Rapid Estrogen Receptor Signaling Mediates Estrogen-Induced Inhibition of Vascular Smooth Muscle Cell Proliferation

Kazutaka Ueda, Qing Lu, Wendy Baur, Mark J. Aronovitz, Richard H. Karas

Objective—The proliferation of vascular smooth muscle cells (VSMCs) plays a crucial role in vascular diseases, such as atherosclerosis and restenosis, after percutaneous coronary intervention. Many studies have shown that estrogen inhibits VSMC proliferation in response to vascular injury in the mouse carotid injury model. However, the mechanisms that mediate these effects remain unclear. Here, we investigated the mechanisms by which estrogen inhibits VSMC proliferation.

Approach and Results—We established a novel transgenic mouse line, referred to as the disrupting peptide mice, in which rapid estrogen receptor (ER)—mediated signaling is abolished by overexpression of a peptide that prevents the ER from forming a signaling complex necessary for rapid signaling. Carotid artery VSMCs from disrupting peptide mice or littermate wild-type female mice were obtained by the explant method. In VSMCs derived from wild-type mice, estrogen significantly inhibited VSMC proliferation. Phosphorylation levels of Akt and extracellular regulated kinase induced by platelet derived growth factor were significantly inhibited by estrogen pretreatment. Estrogen enhanced complex formation between ERα and protein phosphatase 2A (PP2), and enhanced PP2A activity. The blockade of PP2A activity abolished the estrogen-induced antiproliferative effect on VSMCs. In contrast, none of these effects of estrogen observed in the wild-type VSMCs were observed in VSMCs derived from disrupting peptide mice. These results support that rapid, non-nuclear ER signaling is required for estrogen-induced inhibition of VSMC proliferation, and further that PP2A activation by estrogen mediates estrogen-induced antiproliferative effects.

Conclusions—These findings support that PP2A activation via rapid, non-nuclear ER signaling may be a novel target for therapeutic approaches to inhibit VSMC proliferation, which plays a central role in atherosclerosis and restenosis. (Arterioscler Thromb Vasc Biol. 2013;33:1837-1843.)

Key Words: cardiovascular diseases ■ hormones ■ molecular biology ■ signal transduction

Cardiovascular disease (CVD) is the leading cause of death in the United States.1 Premenopausal women have a lower incidence of death attributable to CVD than age-matched men in the United States, supporting that the hormone estrogen may have an important protective effect on the vasculature.2 Although many observational studies have demonstrated that exogenous estrogen therapy is associated with lower primary risk of CVD in postmenopausal women,3-5 randomized controlled trials, primarily in older postmenopausal women, have not shown reductions in CVD events and, in some instances, they have demonstrated harm.6

Numerous animal studies have shown that estrogen significantly protects against the development of atherosclerosis. We and others have repeatedly shown that estrogen inhibits the response to vascular injury in the mouse carotid artery injury model, in which in vivo estrogen treatment dramatically inhibits the proliferation of vascular smooth muscle cells (VSMCs).7-11

Taken together with the clinical data, these findings underscore the complexity of the effects of estrogen on the cardiovascular system and highlight its ability to exert both potentially harmful and potentially beneficial effects. This observation strongly supports the need to better understand the molecular mechanisms by which estrogen exerts its cardiovascular effects.

There are 2 different forms of the estrogen receptor (ER), ERα and ERβ.12 We previously reported that in ERβ knock-out (KO) mice, estrogen was still protective against vascular injury,13 whereas in ERα KO mice, in which ERα is fully deleted, estrogen treatment showed no protective effect on VSMC proliferation after vascular injury,4 supporting that ERα is necessary for the inhibitory effect of estrogen on VSMC proliferation. Importantly, the molecular mechanisms by which estrogen inhibits VSMC proliferation are unknown.

Estrogen, acting via ERs, activates 2 distinct signaling pathways, the genomic pathway and the rapid, non-nuclear pathway.2,14 The genomic pathway has been well studied in which ligand-bound ER translocates to the nucleus and
transcriptionally regulates gene expression. More recent studies have shown that estrogen also signals through a rapid, non-nuclear signaling pathway that involves activation of specific protein kinases but does not directly involve regulation of gene expression (although cross-talk between these pathways has also now been described). Since their discovery, estrogen-induced protection against vascular injury and the inhibition of VSMC growth have been believed to be attributable to long-term genomic effects, whereas little has been investigated about the role of the rapid, non-nuclear pathway in regulating the response to vascular injury and inhibiting VSMC proliferation, although this pathway is known to be involved in regulating endothelial function and NO release.

