Modulation of estrogen signaling in hepatic and vascular tissue

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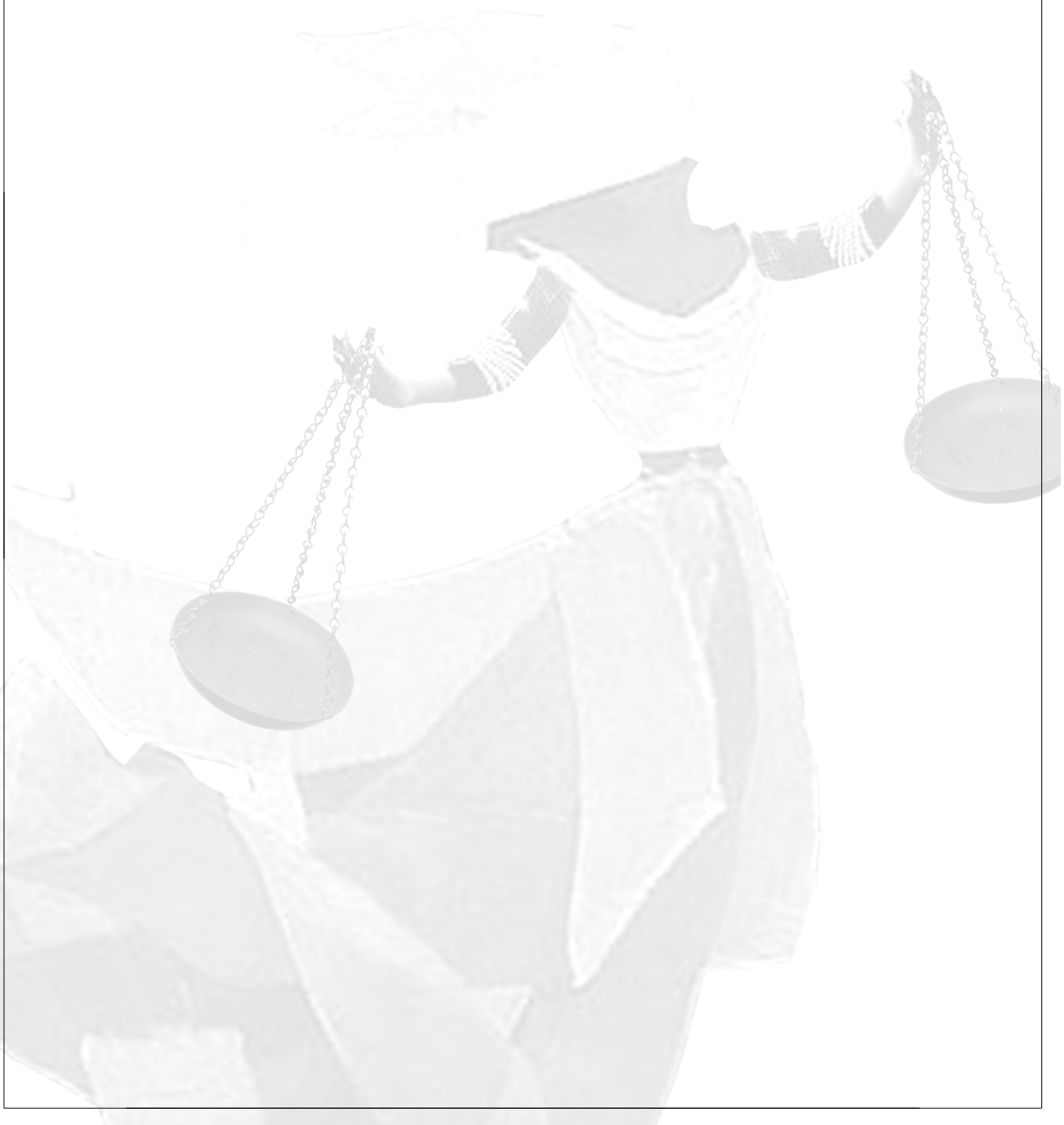
9. Summary, Discussion & Perspectives



Summary

A protective function of E_2 in vascular health has been established [1-3]. Clinical and experimental studies, however, have demonstrated that estrogens are not a magic bullet, but have both beneficial and detrimental effects on different organ systems. Therefore, more insight into the mechanistic actions of estrogens and the respective role of ERα and ERβ in individual cell types is required. To address some of these issues, we set out to modulate ERα and ERβ signaling tissue-specifically.

