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Structure-function properties of the apoE-dependent COX-2 pathway in vascular smooth muscle cells

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Abstract

Apolipoprotein (apoE) E is a multifunctional protein that plays a critical role in atherogenesis, in part by regulating the intimal proliferation of vascular smooth muscle cells. Recently, a novel cyclooxygenase (COX)-2 pathway was shown to contribute to the anti-proliferative action of human apoE3 in vascular smooth muscle cells (VSMC). Here, we provide insight into the structure-function properties by which apoE mediates these effects. ApoE3 is most effective in promoting COX-2 expression as a lipid-free protein and is less active after lipidation. Alterations in the stability of the helix-bundle N-terminal domain of apoE that contains the binding site for the low density lipoprotein (LDL) receptor and heparin do not affect the up-regulation of the COX-2 pathway. In addition, the apoE2, 3, and 4 isoforms are all capable of up-regulating the COX-2 pathway. Finally, the effect of apoE on COX-2 was found to be independent of expression on the VSMC surface of the LDL receptor and heparan sulfate proteoglycans (HSPG). In summary, our data indicates that apoE, particularly in the lipid-free state, can up-regulate COX-2 in murine vascular smooth muscle cells apparently independently of binding to the LDLR, LRP or HSPG.

Keywords

apolipoprotein E; cyclooxygenase-2; LDL receptor; vascular smooth muscle cell; heparan sulfate proteoglycan

Introduction

Apolipoprotein E (apoE) is a component of high density lipoproteins (HDL) and triglyceride-rich proteins (VLDL, chylomicrons) and, as such, is an important regulator of lipid homeostasis. The apoE molecule is composed of 22kDa (residues 1–191) and 10kDa (residues 216–299) domains connected by a hinge region. Each of these domains has distinct functions [1]. The 22kDa N-terminal domain is important for receptor binding while the 10kDa C-terminal domain is involved predominantly in lipid binding [2,3]. The lipid transport functions of apoE depend on its interaction with the LDL receptor (LDLR) family

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and heparan sulfate proteoglycans (HSPG) [4,5]. These apoE receptors are involved in lipoprotein metabolism, cholesterol homeostasis, brain development, vascular integrity, and protection against the development of atherosclerosis [6–8].

Independent of its ability to regulate lipoprotein metabolism, apoE affects the vascular system by modulating inflammation [9], suppressing oxidative stress [10], and inhibiting the proliferation of vascular smooth muscle cells [11–15]. The role of apoE in the proliferation of vascular smooth muscle cells (VSMC) in particular has important implications for atherosclerosis, vascular remodeling and restenosis.

The cyclooxygenase (COX) enzymes convert arachidonic acid to prostaglandin H_2 that eventually leads to the biosynthesis of eicosanoids; these include prostaglandins (PGE_2 , $PGF_{2\alpha}$, PGD_2), prostacyclin (PGI_2) and thromboxane (TX_{A2}). COX exist as two isoforms; COX-1 is constitutively expressed whereas COX-2 is up-regulated by various pro-inflammatory mediators, such as lipopolysaccharide, growth factors, cytokines and apolipoproteins [16]. The major eicosanoids produced by the activation of COX-2 in endothelial cells are prostacyclin (PGI_2) and prostaglandin E2 (PGE_2) [17]. The PGI_2 signaling cascade is dependent upon activation of its prostacyclin receptor (IP), which is generally associated with a G stimulatory protein coupled receptor [18]. The physiological consequences of eicosanoid metabolite production by the COX-2 enzyme are tissue and cell-specific. In vascular systems, COX-2-dependent production of prostacyclin can affect platelet coagulation [19–21] and leukocyte adhesion [22,23]. COX-2 activation in endothelial cells and vascular smooth muscle cells plays a vital role in the development and stability of atherosclerotic plaque. COX-2 is up-regulated in atherosclerotic lesions either by inflammatory cytokines or platelet activation [24,25].