We previously reported that activation of the non-nuclear pathway by estrogen requires binding between the ER and a scaffold protein, striatin, and that a peptide derived from amino acids 176 to 253 of ERα disrupts the binding between ER and striatin, preventing activation of the non-nuclear signaling pathway, whereas leaving the classical genomic signaling pathway intact. We have established a transgenic mouse line in which this disrupting peptide (disrupting peptide mice [DPM]) is overexpressed throughout the body. In these DPM, ERα-mediated non-nuclear signaling is disrupted, whereas the canonical genomic pathway is still intact. In this study, we investigated the molecular mechanisms by which estrogen inhibits VSMC proliferation and explored whether non-nuclear ER signaling is involved in the antiproliferative effect of estrogen on VSMCs.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Estrogen-Induced Antiproliferative Effect on VSMCs Requires ERα-Striatin Binding
E2 significantly inhibited the serum-induced proliferation of VSMCs derived from wild-type (WT) mice but not of VSMCs derived from DPM (Figure 1A). E2 had no effect on VSMC proliferation derived from ERαKO mice, whereas the E2-induced inhibition was restored by ERα overexpression in VSMCs derived from ERTKO mice (Figure 1B). E2 markedly inhibited the increase in mRNA expression of proliferating cell nuclear antigen, a marker of VSMC proliferation, induced by stripped bovine growth serum or platelet derived growth factor (PDGF) in WT VSMCs, but not in DPM VSMCs (Figure 1C). E2 significantly activated an estrogen response element-driven luciferase reporter plasmid to a similar degree in VSMCs from both WT mice and DPM (Figure 1D), supporting that the genomic ER pathway is still preserved in VSMCs from DPM. These data indicate that E2 directly inhibits WT VSMC proliferation in an ERα-dependent manner, and further that the disruption of ER-striatin binding induced by the disrupting peptide abolishes the antiproliferative effect.

Estrogen Inhibits Kinase Phosphorylation Through Activation of Protein Phosphatase 2A
To examine the mechanisms by which estrogen inhibits VSMC proliferation, we assessed phosphorylation levels of kinases that promote cell growth, including Akt and extracellular regulated kinase (ERK). As expected, PDGF stimulation increased phosphorylation levels of Akt and ERK in WT VSMCs (Figure 2A). Meanwhile, E2 pretreatment attenuated PDGF-induced phosphorylation of Akt and ERK in a dose-dependent manner (Figure 2A). E2-mediated inhibition reached statistical significance at 10 to 100 nmol/L of E2 for phospho-Akt and at 50 to 100 nmol/L of E2 for phospho-ERK. Therefore, we used 100 nmol/L of E2 for further experiments. PDGF stimulation increased phosphorylation levels of Akt, its downstream target glycogen synthase kinase 3 α/β, and ERK, not only in WT VSMCs but also in DPM VSMCs (Figure 2B). E2 pretreatment significantly attenuated PDGF-induced kinase phosphorylation in VSMCs from WT mice. In contrast, E2 had no significant effect on PDGF-induced kinase phosphorylation in DPM VSMCs (Figure 2B).

To investigate the mechanisms by which E2 inhibits kinase phosphorylation, we examined whether ER in VSMCs interacts...
with phosphatases. Coimmunoprecipitation experiments revealed that ERα forms a complex with protein phosphatase 2Ac (PP2Ac), but not with either MAPK kinase phosphatase-1 or phosphatase and tensin homolog, all of which are known to dephosphorylate Akt and ERK as their substrate (Figure 1 in the online-only Data Supplement). Therefore, we investigated the role of PP2A in estrogen-mediated inhibition of kinase phosphorylation. Immunoprecipitation of PP2Ac showed that E2 clearly increased ERα–PP2Ac complex formation in VSMCs from WT mice but had no significant effect in DPM VSMCs stimulated with PDGF (5 ng/mL) for 15 minutes with pretreatment of vehicle (V) or E2 for 30 minutes. A, E2 inhibited phosphorylation of Akt and extracellular regulated kinase (ERK) in dose dependency in VSMCs derived from WT mice (n=3). Quantification data are normalized to PDGF(-) vehicle-treated control. #P<0.05, *P<0.01. B, E2-mediated inhibition of phosphorylation of Akt, glycogen synthase kinase (GSK)3α/β, and ERK in VSMCs derived from WT mice or DPM (n=4). #P<0.05.