Hepatic E_2 signaling

Elevated plasma lipid levels are strongly associated with an increased likelihood to develop atherosclerosis. Studies in both humans and animal models indicate that long–term E_2 treatment reduces hyperlipidemia. Post-menopausal hormone replacement therapy (HRT) changes the lipid profile in a potentially anti-atherogenic direction by reducing LDL-cholesterol and triglyceride levels and increasing HDL-cholesterol levels [4]. Mouse models in which E_2 signaling is disrupted, e.g. ovariectomy, ArKO and ERα$^{-/-}$ mice, develop hypercholesterolemia upon aging [5-7].

The liver plays a pivotal role in lipid homeostasis. We hypothesized that the effects of E_2 on lipid metabolism are predominantly regulated by hepatic ERα. ER mediated signaling is known to be complex and controlled via tightly regulated pathways. Therefore, to assess the specific role of hepatic ERα mediated action, subtle modulation is required. In **chapter 2** we approached this by making use of RNA interference (RNAi). Thus far a relatively limited number of studies have described the successful application of RNAi technology in vivo. We obtained efficient intracellular delivery of short hairpin (sh)RNA's that were targeted against mouse ERα (shERα) specifically in liver of adult mice by utilizing Ad vectors. E_2 mediated hepatic ERα activity was reduced 60% by Ad.shERα for at least five days. This was demonstrated by an in vivo reporter mouse model that expresses luciferase driven by a promotor that contains estrogen response elements. Binding of the liganded ERα thus results in luciferase expression that can be detected in vivo using an ultra-sensitive CCD camera. Thus, Ad vectors were demonstrated to be an effective strategy to deliver shRNA molecules to the liver of mice to reduce gene expression. Subsequently, Ad.shERα vectors were applied to further investigate the function of hepatic ERα.

The role of hepatic ERα in lipid metabolism was explored in APOE3-Leiden female mice by applying Ad.shERα (**chapter 3**). While hepatic ERα RNA and protein levels were

reduced by 60%, hepatic and serum TG and Chol levels, as well as the VLDL-TG production rate were not affected. In contrast, expression of some lipid related genes, like Cyp7a, PPARα and SHP gene were changed. Apparently, the changes in the expression of these lipid related genes is compensated for by alternative transcriptional or post-transcriptional mechanisms and does not affect plasma lipid levels. Thus, since reduced hepatic ERα levels did not result in a clear lipid phenotype, our data imply that hepatic ERα is not a limiting factor in hepatic lipid metabolism.

E_2 has also been implicated in the regulation of glucose homeostasis and insulin sensitivity. Interestingly, the risk factors hyperlipidemia and insulin resistance both increase post-menopausally. HRT has been associated with a reduction in the incidence of diabetes [8,9]. Insulin sensitivity was improved by several weeks of E_2 treatment in mice deficient for E_2 [10]. However, the beneficial effects on insulin sensitivity obtained by long-term E_2 treatment could very well be an indirect consequence of the E_2 induced changes in for example adipose tissue. To gain insight in the direct and liver-specific effects of E_2, we determined the acute effects of E_2 on insulin sensitivity. In chapter 4, we performed a hyperinsulinemic/euglycemic clamp study in an insulin resistant mice model, six hours after E_2 treatment. In this short time span, hepatic glucose production was improved, while as expected total bodyweight and hepatic lipid content, known contributors of hepatic insulin sensitivity, were unchanged. Apparently, E_2 influences glucose homeostasis directly, resulting in an improvement of hepatic insulin sensitivity. Since ERα mediated transcription is mainly induced in liver and transcription levels of genes involved in hepatic glucose production were repressed six hours after E_2 treatment, we assume that hepatic E_2 signaling is responsible for these effects. Thus, our data imply a relatively major role for E_2 in regulating hepatic glucose production.