In vitro studies have implicated several molecular pathways by which apoE may directly influence the proliferation of VSMC. Ishigami and colleagues showed that apoE inhibits platelet derived growth factor (PDGF)-induced VSMC proliferation by mitogen-activated protein kinase (MAPK) mediated suppression of the G_1 cell cycle gene, *cyclin D* [14,15]. The initial signal transduction mechanism describing apoE's anti-proliferative activity in VSMC involves binding of the C-terminal domain of apoE to perlecan HSPG through PDGF stimulation to induce iNOS which suppresses MAP kinase activity [13,14]. This suppression subsequently arrests cells in the G_0 phase of the cell cycle (21). We have identified another signaling pathway by which apoE binds to an unknown receptor to up-regulate COX-2 and prostacyclin production [11]. Prostacyclin activates the G_s protein-coupled (IP) receptor which eventually leads to inhibition of the CREBP and pocket protein-E2F of cyclin A, thereby, preventing cell progression to the S-phase [12]. In this study, we sought to further characterize important structure-functional properties of apoE regarding its ability to mediate up-regulation of the COX-2 pathway.

Materials and Methods

VSMC culture

Primary murine aortic smooth muscle cells were isolated from wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, Maine) or LDLR/apobec double knockout mice as described [26] and used between passage three and five. The cells were maintained in 40:40 ratio of Dulbecco's modified Eagle's/Ham F12 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS) and 25mM HEPES, penicillin, streptomycin, and glutamine. The purity of the VSMC population was determined by the expression of α -smooth muscle actin as detected by immunofluorescence. For experiments, VSMC were rendered quiescent when the cells were a near-confluent monolayer in serum-free defined medium [12] for two days followed by re-stimulation in 10% HI-FBS DMEM/Ham F12 media in the absence or

presence of 2 μ M recombinant lipid-free apoE proteins: human apoE3, apoE2, and apoE3, mutants apoE3(K146E), N-terminal domain of apoE-22kDa (apoE22), C-terminal domain of apoE3-10kDa (apoE10), and apoE3 22kDa quadruple mutant (L141K, K143L, K146L, L148K)(4apoE3-22). The expression and purification of recombinant apoE proteins were described previously (27). The 1,2 dimyristoyl phosphatidylcholine (DMPC)-apoE complexes were prepared as described previously [27].

Quantitative real time polymerase chain reaction (QRT-PCR)

VSMC were serum-starved and then stimulated with the 10% HI-FBS media described above in the absence or presence of 2 μ M apoE. After 6 hours (near optimum expression of COX-2), total RNA was extracted by Trizol, treated with DNase and 1.5 μ g total RNA reversed transcribed into cDNA using Superscript II reverse transcriptase enzyme (Invitrogen). mRNA levels were quantitatively determined on an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR-green detector or universal TaqMan. Murine primers COX-2, COX-1 and GAPDH containing unlabeled PCR primers and/or FAM dye-labeled Taqman MGB probe were purchased from assays-on-demand gene expression products (Applied Biosystems). The relative mRNA of COX-2 was normalized to the housekeeping genes, cyclooxygenase-1 (COX-1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then fold-induction was determined above the control levels of 10% FBS conditioned media. There was no expression of COX-2 above control levels in unstimulated VSMC.

Immunoblotting

Subconfluent VSMC were serum-starved for 48 hours and then restimulated as described above in 10% FBS media supplemented with 2 μ M apoE for 24 hours. Total protein was isolated by incubation for 15 minutes at room temperature with MPER lysis reagent (Pierce biotech) supplemented with phosphatase inhibitors and sodium orthovanadate, and the protein concentration determined by BCA assay. An equal amount of total protein (10–20 μ g) was resolved on 4–12% NUPAGE, transferred onto nitrocellulose membrane (BioRad), and blocked with 5% non-fat dried milk in Tween20 phosphate-buffered saline (PBS). The nitrocellulose membrane was incubated with 1:1000 dilution of primary antibody and then with 1:5000 dilution of horseradish peroxidase (HRP) conjugated secondary antibody. The protein bands were visualized with ECL Western blot kit and quantified by densitometric analysis using imaging software. Anti-mouse polyclonal COX-2 (BD Biosciences) and anti-rabbit cdk4 (Santa Cruz Biotechnology) were used to immunoblot for COX-2 and cdk4.