**Figure 2.** E2 inhibits platelet derived growth factor (PDGF)-induced kinase activation in vascular smooth muscle cells (VSMC) derived from wild-type (WT) but not disrupting peptide mice (DPM). Western blotting and quantification of phosphorylated kinases in VSMCs stimulated with PDGF (5 ng/mL) for 15 minutes with pretreatment of vehicle (V) or E2 for 30 minutes. A, E2 inhibited phosphorylation of Akt and extracellular regulated kinase (ERK) in dose dependency in VSMCs derived from WT mice (n=3). Quantification data are normalized to PDGF(-) vehicle-treated control. #P<0.05, *P<0.01. B, E2-mediated inhibition of phosphorylation of Akt, glycogen synthase kinase (GSK)3α/β, and ERK in VSMCs derived from WT mice or DPM (n=4). #P<0.05.

**PP2A Activation Is Required for Estrogen-Induced Inhibition of Kinase Phosphorylation and Antiproliferative Effect on VSMCs**

To determine whether PP2A is involved in E2-mediated inhibition of PDGF-induced Akt and ERK phosphorylation, we next examined whether knock down of PP2A by siRNA attenuated this effect of E2. As shown in Figure 4A, E2 significantly inhibited Akt and ERK phosphorylation in control siRNA–treated WT VSMCs but it no longer inhibited PDGF-induced phosphorylation in WT VSMCs in which PP2A was knocked down.

To further investigate the role of PP2A in E2-mediated inhibition of VSMC proliferation, we next examined the effect of PP2A inhibition with a pharmacological PP2A inhibitor, okadaic acid (OA), on the effects of E2 on VSMC proliferation. OA completely inhibited the E2-induced increase in PP2A activity in WT VSMCs (data not shown). The E2-induced antiproliferative effect observed in stripped bovine growth serum-stimulated VSMCs from WT mice was completely blocked by OA (Figure 4B). Furthermore, OA also significantly blocked the E2-induced antiproliferative effect observed in PDGF-stimulated VSMCs from WT mice (Figure 4C).

Because the kinases Akt and ERK also mediate apoptosis signaling cascades, we also examined the effects of E2 on apoptosis of VSMCs. Terminal deoxynucleotidyl transferase nick end labeling staining showed that E2 had no effect on apoptosis in VSMCs under quiescent, PDGF-stimulated or serum-stimulated conditions (Figure II in the online-only Data Supplement).

**PP2A Activation Is Required for Estrogen-Induced Inhibitory Effect on VSMC Migration**

We further examined whether the rapid, non-nuclear signaling pathway is involved in E2-mediated inhibition of
PDGF-induced VSMC migration, which also contributes to the development of maladaptive response after vascular injury. In vitro scratch wound assays showed that E2 significantly inhibited cell migration promoted by PDGF in VSMCs derived from WT mice, and that this inhibitory effect of E2 on VSMC migration was abolished in cells treated with OA (Figure 5A). In contrast, E2 had no effect on migration in VSMCs derived from ERα mice (Figure 5B) or in VSMCs derived from DPM (Figure 5C).

**Discussion**

Although we and others have previously shown that estrogen inhibits VSMC proliferation in vitro and in vivo, the molecular mechanisms that mediate this effect remain unknown. Recently, we reported that estrogen-induced protective effects against carotid vascular injury are abolished in DPM in vivo, supporting that the rapid, non-nuclear signaling pathway is necessary for the estrogen-mediated antiproliferative/migratory effects in VSMCs (Figure 6).