E_2 signaling in the vasculature

Beyond the effects on metabolic parameters that could account for the beneficial effect of E_2 on vascular diseases, several observations suggest a direct effect of E_2 on the vessel wall. To obtain a better insight in the direct effects of E_2 on the vessel wall and the relative importance of ERα and ERβ, we set out to achieve local and time-controlled modulation of ER signaling within the vascular tissue.

Vascular gene transfer

Studying of the ER subtype specific signaling in vascular cells is challenging. Firstly, in culture most vascular cells lose their ERs. Secondly, gene transfer to introduce exogenous ERs is cumbersome. Vascular cells are notoriously resistant to both non-viral as well as viral gene transfer. In **chapter 5**, the transduction efficiency of vascular cells in vitro has been improved by targeting Ad vectors using a linker protein. This linker construct consisted of the extra cellular domain of the coxsackie virus Ad receptor (CAR) genetically fused to avidin. Via its avidin moiety, a biotinylated cyclic RGD peptide was bound. This resulted in a targeting construct that binds to the Ad vector with one side and to $\alpha_v\beta_{3/5}$ integrins with the other side. This targeting strategy is relatively fast and does not require rederivation of the Ad vectors (as is required for genetically retargeted Ad). The redirection of Ad specificity from cellular CAR to integrins resulted in a significantly enhanced gene transfer to both transformed as well as primary vascular cells in vitro (**chapter 5**). Thus, our retargeting strategy renders Ad vectors exquisitely applicable for the analysis of gene function in vascular cells in vitro.

Next, an extensive effort was made to apply the cRGD-equipped Ad vectors *in vivo* (**chapter 6**). Normally, after systemic application, Ad5 (the most commonly used Ad variant) vectors sequesters in the liver, hampering delivery to alternative target tissues. By using the previously described targeting construct we were able to overcome this barrier, as de-targeting of the liver was consistently found. However, gene delivery to normal and injured carotid arteries was never observed after systemic administration of targeted Ad vectors. This is likely due to rapid clearance of Ad from the bloodstream and the anatomical position of the carotid artery. However, local incubation of cRGD-Ad in both normal and injured carotid artery also did not resulted in enhanced gene transfer. Thus, in principle enhancement of vascular gene transfer should be achievable (**chapter 5**). However unknown parameters have thus far prevented in vivo vascular gene transfer.

Role ER in the vascular wall

An important role in the anti-atherogenic effect of E_2 seems to be fulfilled by the endothelium [11]. In **chapter 7**, we demonstrated the repressive effect of E_2 on cytokine-mediated induction of adhesion molecules like E-selectin and ICAM-1 in two independent vascular cell lines. To determine the role of ERα, we repressed ERα in endothelial cell lines by lentiviral mediated expression of shERα. A significant reduction of ERα mRNA levels (60%) with an equivalent reduction in its activity was observed in the sub-cloned cell lines

that contain near 100% transduced cells. Using these cell lines, we demonstrated that the level of ERα does not limit the repressive effect of E_2 on expression of adhesion molecules.

In vivo, both ERα and ERβ are expressed in the vessel wall. It has been demonstrated that although ERα and ERβ share homologous domains, they can exert distinct and sometimes opposite biological action. Therefore, we postulated that the biological effects of E_2 on vascular remodelling are the result of the expression levels and balance between ERα and ERβ levels in vascular tissue. In **chapter 8,** we addressed the role of both endogenously expressed ERs in vivo on neointima formation by drug-releasing non-constrictive polymeric cuffs. Using these devices, a restenosis-like lesion is induced and ER subtype specific agonists are released. Interestingly, local release of the dual agonist, E_2 and the ERβ selective ligand, DPN both significantly reduced neointima formation. On the other hand, inhibition of intimal hyperplasia when solely ERα was activated was only observed after release of low concentrations. Our results demonstrate distinct, and partly opposite responses of ERα and ERβ on neointima formation. In contrast to the ERα and ERβ knockout studies, which propose that ERα is the receptor responsible for the anti-restenotic and anti-atherosclerotic effects of E_2 [12-15], our data provide evidence for an important, thus far unnoticed role of vascular ERβ in the prevention of restenosis.

