However, in Egr-1−/− mice, CO is unable to suppress serpine-1 induction (Fig. 4C). These data strongly demonstrate that Egr-1 plays a critical role in the biological effects of inhaled CO.

We next measured accumulation of fibrin by immunoblotting lung extracts from rats heparinized immediately ante mortem. Transplantation caused a 5.1-fold increase in fibrin accumulation compared with untreated intact lungs (Fig. 4D). CO treatment of lung donors markedly decreased tissue fibrin accumulation, illustrating that inhaled CO promotes fibrin dissolution.

Because Egr-1 also regulates ischemic induction of inflammatory genes (12), we examined effects of CO on the expression of proinflammatory cytokines (IL-1β) and TNF-α. Both IL-β mRNA (Fig. 5A) and TNF-α protein (Fig. 5B) were up-regulated by transplantation, and their induction was suppressed by CO. CO significantly reduced leukocyte recruitment after transplantation as measured by graft myeloperoxidase activity (Fig. 5C). These data indicate that CO potently suppresses the induction of proinflammatory cytokines and graft leukostasis after lung transplantation.

Effect of CO on Lung Function and Pulmonary Graft Survival. To further determine the physiological consequences of Egr-1 suppression by CO, we used a stringent model of lung ischemia (lung transplantation), because it enables instrumentation of animals for physiological measurements. Reduced graft microvascular thrombosis and leukostasis induced by CO results in distinct functional improvements after orthotopic lung transplantation. CO significantly reduced pulmonary vascular resistance of transplanted lungs and increased arterial blood flow (Fig. 6 A and B) in a dose-dependent manner. In addition, inhaled CO improved arterial oxygenation and recipient survival (Fig. 6 C and D). ODQ treatment (2 mg/kg) abolished CO’s beneficial effects on both lung graft function and recipient survival. These data emphasize that the beneficial effects of CO are likely mediated by activation of sGC.

Discussion

The major finding of the current study is that CO inhibition of ERK-dependent Egr-1 expression interrupts proinflammatory and prothrombotic mediators in ischemic lung injury. In lung transplantation, levels of NO, the dominant beneficial gaseous mediator, fall precipitously because of quenching by superoxide. However, because CO is more stable and shares similar reactivity with heme prosthetic groups, some biological activities ascribed to NO during ischemia could be attributed to increased CO. Moreover, despite the primary recognition of CO as a lethal inhalant or respiratory asphyxiant, data from this study indicate...
that, at low doses, CO can be therapeutically useful in a clinically relevant model. Most notably, the antiatherogenic and antiinflammatory activities of CO appear to confer functional benefit under ischemic stress.

We further demonstrate ERK1/2 phosphorylation as a proximal event after lung ischemia that leads to Egr-1 induction. The involvement of ERK1/2 in lung transplant injury is not surprising, given that, in addition to hypoxic/ischemic stress, MAPK pathways can be rapidly activated by other triggers of cellular stress, including heat shock, UV light, inflammatory cytokines, and endotoxin. Although three primary MAPKs have been reported in mammalian cells (ERK, JNK, and p38 MAPK), data shown here support a primary role for ERK1/2.

The signaling effects of CO through cGMP demonstrated here are similar to NO/Egr-1 signaling. NO down-regulates shear stress induction of Egr-1 by inhibition of ERK1/2 phosphorylation (21). NO donors not only suppress Egr-1 mRNA but also reduce Egr-1 protein expression and transcriptional activity (22). Moreover, NO also reduces Egr-1 mRNA induction in rat macrophages treated with LPS (23). cGMP-dependent CO suppression of ERK1/2 phosphorylation in RAW cells shown here is similar to the cGMP-dependent NO effects in human platelets and rat vascular smooth muscle cells (24). These findings are concordant with our data showing a cGMP-dependent CO suppression of ERK1/2. Regulation of ERK1/2 by CO results in Egr-1 attenuation and a consequential decrease in proinflammatory and prothrombotic gene targets of Egr-1 in ischemic tissue. Although CO increases NO under certain experimental conditions (18), here the pan-NO synthase blocker L-NNAME failed to inhibit the suppressive effect of CO on ERK phosphorylation in hypoxic RAW cells. This finding indicates that cGMP-dependent CO effects on ERK activation are NO-independent, and generation of NO by CO does not appear functionally important in the models presented here.