PP2A is a major serine/threonine protein phosphatase that has been highly conserved in all eukaryotes. PP2A phosphatase activity is associated with growth inhibition and cell cycle arrest, so that PP2A is known to be a key mediator of tumor growth suppression. Because proliferation of VSMCs is also enhanced in large part by protein kinase–dependent pathways, we explored the role of PP2A activation in E2-mediated inhibition of VSMC proliferation. We previously showed that ER binds to PP2Ac, which is a catalytic subunit of PP2A complex, and that estrogen enhances the interaction between PP2Ac and ERα in endothelial and COS-1 cell lines, respectively. In that study, inhibition of PP2A activity by OA increased ER-mediated transcriptional activity, which is consistent with our current data showing a trend toward increased transcriptional activity of ERα in VSMCs from DPM compared with WT mice, although the difference was not statistically significant (Figure 1D). In this study, we further show that in VSMCs derived from WT mice, E2-treatment increases the binding between ERα and PP2Ac and increases PP2A activity in VSMCs derived from WT mice. These effects of estrogen were diminished or absent in VSMCs derived from DPM, supporting that estrogen enhances PP2A activity, possibly by increasing the interaction between ERα and PP2Ac, and that this is dependent on the rapid, non-nuclear pathway. In VSMCs derived from DPM, where ERα no longer

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**Figure 3.** E2 enhances estrogen receptor--protein phosphatase 2A (ER--PP2A) complex formation and increases PP2A phosphatase activity in wild-type (WT) mice but not in disrupting peptide mice (DPM) vascular smooth muscle cells (VSMCs). **A**, PP2Ac was immunoprecipitated from VSMCs treated with vehicle (V) or E2 for 30 minutes. Immunoblotting with striatin, ERα, and PP2Ac and quantification are shown. Normal mouse IgG was used as negative control (n=3). #P<0.05. **B**, PP2A activity assay. VSMCs were treated with vehicle (V) or E2 for specific time (n=4 for each condition). #P<0.05. **C**, Western blot of PP2Ac in VSMCs treated with vehicle (V) or E2 for 30 minutes. Three repeated experiments showing similar results were performed.
binds to striatin. E2-induced ERα–PP2A complex formation was significantly attenuated, suggesting that striatin plays a critical role for ERα–PP2Ac complex formation mediated by E2. Furthermore, the inhibition of PP2A activity resulted in loss of the ability of estrogen to inhibit kinase phosphorylation and cell proliferation, suggesting that estrogen-induced antiproliferative effect on VSMCs is dependent on PP2A activation. Estrogen regulates physiology and pathophysiology in both reproductive and nonreproductive target tissues, including the cardiovascular system.12 Estrogen exerts diverse actions in
The evidence from randomized controlled trials suggests that treatment with estrogen early after menopause may be more effective in reducing the risk of CVDs than late treatment with estrogen. However, although PP2A might play a role in the different effects of estrogen in early versus late, there is no direct evidence that the function of PP2A in the vasculature is altered after menopause. Further studies will be needed to address this specific point.

Several limitations of the current findings are worthy of mention. First, although the data presented support that E2, acting via ERα, activates PP2A catalytic activity, the molecular mechanisms that mediate this effect are unclear. Second, E2 has also been reported to interact with and regulate activity of phosphatases in addition to PP2A, such as MAPK kinase phosphatase-1 and phosphatase and tensin homolog, and the extent to which effects on other phosphatases might also contribute to regulation of VSMC proliferation has not been tested here. Finally, although our data indicate that estrogen inhibits VSMC proliferation via PP2A activation through the rapid, non-nuclear pathway in cultured VSMCs, and the relevance of the rapid pathway in vivo was observed in our previous study using DPM, further in vivo studies are needed to assess whether PP2A activation by estrogen is also relevant in vivo.

In conclusion, our results support that the rapid, non-nuclear ER signaling is required for estrogen-induced inhibition of VSMC proliferation, and further that PP2A activation by estrogen mediates estrogen-induced antiproliferative effects in these cells. Taken together, our findings support that rapid, non-nuclear ER signaling may be a novel target for therapeutic approaches to inhibit VSMC proliferation, which plays a central role in vascular diseases such as atherosclerosis and restenosis.

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Disclosures
None.

References
Inhibitory Effect of Rapid Estrogen Signaling

The proliferation of vascular smooth muscle cells plays a crucial role in vascular diseases, such as atherosclerosis and restenosis, after percutaneous coronary intervention. Although estrogen is known to inhibit vascular smooth muscle cell proliferation in response to vascular injury, the mechanisms that mediate these effects remain unclear. Here, we demonstrate that protein phosphatase 2A activation via the noncanonical estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury. Circulation. 2012;126:1993–2004.