Discussion & Perspectives

ER mediated cellular processes are very complex. To unravel the role of either ERα or ERβ, whole body ER deficient mice have been generated. However, information obtained from whole body ER deficient mice needs to be interpreted with caution. The complex phenotype of knock-out mice could obscure the role of ER at later stages of development or in specific tissues. In addition, back-up mechanisms that counterbalance the ER deficiency might be induced. With the development of conditional knock-out technology, these problems can be addressed [16]. However, this technology requires considerable effort and time. As an alternative method for inhibiting target gene expression, we have generated shERα constructs that are described in this thesis. These shERα constructs can be applied at a specific time point during development. By using a suitable vector, tissue specificity can be achieved. However, for all RNAi based approaches, the percentage and type of cells that can be transduced with a specific vector and the knock-down efficiency are variables that need to be taken into account when interpreting the results. In this respect, adenovirus vectors are highly

suitable for both dividing and non-dividing cells and in general are capable of inducing high levels of transgene expression.

The vessel wall seems to be an important target tissue for E_2. However, low efficiency of available gene-transfer systems, limits the applicability to dissect the role of ERs in vascular cells. Generally, viral vectors are more successful as compared to non-viral vectors in transducing vascular cells. In **Chapter 5**, it has been demonstrated that by re-targeting Ad vectors to integrins, gene transfer to primary vascular cells could be enhanced considerably. However, although, efficient gene transfer was accomplished in primary VSMC and ECs, gene transfer was not enhanced in the vessel wall in vivo. Even after local incubation in an injured artery, enhanced gene transfer was not observed (**chapter 6**). It is possible that the physical size of the targeted Ad vector, in combination with the dynamics of integrin expression in injured vessels is incompatible with the incubation time of Ad (10 minutes). However, it should also be noted that approaches to enhance Ad mediated gene transfer to vascular cells in vivo, thus far has resulted in very few successful applications. Therefore, we believe that essential knowledge regarding the fate and mechanism involved in the uptake of Ad in tissues other than liver in vivo is missing. This hampers the construction of an efficient, specific and non-toxic delivery device. Thus, basal research on Ad vectors should continue.

The understanding of E_2 action is incomplete and much remains to be discovered with respect to the effects of the large changes in E_2 concentrations and ER levels in development and aging. Thus far, the effect of different ERα levels on metabolic parameters and in vascular tissue is unknown. In this thesis, the role of ERα levels has been addressed by use of a shERα construct (**chapter 2**). This shERα construct allowed studying the response of E_2 in specific target tissues in the presence of altered ER levels. It revealed that hepatic ERα levels are not rate-limiting in determining plasma and liver lipid parameters (**chapter 3**) and that vascular ERα levels do not limit the repressive effect of E_2 on adhesion molecule expression (**chapter 7**). In human vascular tissues, it has been reported that the expression of ER changes with pathological conditions such as atherosclerosis [17,18]. Although ER knock-out mouse models have shown that ER deficiency leads to abnormal vascular function [19], it is not known whether reduced ERα levels cause a predisposition towards vascular dysfunction. The results obtained from our in vitro experiments, imply that reduced ERα levels are not a causative factor. However, more research is required to verify this hypothesis. As a follow-up

study, it would be interesting to screen for genes whose expression levels are more susceptible to ERα quantities. These genes can be identified by performing micro-array analysis using the shERα expressing endothelial cell lines. Furthermore, the effect of reduced ERα levels on expression of adhesion molecules and its effect on the development of the atherosclerotic process remain to be addressed in an in vivo model.