Prolonged CO exposure may influence other signaling pathways, such as cAMP/PKA, through elevation of intracellular cAMP levels (17), resulting in secondary ERK regulation. Our data demonstrate that increasing cAMP-activated ERK in RAW cells and CO was unable to abrogate cAMP-mediated ERK activation. Furthermore, inhibition of PKA did not affect CO-mediated regulation of ERK phosphorylation after hypoxia while effectively inhibiting cAMP-mediated ERK activation. Together, these data support a cAMP/PKA-independent signaling by CO.

Opposing effects of hemin (inducer) and ZnPP IX (inhibitor) of Hmox-1 on endogenous CO production and regulation of Egr-1 demonstrated in this study illustrate how Hmox-1 may reduce postischemic inflammation. Our data are consonant with reports of potent antiinflammatory effects of Hmox-1 in other model systems. In vivo transfection of rat lungs increased Hmox-1 protein, attenuated neutrophil infiltration, and conferred cytoprotection in hypoxic injury (9). In a microvascular model of venular leukocyte adhesion, Hmox-1 induction reduced leukocyte adhesion, and blockade of its activity with ZnPP IX increased adhesion (1). Although this effect was ascribed to the bile pigment antioxidants generated by Hmox-1 activity, a role for CO was not excluded. The cytoprotective role of Hmox-1 was further substantiated in a rat model of endotoxemia, in which Hmox-1 induction before endotoxin challenge was beneficial, and exacerbation of outcome was observed after Hmox-1 inhibition (25).

Because beneficial effects of CO are cGMP-dependent, hypothetically, a brief exposure of CO should suffice in conferring protection. In this study, longer duration (up to 16 h) of CO stimulation was required to block downstream signaling through ERKs to suppress Egr-1. The exact reasons for this result are presently unclear. Arguably, effective attenuation of Egr-1 gene expression is likely dependent on sustained and sufficient levels of cGMP to adequately suppress ERK activation after hypoxia/ischemia. In contrast to known NO-mediated rapid but transient increases in cGMP, the effects of CO, which is more stable than NO, are likely temporally delayed but may remain sustained.

Our data indicate that cGMP levels rise substantially in lungs preserved with inhaled CO, in parallel with potent suppression of postischemic lung inflammation. Because functional benefits of CO were blocked by ODQ, cGMP formation likely represents an important intermediary step in the antiinflammatory and antiatherogenic effects of CO in lung transplantation. These data are in contrast to in vitro evidence of cGMP-independent antiinflammatory effects exerted by CO in LPS-challenged macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear. In a recent murine study, although both lung ischemia and LPS administration triggered induction of a similar profile of proinflammatory cytokines, adhesion receptors, and procoagulant mediators, only ischemia (not LPS) induced Egr-1 (12). Model-related differences may also underlie the divergent effects observed with CO administration and MAPK induction between the LPS-mediated effects versus ischemia. Although CO apparently did not affect ERK1/2 phosphorylation after LPS challenge in RAW cells (10), it potentiated the Egr-1 protein expression in LPS cultured macrophages (10). The reasons for the differences in our findings are not entirely clear.

