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The role of the sigma-1 receptor ligands in the arachidonic acid metabolism of healthy and diabetic rat platelets or abdominal aorta

PhD Thesis

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SCIENTIFIC PUBLICATIONS ON THE SUBJECT OF THE THESIS:

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INTRODUCTION

The sigma-1 receptor (S1R) is expressed in the cells of both the central nervous system and peripheral organs. S1Rs are mainly located in the mitochondria-associated membrane of the endoplasmic reticulum (ER) but can also be found in other cellular membranes. S1Rs are involved in the regulation of intracellular signalling pathways by modulating the activity of ion channels, inositol phosphatases and protein kinases. The substrate for eicosanoids, arachidonic acid (AA), is released from membrane phospholipids (PL) by phospholipase A_2. The quantity of free AA depends on the cell membrane phospholipid (PL) content, re- or de-acylation of PLs, intracellular calcium ion concentration ($[Ca^{2+}]_i$), and the activation of cyclooxygenase-1 or -2 (COX-1 and -2) and lipoxygenases (LOXs) enzymes. The prostaglandins ($PGF_{2\alpha}$, PGE_2 and PGD_2), thromboxane A_2 (TxA_2) and prostacyclin (PGI_2), are produced by tissue-specific synthases from endoperoxide (PGH_2), which is formed by cyclooxygenase, while the activation of lipoxygenases results in the formation of hydroxyl-eicosatetraenoic acids (HETEs).

S1Rs are involved in the regulation of not only physiological but also pathological cellular functions. S1R gene expression is known to be involved in the prevention of diabetic complications. Diabetes leading to the formation of glycation end products (AGEs) and reactive oxygen species (ROS) results in endothelial dysfunction and platelet activation.

Platelets are involved not only in haemostasis, but also in inflammatory and immune processes and in the regulation of microcirculation. Bioactive lipid mediators synthesized by platelets and endothelial cells play an important role in these processes.

OBJECTIVE

Our primary aim was to prove our hypothesis that rat platelets can express S1R. We hypothesized that S1R may play a part in the physiological function of platelets. To confirm this, we performed preliminary experiments to investigate the effects of a known S1R agonist ligand, PRE-084, on platelet AA metabolism and platelet aggregation *in vitro* in healthy rats. We also considered it important to clarify that S1R ligands administered sub-chronically to rats i.p., a known S1R agonist PRE-084, an antagonist NE-100 and a new S1R ligand, *(S)*-L1 selected by Dvorácskó et al. (2021), are able to induce *in vivo* changes in the metabolism of healthy rat platelets and abdominal aortic AA that can be detected *ex vivo* even when the ligand is no longer present in the test medium. We hypothesized that S1R ligands may affect not only the quantity of free AA substrate and enzyme activity, but also the mRNA levels of genes encoding S1R and COX (*Sigmar1* and *Ptgs*) derived from megakaryocytes and stored

in platelets. We hypothesized that eicosanoids synthesized by platelets and endothelial cells may play a mediating role in the beneficial effects of S1Rs in diabetes. Our aim was to clarify whether STZ-induced diabetes affects platelet *Sigmar1* and *Ptgs* mRNA levels, and platelet and aortic AA metabolism. We then sought to answer whether i.p. sub-chronic S1R ligands could restore the parameters altered by diabetes noted above and whether there is a difference in the effect of each ligand.

METHODS

The expression of S1R on rat platelets was detected by RT-qPSR and visualized by immunostaining and confocal laser scanning microscopy. Radioactive substrate and ELISA assays were used to study eicosanoid synthesis in both healthy and diabetic animals. Platelet aggregation assays were performed using whole blood, ADP and AA inducers by multiple-electrode aggregometry. LC-MS was used to determine blood levels of sub-chronic i.p.-administered S1R ligands in healthy and diabetic rats.

The effect of S1R ligands on platelet *Sigmar1* and *Ptgs* gene expression was examined by RT-qPSR. Diabetic rats were generated to study the effect of S1R under pathological conditions.

RESULTS

Sigmar1 gene expression in non-activated, healthy rat platelets was detected by both RT-PCR and qPCR (17.3 ± 0.22) and quantified as relative expression normalized to Gapdh and Actb control genes. S1R was also present at the protein level in non-activated rat platelets: the punctate staining pattern was co-localized with the F-actin structure or with the wheat germ agglutinin lectin staining of rat platelets as visualized by confocal microscopy.