Measurement of TxB₂, 6-keto-PGF_{1 α} , and PGE₂ in cell culture medium

Eicosanoids were quantitated by liquid chromatography/tandem mass spectrometry (LC/MS/MS), using reverse phase chromatography, negative ion electrospray introduction and selected reaction monitoring (SRM) techniques. Tetradeuterated analogues of PGE₂, TxB₂, and 6-keto-PGF_{1 α} , 5 ng each (Cayman Chemical Co., Ann Arbor, MI), were added to 1.0 ml of cell culture medium. The conditioned medium was collected after 24 hours of apoE stimulation. The methoxime derivative was formed by adding 0.5 ml methoxyamine HCl in water, 1g/ml, and eicosanoids were extracted by solid phase extraction (SPE) using StrataX SPE cartridges (Phenomenex, Torrance, CA). Samples were then dissolved in 200 μ l of 20% acetonitrile in water for analysis by LC/MS/MS. The instrument used was a Thermo-Finnigan Quantum Ultra AM interfaced to a Shimadzu Prominence HPLC system. A 150 mm $\times$ 2 mm $\times$ 5 μ m HyperClone C18 BDS HPLC column (Phenomenex) was used. The mobile phase was generated from (A), HPLC-grade water and (B) 5% methanol/95% acetonitrile, both containing 0.005% acetic acid adjusted to pH 5.7 with ammonium hydroxide. The flow rate was 200 μ l/min using a gradient starting at 20% B and ramping to 45% B in 15 min. The transitions monitored were m/z 384 $\rightarrow$ 272 (d₄-PGE₂), m/z 380 $\rightarrow$

268 (PGE₂), m/z 402 → 173 (d₄-TxB₂), m/z 398 → 169 (TxB₂), m/z 402 → 372 (d₄-6-keto-PGF_{1α}), and 398 → 368 (6-keto-PGF_{1α}). The collision gas was argon, 1.5 mTorr. The collision energy was 18 eV for 6-keto-PGF_{1α} and TxB₂ and 17 eV for PGE₂. Source collision-induced dissociation was 10 eV. Quantitation was by peak area ratios.

Statistical Analysis

Results were analyzed by one-way ANOVA (Bonferroni) using Prism 3.0 software (GraphPad). Statistical significance is shown when P<0.05.

Results

The ability to up-regulate COX-2 is confined to the N-terminal 22kDa domain of apoE3

Previous experimental data indicated that anti-mitogenic effect of apoE3 was contained within the 22 kDa N-terminal domain but not the C-terminal 10kDa fragment of apoE [11]. Hence, to confirm that the anti-mitogenic effect of apoE3-22kDa domain was up-stream of its ability to inhibit cell cycle progression, COX-2 mRNA and prostaglandin production were analyzed after stimulation of primary murine VSMC with apoE3 variants. As shown in Figure 1A, the 22kDa domain of human apoE3 exhibited similar COX-2 induction to full length apoE3 (1.5–2 fold increase), whereas the 10kDa domain of apoE3 had no additional effect over control background media. The increase in COX-2 mRNA production by the 22kDa domain also translated to significant increase in prostaglandin production (2.5-fold 6-keto PGF_{1α} and 8–10 fold PGE₂) (Figure 1B–C), but no change in thromboxane metabolite (Figure 1D). The 10kDa domain had no effect on production of these prostaglandins. Therefore, the 22kDa but not the 10kDa domain of human apoE3 can regulate COX-2 signaling up-stream of the activation of the IP receptor.

Effect of lipidated apoE on COX-2 pathway

Lipid-free human apoE3 can up-regulate COX-2 expression and inhibit serum-dependent VSMC proliferation [11]. ApoE is known to change conformation upon lipidation [27–30]. The apoE complexed with DMPC in a discoidal HDL-like particle, allows apoE to adopt a conformation in which the N-terminal helix bundle is open so that binding to the LDL receptor is enhanced [31]. Thus, we wanted to assess whether lipidation of apoE3 would have a functional consequence for the COX-2 signaling pathway. ApoE variants were complexed with DMPC and the effects on COX-2 and eicosanoid synthesis were analyzed. The lipidation of full-length apoE3 decreased the level of COX-2 mRNA induction by a factor of two from 3-fold to 1.5 fold (Figure 2A). A similar trend for the levels of COX-2 protein was observed; lipid-free apoE3 increased ~ 5-fold whereas lipidated apoE3 gave only a 3-fold increase (Figure 2B). In experiments of this type, the addition of DMPC- small unilamellar vesicles (SUV) alone gave changes in COX-2 mRNA or protein levels comparable to the controls. There was no change in eicosanoid production compared to control media when DMPC-SUV alone were added to the medium (Figure 2C). Lipidation of wild-type apoE3 also significantly decreased, about 2–3 fold, the production of PGE₂ and 6-keto PGF_{1α} compared to lipid-free apoE3 (Figure 2C–D). Thus, these results indicate that lipid-free apoE3 is the more effective modulator of the COX-2 pathway.