With regard to the physiological effects of endogenous and exogenous CO, the current experiments establish a paradigm to explain how CO suppresses thrombosis and inflammation. Here we demonstrate that CO suppressed ischemic induction of Egr-1 as well as Egr-1/DNA binding and abrogated expression of a prototypical Egr-1-responsive gene, serpine-1. In Egr-1 gene null mice, CO failed to suppress ischemia-induced serpine-1 gene expression. A cascade of events leading from Hmox-1 induction to CO generation, through an intermediary step of ERK phosphorylation,
represses the Egr-1 “master switch” (12). Induction of Egr-1 by hypoxia (19) or ischemia (12) triggers procoagulant and inflammatory genes (12, 13, 19, 26) such as TF, serpine-1, and IL-1β. Modulation of these genes by CO (5, 10) may contribute to its clinically beneficial actions in lung transplantation (27). The present study underscores a cGMP-dependent mechanism by which CO prevents ischemic induction of downstream target genes through ERK/Egr-1 suppression, thereby reducing inflammatory and thrombotic mediators during ischemia.

**Methods**

**Hypoxia and CO Exposure.** Mouse macrophage cells (RAW cells) were placed in a normobaric normoxic environment. See Supporting Text, which is published as supporting information on the PNAS web site, for more details. For the analysis of cell signaling the following were used: MAPK kinase/MEK (upstream of ERK) inhibitor PD98059 (10 μM/10 min; Calbiochem); p38 MAPK inhibitor, SB202190 (100 nM; Calbiochem); adenylate cyclase activator forskolin (10 μM/20 min; Sigma); PKA inhibitor H89 (20 μM/20 min; Sigma); NO synthase inhibitor L-NAME (1 mM/24 h; Sigma); guanylate cyclase activator (28) YC-1 [1-benzyl-2-furyl]indazole (30 μM/30 min); 8-bromo-cGMP (0.1 mM/30 min to 2 h; Sigma); and guanylate cyclase inhibitor ODO (24) (10 μM/2 h; Sigma) were added before hypoxia (1 h). Exposure to CO was accomplished by using a similar lung transplant model was used, with donor preexposure to room air or CO (0.1% or 1.0 ppm for 16–24 h before surgery) as indicated. See Supporting Text for more details.

**Rat Lung Transplant Experiments.** An orthotopic, isogeneic rat left lung transplant model was used, with donor preexposure to room air or CO (0.1% or 1.0 ppm for 16–24 h before surgery) as indicated. See Supporting Text for more details.

**Murine Lung Ischemia Experiments.** The effect of inhaled CO on expression of serpine-1 was examined in 8- to 10-week-old male Egr-1−/− mice (12, 29) and compared with Egr-1+/+ littermate controls (genotype confirmed by Southern blotting as described in refs. 5, 30, and 31). CO exposure of mice was similar to rat experiments.

**Electrophoretic Mobility Gel Shift Assay.** Gel shift assays were performed on nuclear extracts prepared immediately after lung harvest, as described in refs. 31 and 32.

**Northern Blotting.** Twenty micrograms of total RNA from tissue or cell culture samples per lane was resolved on 0.8% agarose/ formaldehyde gel, electrophoresed, and transferred to Duralon-UV membranes (Stratagene). Membranes hybridized with 32P-labeled cDNA probes for Egr-1 (35, 34), IL-1β (12), TF, or serpine-1. Human β-actin cDNA served as an RNA loading control, and quantification was performed with National Institutes of Health Image analysis.

**Western Blotting.** Immunoblotting was performed by using standard methods with primary antibodies; rabbit anti-phospho- or total p42/p44 MAPK IgG, rabbit anti-phospho- or total JNK IgG, rabbit anti-phospho- or total p38 MAPK IgG (Cell Signaling Technology, Beverly, MA), rabbit anti-Egr-1 IgG, goat anti-TNF-α (Santa Cruz Biotechnology), or mouse anti-fibrin IgG (Biodiagnostic International, Saco, ME).

**Myeloperoxidase and cGMP Assays.** Tissue myeloperoxidase activity was measured as an index of graft leukocyte accumulation as described in ref. 35.

**Statistical Analysis.** The product limit (Kaplan–Meier) estimate of cumulative survival was assessed by the log-rank test; *, P < 0.05. Statistical significance was analyzed by ANOVA by using commercial software (STATVIEW; Cary, NC). For all experiments, data are shown as mean ± standard error of the mean; *, P < 0.05.

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