Subsequently, we investigated the *in vitro* effect of PRE-084, already known as an S1R agonist, on non-activated, healthy rat platelet function. *The in vitro AA metabolism of non-activated rat platelets* was enhanced by all concentrations of PRE-084 that we tested, via both the COX and LOX pathways. The total quantity of metabolites formed by the COX pathway and the synthesis of TxB_2, PGD_2 and PGE_2 were enhanced most significantly by a 2 μM concentration of PRE-084. The dose response curves for PRE-084 were sigmoidal for all COX products noted above. *Through a quantitative analysis with ELISA*, we were able to detect the effect of *in vitro* application of 2 μM PRE-084 in platelets to increase COX-1 (48.4 ± 8.3 ng/ml vs. 11.3 ± 2.5 ng/ml) and TxB_2 (0.528 ± 0.16 ng/ml vs. 0.194 ± 0.038) concentrations. PRE-084 enhanced ADP- and AA-induced *platelet aggregation in healthy rats* compared to the control sample. The effect of PRE-084 on aggregation resulted in a

sigmoidal dose-response curve with a lowest effective concentration of 2 μM. No significant difference was found between AA- or ADP-induced platelet aggregation with the same agonist. Our preliminary experiments demonstrated the platelet function enhancing effect of PRE-084 in rats. Further, we wanted to extend our studies to NE-100, known as an S1R antagonist, and a new ligand, *(S)*-L1, selected from the in-house compound library of the Institute of Pharmaceutical Chemistry, University of Szeged, with in silico screening in collaboration with Dvorácskó et al. Using molecular modelling to investigate ligand binding to S1R, we found that *(S)*-L1 and NE-100 have very similar binding positions.

Our *in vitro* experiments demonstrated the acute effect of PRE-084 in healthy rat platelets.

We chose intraperitoneal ligand administration to study the *in vivo/ex vivo*, sub-chronic effects of S1R ligands. In our preliminary experiments, we demonstrated the entry of ligands into the circulation and their complete elimination 20 hours after treatment. Prior to our direct *ex vivo* studies, plasma ligand concentrations in all healthy and diabetic animals were around the limit of detection or quantification. That is, the results of these experiments cannot be considered as acute ligand effects.

During sub-chronic, *in vivo* treatment, none of the S1R ligands resulted in changes in the *ex vivo* total quantity of radioactive AA metabolites (COX+LOX) in healthy rat platelets *ex vivo*. However, both PRE-084 and *(S)*-L1 decreased COX metabolites and increased LOX metabolites, whereas NE-100 induced the opposite effect, resulting in a product shift within the AA cascade. The reduction in the ratio of vasoconstrictor, platelet aggregator (CON) and vasodilator, platelet anti-aggregator COX metabolites was only induced by the *(S)*-L1 S1R ligand. While the formation of CON and DIL COX metabolites was reduced to the same extent by PRE-084, it was increased by NE-100.

The quantity of radioactive AA metabolites produced by COX and LOX pathways in platelets of diabetic rats was similar to that of the whole rats. However, the AA metabolism of platelets from diabetic rats was shifted by PRE-084 and *(S)*-L1 towards the LOX pathway, whereas NE-100 was shifted towards the COX pathway. In diabetic rat platelets, greater DIL COX product formation was detected with unchanged CON COX metabolite synthesis. DIL COX dominance of diabetic platelets was not affected by NE-100, but was reduced by both PRE-084 and *(S)*-L1.

In both healthy and diabetic rat platelets, we were able to detect both COX-1 and COX-2 enzymes using ELISA. In healthy rat platelets, *(S)*-L1 and NE-100 significantly raised the concentrations of both COX-1 and COX-2 and consequently the total COX (COX-1+COX-2) concentration. The rise in the concentration of the two enzymes was different, as evidenced

by the dop in the COX-1/COX-2 ratio. The concentration of constitutive COX-1 was not different, while inducible COX-2 was higher in platelets from vehicle-treated diabetic rats than that in healthy platelets from vehicle-treated rats. PRE-084 and *(S)*-L1 increased platelet COX-1 and COX-2 levels in diabetic rats to the same degree compared to both the vehicle-treated healthy and diabetic groups. Although NE-100 also raised the total quantity of COX enzymes in diabetic platelets, this was due to a rise in COX-2 alone, as evidenced by a fall in the COX-1/COX-2 ratio.

When investigating the *ex vivo* effects of S1R ligands on *Sigmar1*, *Ptgs1* and *Ptgs2* transcripts, we did not detect *Ptgs2* mRNA in either healthy or diabetic rat platelets by RT-qPCR. The levels of *Sigmar1* and *Ptgs1* mRNA in healthy rat platelets were not altered by one week of treatment with S1R ligands.