All three common apoE isoforms modulate the COX-2 pathway

ApoE exist as three isoforms, apoE2, apoE3, and apoE4, which differ by cysteine and arginine interchanges at residues 112 and 158 [1]. The exchange of cysteine and arginine residues affect the stability of the N-terminal helix bundle in the apoE molecule [3,32]. These three isoforms have differential effects on lipoprotein metabolism, the development of atherosclerosis, and Alzheimer disease [33,34]. To determine whether there is selective up-

regulation of the COX-2 pathway by the apoE isoforms, the lipid-free human apoE isoforms were examined for their effects in WT VSMC. All three apoE isoforms up-regulated COX-2 mRNA 2-fold, protein from a range of 2–4 fold with fold induction values of 1.0 ± 0.0 (control), 4.3 ± 2.0 (apoE2), 2.4 ± 0.7 (apoE3), and 2.7 ± 0.4 (apoE4), and prostaglandin production (3-fold 6-keto PGF_{1 α} and ~7.5 fold PGE₂) as compared to control (Figure 3). Therefore, there is no selective regulation of the COX-2 pathway with apoE isoforms.

Disruption of the apoE receptor-binding domain has no effect on COX-2 signal

As mentioned above, signal transduction via the COX-2 pathway is confined to the N-terminal region (residues 1–191) of apoE. The N-terminal region folds into a four-helix bundle structure containing amphipathic alpha helices [1,35]. Residues 140–150 are important for binding to both the LDL receptor (LDLR) [36] and heparin (1, 39, 40, 42,43). To determine if residues 140–150 are important for COX-2 signaling, a quadruple N-terminus 22kDa mutant (L141K/K143L/K146L/L148K)-4E3-22 was assessed. This interchange of lysine and leucine residues abolishes the amphipathicity of the helix and the four-helix bundle is destabilized. This destabilization eliminates binding to the LDLR [36] but does not prevent binding to heparin [37,38]. As shown in Figure 4A–B, there was a trend towards up-regulation of COX-2 mRNA with the 4apoE3-22 mutant; the increase in protein expression was about 2-fold (2.3 ± 0.5) and similar to the increase created by the wild-type 22kDa fragment (2.3 ± 0.2). This observation is validated by the ~2.5-fold increase in production of the stable 6-keto PG F_{1 α} metabolite (Figure 4C), which is the major prostaglandin product, and also by the increase in the PGE₂ metabolite (8-fold) (Figure 4D). No thromboxane production was detected above background levels (data not shown). These results suggest that the organization of the alpha helices in the of 22-kDa fragment is not critical for receptor interaction or activation of COX-2 signaling pathway. Moreover, the finding implies that apoE-LDLR interaction is not involved, although, does not exclude an apoE-HSPG interaction.

Involvement of the LDLR and HSPG in the COX-2 pathway

ApoE can bind to HSPG and to members of the LDL receptor family, which includes the LDL receptor-related protein receptor (LRP), the very low density lipoprotein receptor (VLDLR), and receptor 2 (apoER2) [39]. Previous studies suggested that apoE3 inhibits PDGF-dependent VSMC proliferation via up-regulation of perlecan HSPG through activation of iNOS [13,15,40]. In contrast, our data suggest that anti-proliferative properties of apoE3 can be mediated via the COX-2 pathway; although, the receptor(s) involved are not known. To further investigate the nature of the receptor that mediates apoE signaling through the COX-2 pathway, wild-type (WT) and knockout LDLR VSMC were stimulated with wild-type apoE3 and a K146E-apoE3 mutant. Residue K146 is important for ionic interaction both with heparin and LDLR, so that binding of the K146E mutant is defective [1,36–38,41]. Similarly to wild-type apoE3, stimulation of wild-type VSMC with the apoE3 (K146E) mutant resulted in a 2-fold induction of COX-2 mRNA and an average 3-fold up-regulation of protein level with values of 3.5 ± 0.8 apoE3 and 3.1 ± 0.4 K146E versus 1.0 ± 0.0 control (Figure 5A–B). In addition, the levels of prostaglandins with the apoE3 (K146E) mutant were similar to those with WT apoE3, a ~2-fold increase of 6-keto PG F_{1 α} , 6-fold increase in PGE₂ (Figure 5 C–D), and no difference in TXB₂ compared to control media levels in wild-type VSMC (data not shown). Strikingly, the K146E apoE mutant induced COX-2 above control background levels in LDLR-null VSMC (Figure 5E–F). The COX-2 mRNA was increased by WT and K146E apoE3 2–3 fold (Figure 5E). In the case of COX-2 protein expression (Figure 5F), the effects of WT and K146E apoE3 were variable but, in all cases, both proteins increased the levels of COX-2. These results provide clear evidence that the LDLR is not involved in the apoE COX-2 pathway. This conclusion is consistent with the observation that lipid-free apoE is not recognized by the LDLR [42]. Furthermore, the