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Materials and Methods

Animals

All animal–related procedures described were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. Female (C57Bl/6 background, 10 week old) mice were used in this study. The generation of DPM and ERαKO(st) mice have been described previously (1, 2). Littermate transgene-negative wild type (WT) mice were used as controls.

Cell lines and culture methods

Carotid artery smooth muscle cells were cultured from carotid artery explants from WT, DPM and ERαKO(st) mice, as previously described (3), and grown in phenol red-free DMEM with 10% charcoal-stripped bovine growth serum (sBGS) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. VSMC from passage 7 to 10 were used for the experiments.

For VSMC proliferation assays, cells were plated in 96-well plates at a density of 2500 cells / well with DMEM containing 10% sBGS. Four hours after plating, the media was replaced by DMEM with either 3% sBGS or 5ng/ml PDGF (Life Technologies, Grand Island, NY), and treated with 100 nmol/L 17β-estradiol (E2, Sigma-Aldrich, St. Louis, MO) or EtOH vehicle. In a subset of experiments, okadaic acid (50nmol/L) was added to the culture medium 15 minutes prior to E2 treatment, then PDGF (5 ng/ml) was added 30min after the E2 treatment. The VSMC proliferation was assessed at the specified time points by the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacture’s instructions. Subculture was
performed in WT and DPM in parallel, and these assays were repeated in three subclones in each genotype.

For migration assays, VSMC \((2 \times 10^5)\) were plated in 6 well plates with DMEM containing 10% sBGS. Twenty four hours after plating, the media was replaced with DMEM with 0.5% sBGS, and 18 hr after starvation, a single scratch was made with a plastic p200 pipette tip. Cells were rinsed once with PBS to remove non-adhered cells, and incubated in DMEM with 0.5% sBGS containing E2, EtOH vehicle, PDGF or okadaic acid. At 24 hr after the scratch was made, bright-field images of 4 different positions for each condition were captured, and cell migration was measured as the number of cells that had entered the scratch. Each experiment was repeated 4 times.

To assess the effect of E2 on phosphorylation levels of kinases, VSMC \((2 \times 10^5)\) were plated in 6 well plates with DMEM containing 10% sBGS. Twenty four hours after plating, the media was replaced with DMEM without sBGS, and 6 hr after starvation, cells were treated with okadaic acid, 5-100 nmol/L E2 and PDGF as described above. Cells were washed with cold PBS 15 min after PDGF stimulation and then frozen immediately at -80 degrees.

For apoptosis assays, VSMC \((2 \times 10^4)\) were plated with DMEM containing 10% sBGS in 6 well plates where gelatin-coated coverslips were put on the bottom of each well. Twenty four hours after plating, culture media was replaced with DMEM with 0.5% sBGS, and 16 hr after starvation, cells were subjected to treatment with E2 or EtOH followed by stimulation with PDGF (5ng/ml) or sBGS (3%). At 24 hr after stimulation, cells were fixed by 4% paraformaldehyde and subjected to TUNEL staining using a
commercially available kit (In Situ Cell Death Detection Kit; Roche, Basel, Switzerland) as directed by manufacturer. DAPI staining was used for detection of nuclei.

**qRT-PCR**

To assess the effect of E2 on mRNA expression level of Proliferating Cell Nuclear Antigen (PCNA) in VSMC, cells were plated in 6 well plates with DMEM containing 10% sBGS for 24 hr. Then cells were switched to media containing 0.5% sBGS for 18 hr, treated with 100 nmol/L E2 or EtOH vehicle for 30 min and then with 5ng/ml PDGF, 100 mmol/L acetic acid with 0.1% BSA as vehicle control, or 3% sBGS for 24 hr before RNA was extracted using the RNeasy plus kit (Qiagen, Valencia, CA). cDNA was prepared using the Super Script VILO kit (Life Technologies), and qRT-PCR was performed using Quanti Tect SYBR Green (Qiagen) and the primers: **PCNA:** forward CTAGCCATGGGCGTGAAC, reverse GAATACTAGTGCTAAGGTGTCTGCAT, and **GAPDH:** forward CACTGAAGGGCATCTTGG, reverse CATTGTCAATACCAGGAAATGAG.