In vivo treatment with PRE-084 and *(S)*-L1 ligands increased *Sigmar1* transcript levels in platelets from diabetic rats, compared to both vehicle-treated diabetic and healthy rat samples. *Ptgs1* transcript levels were higher in the platelets of vehicle-treated diabetic rats than in vehicle-treated healthy animals. In diabetic rats, PRE-084 treatment did not alter *Ptgs1* mRNA levels compared to samples from vehicle-treated diabetic animals. *Ptgs1* mRNA levels were lower in the *(S)*-L1 group than in the vehicle- or PRE-084-treated diabetic rat group, but were not significantly different from the vehicle-treated healthy group. NE-100 treatment reduced platelet *Ptgs1* levels in diabetic rats compared to the vehicle-treated diabetic group. Similarly to PRE-084, the *(S)*-L1 ligand raised *Sigmar1* mRNA levels in both healthy and diabetic controls, while, similarly to NE-100, it lowered *Ptgs1* mRNA concentration in vehicle-treated diabetic rat platelets.

Following *in vivo* treatment in the abdominal aorta of healthy rat, both PRE-084 and *(S)*-L1 decreased the total quantity of eicosanoids (COX + LOX) synthesized *ex vivo* from radioactive AA, whereas NE-100 increased it. The changes in the quantity of COX and LOX metabolites were similar in response to PRE-084 and NE-100, but *(S)*-L1 treatment shifted eicosanoid synthesis towards the COX pathway by reducing LOX metabolite formation, as evidenced by the rise in the COX/LOX ratio. In the aorta of healthy rats, both PRE-084 and NE-100 resulted in similar drops in *ex vivo* CON- and DIL-COX metabolite synthesis, and therefore no CON/DIL ratio shifts were observed in this case. In the aorta of healthy rats, both PRE-084 and *(S)*-L1 led to a similar reduction in the synthesis of CON- and DIL-COX metabolites *ex vivo*, and therefore no CON/DIL ratio shift was observed in this case. However, *in vivo* treatment with NE-100, although it boosted both CON-COX metabolite formation and DIL-COX metabolite formation, resulted in a fall in CON/DIL ratio, i.e. DIL-

COX dominance. In the healthy rat aorta, the synthesis of 6-k-$PGF_{1\alpha}$ was increased by *(S)*-L1 and NE-100 and decreased by PRE-084 compared to the vehicle-treated sample.

Neither the quantity of COX nor LOX metabolites nor their total quantity (COX+LOX) nor their ratio (COX/LOX) changed in the aorta of *diabetic rats* compared to healthy rats treated with vehicle. We detected no any difference in the formation of either CON-COX or DIL COX metabolites in the aorta of vehicle-treated diabetic and vehicle-treated healthy rats.
In vivo treatment with PRE-084 and *(S)*-L1 reduced the formation of LOX products in diabetic rat aorta, whereas NE-100 enhanced it compared to vehicle-treated diabetic animals. In the aorta of diabetic rats, *(S)*-L1 treatment decreased the synthesis of CON-COX metabolites, whereas NE-100 treatment increased DIL-COX formation compared to both healthy and diabetic vehicle-treated samples, resulting in a fall in the CON/DIL ratio, leading to DIL-COX dominance. The synthesis of TxB_2 was unchanged, whereas the production of 6-k-$PGF_{1\alpha}$ was significantly reduced in the aorta of vehicle-treated diabetic rats compared to healthy animals. *(S)*-L1 induced a drop in the synthesis of TxB_2 in diabetic aorta compared to vehicle-treated healthy and diabetic animals. However, NE-100 both lowered the production of TxB_2 and increased the synthesis of 6-k-$PGF_{1\alpha}$.
Higher concentrations of COX-2 than COX-1 were detected in the abdominal aorta of healthy rats by ELISA. *In vivo* treatment of healthy rats with PRE-084 induced an increase in COX-1 concentration, whereas NE-100 treatment caused a decrease in COX-2 concentration in the aorta. Treatment with *(S)*-L1, on the other hand, raised both COX-1 and COX-2 levels, but not to the same degree, as it resulted in a rise in the COX-1/COX-2 ratio.
Neither the concentration of COX-1, nor the concentration of COX-2, nor their sum (COX-1+COX-2) in the aorta of vehicle-treated diabetic rats differed from that of vehicle-treated healthy animals. The concentration of these parameters in the aorta of diabetic rats was not changed by any of the S1R ligands tested. However, the COX-1/COX-2 ratio was higher in the aorta of diabetic rats treated with vehicle or PRE-084 than in the aorta of healthy rats treated with vehicle.