fact that the apoE3 (K146E) variant, which does not bind to HSPG, is active suggests that HSPG is not the receptor involved in mediating COX-2 signaling pathway.

Discussion

In this study, some structure-function features of apoE were analyzed to determine their influence on COX-2 signaling pathway in VSMC. The results demonstrate that the receptor binding domain of the apoE molecule (spanning residues 140–150) is important, but proper orientation of the α -helix spanning residues 140–150 in the N-terminal domain is not necessary for appropriate receptor interactions. Furthermore, lipid-free apoE is more effective than lipid-bound apoE in the induction of COX-2 and there is no selective recognition of the apoE isoforms. In addition, the LDLR clearly is not involved and HSPG does not seem to play a vital role in the apoE-dependent COX-2 signaling pathway.

The present data suggest that the LDL receptor family may not mediate this novel COX-2 anti-proliferative pathway of apoE in VSMC. The LDLR belongs to a lipoprotein receptor family which includes LRP, VLDLR, and apoER2 [39]. The apoE isoforms have different binding affinities for the LDL, LRP, and VLDL receptors. The LRP and VLDLR can recognize all apoE isoforms, however, it is known that, like the LDLR, LRP does not recognize lipid-free apoE [44]. The current data shows that lipid-free apoE can up-regulate COX-2 effectively, suggesting that the LDLR and LRP are not plausible receptor candidates for this novel apoE3-dependent COX-2 signaling pathway. Furthermore, while LRP is known to mediate apoE signaling, its role is thought to be in the anti-migratory function of apoE and not in its anti-proliferative activity [43]. Perhaps the VLDLR may be involved since it can bind lipid-free apoE and is expressed in VSMC [44]. HSPG can also mediate apoE signaling and perlecan HSPG has been implicated as the receptor mediating the anti-proliferative action of apoE although via the iNOS pathway [14,40]. Moreover, iNOS signaling is dependent on the C-terminal region of apoE for its interaction with HSPG [13]. However, our data clearly demonstrates that the N-terminal domain of apoE and not the C-terminal domain mediates the COX-2 signaling pathway (Figure 1) and anti-proliferative activity. This suggests that HSPG is not involved in the COX-2 pathway. The identification of the receptor involved is a crucial step to determine whether modulation of apoE dependent COX-2 pathway is plausible target for therapies of vascular proliferative diseases as well as clearly distinguishing apoE's anti-proliferative properties via COX-2 versus iNOS signaling pathways.

Currently, there are two possible mechanisms to explain apoE's anti-proliferative and anti-atherogenic activity during the progression of atherosclerosis. The first pathway involves binding of apoE to HSPG, up-regulation of iNOS, and eventually suppression of the cyclin D cell cycle gene [14,15]. The other identified pathway involves COX-2 signaling affecting the down-stream suppression of the cyclin A gene [11]. Our data suggest that these pathways are distinct despite documented studies showing these inducible enzymes can crosstalk between each other and that iNOS can regulate COX-2 expression [45]. Under our conditions, inducible NOS is up-regulated with human apoE3 and its 22kDa domain (data not shown), but selective iNOS inhibitors do not prevent the anti-proliferative action of apoE [11]. Further studies are needed to distinguish these two pathways which would involve understanding the up-stream and down-stream pathways for activation of COX-2, as well as the receptor involved.

VSMC migrate and proliferate into the intima of the vessel wall upon atherogenic stimulation which is an important process during the development of atherosclerosis. Here, we have shown that the structural organization of the apoE molecule influences its ability to up-regulate COX-2 which is the signaling pathway required for apoE's anti-proliferative

activity. Overall, continued molecular dissection of these apoE-dependent anti-proliferative pathways will be beneficial in understanding the role of apoE in modulating vascular abnormalities of restenosis and atherosclerosis.