**Gene transfer**

To test the role of ERα in VSMC on E2-mediated anti-proliferative effect, the adenovirus encoding both ERα and green fluorescent protein (GFP) (adeno-GFP-ERα) was introduced to VSMC derived from ERα KO mice. Adenovirus encoding GFP only (adeno-GFP) was used as a control. The generation of the adeno-GFP-ERα and -GFP was described previously (4). VSMC (2×10^5) were plated in 6 well plates with DMEM containing 10% sBGS and cultured for 24 hr, and then infected by adeno-GFP-ERα or -
GFP. Sixteen hours later, the media was replaced by DMEM containing 10% sBGS to wash out the adenovirus, and cells were then cultured for 8 hours. The cells were then re-plated in 96 well plates as described above for the VSMC proliferation assay. The efficacy of infection was assessed by counting cells expressing GFP fluorescent. Approximately 60-80 % of cells expressed GFP 24 hr after adeno adeno-GFP-ERα or -GFP infection (data not shown).

To examine the transcriptional transactivation potential of the E2 receptors, VSMC were plated in 12-well plates and cultured in DMEM with 10% sBGS overnight and were transfected with the reporter plasmid ERE-Luc (containing an estrogen response element (ERE) driving expression of the luciferase gene) and β-galactosidase by using Fugene HD (Roche). Following transfection, cells were treated with E2-containing media 24 h prior to harvest for determination of luciferase activity. Cells were lysed in passive lysis buffer (Promega), and luciferase assays (luciferase assay system, Promega) and β-galactosidase assays (Tropix, Bedford, MA) were performed according to the manufacturer's guidelines.

To test the role of protein phosphatase 2 (PP2) Ac in E2-induced inhibition of phosphorylation of Akt, VSMC (1×10^5) were plated in 6-well plates and cultured in DMEM with 10% sBGS, and siRNA (30 pmol) targeting PP2Ac and negative control RNA (Dharmacon, Chicago, IL) were transfected into VSMC by using Lipofectamine RNAiMAX (Life Technologies) according to the manufacture’s instructions. Forty-eight hours after transfection, cells were treated with E2 followed by PDGF stimulation as described above, and then cells were harvested 15 min after PDGF stimulation.
**Western blotting assay**

Western blotting was performed as described previously (4). VSMC were lysed in RIPA buffer or IP buffer (Thermo Scientific, Lafayette, CO) containing protease inhibitor and phosphatase inhibitor cocktail (EMD, LaGrange, IL). Lysates were subjected to SDS-PAGE and then transferred onto PVDF membranes (Thermo Scientific). The membranes were probed using a primary antibody against, phospho-Akt, phospho-ERK, phospho-GSK3α/β, PTEN (Cell signaling, Danvers, MA), α-tubulin (EMD), ERK (Life Technology), GAPDH, ERα (MC20), Akt, PP2Ac, (Santa Cruz Biotechnologies, Santa Cruz, CA), MKP-1, and striatin (BD bioscience, San Jose, CA). The ECL-plus system (GE healthcare, Pittsburgh, PA) was used for detection.

**PP2A activity assay**

PP2A activity was measured by using PP2A immunoprecipitation phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY). Cells were lysed by M-PER lysis buffer (Thermo Scientific) containing protease inhibitor cocktail. Phosphatase inhibitor was not added into the samples. Each sample (150 µg) was subjected to immunoprecipitation with PP2Ac antibody. Beads containing precipitated PP2Ac were added to a phosphatase reaction with threonine phosphoprotein in a shaking incubator. Samples were then aliquoted into two wells of a 96-well plate, into which malachite green detection solution was added. Plates were incubated for 15 min at room temperature and then read at 650 nm on an automated plate reader. Absorbance data was calculated by using the standard curve which ranges between 0-2000 pmol/L.
Statistical analysis

All data are shown as mean±SEM. Comparison between two groups was analyzed by the two-tailed Student’s t-test. Multiple group comparison was performed by ANOVA followed by the Tukey procedure for comparison of means. Values of $P<0.05$ were considered statistically significant.