DISCUSSION

The presence of S1R and its role in physiological and pathological processes have been demonstrated in a number of cell types, but its presence and role in platelets have not been studied. Intracellularly, S1R is mainly found in the mitochondria-associated membrane of the ER, but it is able to migrate from the ER to the plasma membrane.

Although platelets are anuclear cells, they possess a number of other intracellular cellular components that provide the structural conditions for the presence of S1R. They are even capable of protein biosynthesis through megakaryocyte-derived cytoplasmic mRNA. Using RT-PCR, qPCR and immunocytochemistry at both mRNA and protein levels, we were able to confirm our hypothesis that non-activated rat platelets can also express S1R.

Platelets are involved not only in haemostasis, but also in the body's defence responses. Their activation results in platelet aggregation, degranulation, aggregation, expression of receptors and increased AA metabolism.

S1R can also influence a number of intracellular processes (ROS metabolism, abnormal protein elimination, ATP production, ion channel function, lipid transport and enzyme activity). The membrane structure and function modifying effect of S1R has been extensively studied and S1R has been successfully detected in non-activated rat platelets. We thus hypothesized that the S1R agonist PRE-084 can affect the metabolism of membrane phospholipids to AA and eicosanoids *in vitro*.

We used sex- and age-matched male rats in our experiments to correctly interpret the effect of S1R ligands. An important aspect was to prevent spontaneous platelet activation and endothelial damage and to use them rapidly after sample collection. In order to prevent platelet activation during separation, we used plastic instruments, a thick needle for draining blood, and EDTA, a calcium chelating anticoagulant that inhibits platelet aggregation. In the study of platelet eicosanoid synthesis, we used EDTA- and plasma protein-free tissue culture medium, which provided the Ca^{2+} concentration required for physiological platelet function. In order to avoid platelet activation, the concentration of radioactive substrate used as a marker (0.172 nM 1-^{14}C-AA) was significantly lower than the quantity of AA (0.5 mM), the inducer that causes aggregation.

In vitro pre-treatment with the S1R agonist PRE-084 enhanced AA metabolism in non-activated platelets of healthy twelve-week-old male rats, which was detected semi-quantitatively with the application of radioactive substrate and quantitatively with ELISA. This effect could be explained by the fact that PRE-084 increased PL content in the cell membrane through its intracellular lipid transport effect, while its phospholipase-activating ability raised the quantity of free AA, the substrate of eicosanoids, by inducing PL de-acylation. Although both the total quantity of AA metabolites formed by the COX pathway and the total quantity of AA metabolites formed by the LOX pathway were increased by PRE-084 treatment *in vitro*, the rate and dose dependence of their increase were not the same. These results raise the possibility that this S1R ligand may have a stimulatory effect, not only

by raising free AA levels, but also directly on COX and/or LOX expression and/or activity. The different effects of PRE-084 ligand on COX and LOX expression and/or activity may be explained by the different exposure times and the different sensitivities of the enzymes. The constitutively expressed isoform of the cyclooxygenase enzyme is COX-1, whereas COX-2 is an inducible isoenzyme. Despite the fact that platelets are anuclear cells, they are still able to synthesize *de novo* COX-1 from cytoplasmic mRNA derived from megakaryocytes. COX-2 mRNA and protein have been detected in human platelets, but in much lower quantities than COX-1. *In vitro*, an increase in COX-1 concentration was detected with ELISA in non-activated rat platelets after PRE-084 treatment, consistent with a rise in the total quantity of COX metabolites obtained using the radioactive substrate. Under the present assay conditions, the synthesis of each COX product (TxB_2, PGD_2 and PGE_2) was increased equally but to different extents. These results suggest that pre-treatment of non-activated platelets *in vitro* with PRE-084 can enhance not only the expression and/or activation of PLA_2, COX and LOX enzymes, but also specific enzymes involved in the formation of eicosanoids synthesized by the COX pathway. The exact mechanism of these effects is not yet known, although several studies have reported on the role of S1R in modifying the ion channel and enzyme function. *In vitro*, under the influence of PRE-084, non-activated rat platelets synthesized several aggregating and vasoconstrictor TxA_2 stable metabolites, i.e., TxB_2, aggregation inhibitor and vasodilator PGD_2, and PGE_2, whose effects on platelets are concentration-dependent.