Our observation that lipid parameters were not changed upon shERα treatment, is in line with the fact that the changes with respect to lipid metabolism have not been reported in ERα heterozygous knockout mice. In homozygous ERα knockout mice the effects are only apparent under stressed conditions and/or upon aging [5,7]. Our data imply that the absence of a lipid phenotype in young mice is not due to compensatory changes, like for instance up-regulation of ERβ as a result of the ERα deletion, but truly indicate that hepatic ERα levels are not limiting. In addition, changes in lipid parameters induced by systemic E_2 administration or after ovariectomy are only apparent after a time lag of at least two weeks (personal communication, d'Olivera, Hoekstra). Therefore, it seems likely that the changed plasma lipid levels induced by prolonged E_2 administration are initiated by a cascade of events, in which non-hepatic tissues, like brain and adipose tissue play an important role. To address these issues, E_2 signaling should be modulated in a tissue specific manner. At the moment, techniques to apply drugs or RNAi locally are available. In rat models it has been shown that RNAi can be applied into certain regions of the brain [20]. In adipose tissue, E_2 signalling could be modulated by transplantation of fat from either $ER\alpha^{-/-}$ or $ER\beta^{-/-}$ mice.

The observed changes in lipid metabolism induced by long-term modulation of E_2 signaling could also be an indirect consequence of the short-term and perhaps prolonged changes in hepatic insulin sensitivity (**chapter 4**). The dissection of these cause and effect relations would require a substantial effort. As an initial study, the hepatic glucose pathway that is targeted by E_2 should be mapped using both transcriptomic (gene expression levels) as well as proteomic approaches (protein levels and modifications). By blocking parts of pathways that are thus revealed, the effect of prolonged E_2 administration on hepatic glucose production and subsequent changes in lipid parameters could be assessed.

The doses of E_2 that have been applied in reported experimental as well as in clinical studies are highly variable. However, the effect of these different E_2 levels in vascular tissue is unclear. In **chapter 8**, different concentrations of ER subtype selective ligands were

released to locally activate either ERα or ERβ. These data revealed that both a low and high dose of the dual agonist E_2 and the ERβ selective agonist, DPN, inhibited neointima proliferation (**chapter 8**). Thus for these two ligands, a dose-dependent effect was not observed. However, an inverse dose-dependent effect was observed when the ERα specific agonist, PPT was applied. PPT significantly inhibited neointima proliferation at low dose but not at high dose. Inverse dose-dependent effects could be explained by a dose-dependent shift in ERα:ERβ activity. This seems unlikely, since a dose dependent effect was only observed with the ERα specific agonist, PPT and not with the dual agonist E_2. An alternative exlanation for the inverse dose-dependent effect of PPT could be PPT mediated down-regulation of ERα expression. A high dose of PPT would result in very low ERα expression levels and thus a decrease in ERα-mediated effects.

The dose-dependent effects of PPT can also be explained by ER subtype specific biological effects. ERα might play an important role in the balance between pro- and anti-restenotic pathways. At low ERα activity, the anti-restenotic effects could be dominant, but also maximally induced. Increasing ERα activity by applying a high dose of PPT will then only enhance pro-restenotic effects and not the anti-restenotic effects.

It is obvious that there is a complex interplay between pathways induced by either ER subtype. Most likely, the interplay between the ER induced pathways in the vasculature is also affected by devevelopmental stage, aging and pathology such as restenosis and atherosclerosis. In conclusion, this study indicates that the dosing of ER ligands may be critical in determining the magnitude and direction of the biological effects.