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Figure 1. Influence of apoE3 domain structure on its ability to up-regulate the COX-2 signaling pathway

Quiescent primary murine VSMC were stimulated in 10% HI-FSC media with or without 2 μ M of full length apoE3, apoE3-10kDa or apoE3-22kDa domains for 6 or 24 hours. Unstimulated or quiescent cells were maintained in serum-free medium as described in methods A). mRNA fold induction above control levels after 6 hours B–D). Fold-increase of 6-keto PGF $_{1\alpha}$, PGE $_2$, and TXB $_2$ production compared to control after 24 hours. The control value of PGF $_{1\alpha}$ was 15.5 ± 2.0 ng/mL, PGE $_2$ was 0.6 ± 0.2 ng/mL, and TXB $_2$ was 1.2 ± 0.0 ng/mL. All data represents the mean $\pm$ S.E.M of three independent experiments, statistical significance $p < 0.05$. * is compared with control.

Figure 2. Lipidation of apoE3 reduces its ability to up-regulate the COX-2 pathway

Relative COX-2 mRNA, protein expression levels and eicosanoids production after 6 or 24 hours stimulation of quiescent primary murine VSMC with 2 μ M of apoE3 with or without DMPC (2:1 w/w ratio) A). mRNA fold induction above control after 6 hours B). COX-2 protein expression C–D). The fold induction of 6-keto PGF_{1α} and PGE₂ production compared to control after 24 hours. The control value of 6-keto PGF_{1α} was 3.6 $\pm$ 0.7 ng/mL and 1.9 $\pm$ 0.5 ng/mL for PGE₂. The data represents the mean $\pm$ S.E.M of three independent experiments except for the COX-2 protein expression, which is the mean from two independent experiments. Statistical significance is a $p < 0.05$. * is compared with control and # compared with apoE3 without DMPC.

Figure 3. The influence of apoE polymorphism

Quiescent primary murine VSMC were stimulated with 2 μ M of apoE isoforms in 10% HI-FCS media for 6 or 24 hours. A). Fold-induction of COX-2 mRNA after 6 hours B). COX-2 protein after 24 hours C–D). Eicosanoids production after 24 hours. The control values were 8.2 ± 2.8 ng/mL of PGF_{1 α} and 0.5 ± 0.2 ng/mL of PGE₂. The data in the figures represents the mean $\pm$ S.E.M of three independent experiments, statistical significance * $p < 0.05$ as compared to control.

Figure 4. Disruption of LDL receptor binding activity of apoE3

WT VSMC were quiescent for 48 hours followed by stimulation in 10% HI-FCS media supplemented with 2 μ M of WT apoE3, 10kDa or 22kDa domains of apoE3, or a quadruple 22kDa apoE3 mutant. A). COX-2 mRNA fold induction after 6 hours stimulation. B–D). COX-2 protein expression and fold-induction of 6-keto PGF_{1α} and PGE₂ production after 24 hours. The control values were 16.4 $\pm$ 5.2 ng/mL of PGF_{1α} and 0.7 $\pm$ 0.3 ng/mL of PGE₂. The data in the figures represents the mean $\pm$ S.E.M of three independent experiments, statistical significance $p < 0.05$. * is significant difference to control.

Figure 5. Effects of disruption of LDLR and HSPG binding of apoE3 on COX-2 signaling pathway

COX-2 mRNA and protein expression as well as eicosanoids production after stimulation of quiescent wild-type or LDLR null VSMC in 10% HI-FCS with and without 2 μ M of apoE3 variants. A). COX-2 mRNA fold-induction after 6 hours stimulation in WT VSMC. B.) 24 hours COX-2 protein expression in WT VSMC. C–D). Eicosanoid fold-induction after 24 hours. The control value of 6-keto PGF_{1 α} was 15.5 $\pm$ 2.0 ng/mL and PGE₂ was 0.6 $\pm$ 0.2 ng/mL. E–F). COX-2 mRNA and protein expression in apoE3-stimulated LDLR null VSMC. All data represents the mean $\pm$ S.E.M of three independent experiments, statistical significance * $p < 0.05$ as compared to control.