The results obtained from **chapter 7 & 8** imply that E_2 prevents atherosclerosis by interfering either prior to injury or very early post-injury. In **chapter 7** we observed that pre-treatment with E_2 significantly reduces the cytokine-induced expression of the endothelial adhesion molecules E-selectin and ICAM-1. Suggesting that due to E_2, the endothelial cells are less responsive to inflammatory signals. Opposite results have also been published [21,22]. However, in those studies, E_2 was added simultaneously with the cytokine instead of before treatment. In **chapter 8**, E_2 and DPN both significantly inhibited neointima proliferation. When the drug release profiles are taken into account, both E_2 and DPN are only released in the first week. In addition, the half-life of both compounds is less than a day [23,24]. Thus, from these studies it seems likely that the anti-inflammatory and anti-restenotic effects are exerted pre-injury. Our data do confirm earlier studies in primates, rats and rabbits,

which have demonstrated that the protective effects of E_2 are only apparent if E_2 was administered prior to the development of atherosclerosis and not when arterial damage was present prior to hormone treatment [25-29]. For future study, it would be interesting to address this point in more detail. Because of the applicability of local drug treatment, our mouse model would offer the opportunity to perform such a time range. In addition to releasing ER subtype specific ligands simultaneously with the induction of restenosis (**chapter 8**), restenosis can first be induced by applying an empty cuff, which is then followed by a drug-releasing cuff. To address the local and time dependent effect of E_2 on atherosclerosis, these cuffs should be used in an atherosclerotic mice model, such as $ApoE^{-/-}$ and ApoE3Leiden mice.

Clinical perspectives

Although caution should be taken when extrapolating results obtained by *in vitro, ex vivo* or animal models to humans, findings obtained by numerous experimental studies clearly indicate the importance of E_2 status on vascular endothelial function**.**

Atherosclerosis is multi-factorial by nature, caused by a wide variety of genetic as well as environmental factors, and develops decades earlier than its clinical manifestation. Therefore, drug therapy alone to treat the atherosclerotic vessel wall is not likely to be sufficient. Management of risk factors, like obesity, hyperlipidimea, hypertension and insulin resistance should be the primary approach to decrease the development of atherosclerosis. This can be achieved by lifestyle modifications (ie, weight control, change in diet, regular exercise and smoking cessation). Whenever this is not feasible, because of for example a genetic predisposition to hyperlipidemia, drug therapy becomes the primary approach.

E_2 has been suggested as a possible drug to prevent vascular diseases by reducing metabolic risk factors. However, from the clinic there is a justifiable question; should hormone therapy be continued beyond management of menopausal symptoms? With the current knowledge the answer is no, because the beneficial effects do not outweigh the adverse side effects. Side effects however could be minimized by local treatment. In this thesis we tested whether changes in lipid and glucose metabolism are induced by a direct effect of E_2 on liver. This seems to be true for regulation of glucose- but not for lipid metabolism (**chapter 3 & 4)**. In agreement with the fact that the effect on lipid metabolism takes a significant time to develop, it suggests an indirect effect of E_2 on lipid metabolism. Therefore, it seems unlikely that E_2 will become serious competition for the commonly available and effective lipid-lowering drugs. Alternatively, the beneficial effect of E_2 on

hepatic insulin sensitivity obtained within six hours after treatment is interesting and deserves further study. Moreover, the simultaneously induced peripheral insulin resistance implies the importance of tissue specific modulation.

Locally in the vessel wall, our data indicate that E_2 and ER subtype specific ligands form an attractive drug to prevent in-stent restenosis after PTCA. In the clinic, introduction of drug eluting stents has led to a tremendous reduction of in-stent restenosis. E_2 has been shown to prevent restenosis (chapter 8) and in comparison to alternative drugs which solely reduce VSMC proliferation, E_2 has also been shown to promote re-endothelialization [30]. However, we have not addressed this issue in our model and this requires further study. For the improvement of therapeuty, a thorough understanding of the effects of E_2 and the interplay between ERα and ERβ in the vasculature is required. By use of ER subtype specific ligands, we have demonstrated that both ERα and ERβ are involved in the vascular protective effects of E_2. A future challenge will be to determine to what degree the ERα versus ERβ are involved during the different stages of injury during the development of the restenotic process as well as during the development of atherosclerosis. Thus, selective pharmacological targeting of ER subtypes may represent a novel and promising approach in the treatment of in-stent restenosis.

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