In addition to these experimental results, in order to clarify the effect of PRE-084 on platelet aggregation, we investigated the direct effect of this S1R ligand on platelet aggregation induced by ADP and AA. Concentrations of PRE-084 that enhanced eicosanoid synthesis in non-activated platelets enhanced both ADP- and AA-induced platelet aggregation using whole blood aggregometry. In this procedure, fibrinogen, calcium ion and other circulating blood-forming elements that promote platelet aggregation are also available. The effect of the S1R agonist on the rate of AA-induced aggregation was most pronounced compared to the control group. This may be explained by the fact that AA-synthesized thromboxane, when bound to its own receptors, induces autocrine and paracrine platelet activation, and AA-induced platelet activation induces endogenous (granule-derived) ADP inducer release. The dose-response curve of PRE-084 on ADP- or AA-induced platelet aggregation did not differ significantly, despite the fact that the primary effect of ADP on platelets is receptor-mediated, whereas the primary effect of AA is not. However, their secondary intracellular signalling pathways are similar, as both are able to induce elevations in $[Ca^{2+}]_i$ and secretion

of endogenous inducers such as thromboxane, serotonin and ADP. It was hypothesized that PRE-084 could modulate this common intracellular signalling pathway of ADP and AA. However, the magnitude of the rise in platelet aggregation induced by PRE-084 was smaller than that of thromboxane production. This difference could be explained by an increase in the formation of anti-aggregator eicosanoids (PGD_2, and PGE_2) and the concomitant presence of PRE-084-binding cells and plasma proteins. However, the mechanism of these latter processes and their presence in platelets is not yet clearly understood.

Following the demonstration of *in vitro* rat platelet AA metabolism and the aggregation enhancing effects of the S1R agonist PRE-084, we extended our studies to other S1R ligands, such as NE-100, known as an S1R antagonist, and a novel ligand, *(S)*-L1.

The endothelial cell layer lining the inner surface of blood vessels and platelets form a functional unit. The platelets mainly synthesize vasoconstrictor, platelet-activating thromboxane, while the endothelium mainly synthesizes vasodilator, platelet aggregation-inhibiting prostacyclin, which ensure normal microcirculation in equilibrium under physiological conditions.

With this knowledge, we investigated the *in vivo/ex vivo* effects of the S1R ligands noted above on platelet and aortic eicosanoid synthesis following sub-chronic i.p. treatment in both healthy and STZ-induced 16-week-old diabetic male rats. The S1R ligands to be applied were selected based on their binding strength to the receptor and their binding site in the S1R binding pocket. The main difference between S1R agonist and antagonist binding is whether the ligand can interact with the C-terminal helix of the S1R. The cyclohexane ring of PRE-084 is the only moiety that can interact with this helix. *(S)*-L1 has a very similar binding position to the S1R antagonist NE-100. The duration of treatment was determined by platelet lifetime (one week).

All of the S1R ligands administered i.p. were absorbed into the circulation, but the difference between their 30-min serum levels may indicate a difference in the rate of absorption. The difference in the time-dependent serum levels of the ligands may be explained by their different metabolism and excretion, the mechanism of which is not yet fully understood. In order to exclude a direct effect of S1R ligands, our *ex vivo* studies were started 20 h after the last ligand treatment, when serum levels of ligands were already below the limit of detection or quantification. In other words, in this case, the changes in AA metabolism detected *ex vivo* in the platelets and aorta were due to the effects induced by ligands *in vivo*.

Upon *in vivo* treatment, none of the S1R ligands we tested resulted in changes in the total quantity of *ex vivo* radioactive AA metabolites in healthy rat platelets. Nevertheless, a drop in

the COX/LOX ratio was detected in platelets in response to PRE-084 and *(S)*-L1, whereas an rise in this ratio was detected in response to NE-100. In healthy rat aorta, however, the total quantity of AA metabolites was decreased by PRE-084 and *(S)*-L1, whereas it was increased by NE-100. In addition, a product shift towards the COX pathway within the AA cascade was observed upon *(S)*-L1 treatment. In healthy rat platelets, none of the ligands induced changes in either S1R or COX mRNA levels; that is, the difference in AA metabolism cannot be explained by the effect of ligands on S1R or COX enzyme transcription. However, despite the fact that small quantities of COX-2 mRNA and protein have been detected in human platelets, we were unable to detect COX-2 (*Ptgs2*) mRNA in rat platelets by 40-cycle RT-qPCR under our current experimental conditions. This may be explained by the fact that a limited pool of mRNA transcripts from megakaryocytes can be used for protein (e.g. COX-2) synthesis, and thus increased AA metabolism may lead to depletion of i.c. reserves (mRNA, enzyme pool and $[Ca^{2+}]_i$).

Although PRE-084 and (S)-L1 treatment in vivo lowered the total quantity of radioactive COX metabolites in platelets of healthy rats after treatment with PRE-084 and NE-100 raised it, we did not detect changes in COX enzyme (COX-1+COX-2) concentrations with ELISA. However, a rise was observed with (S)-L1 and NE-100 treatment. Both (S)-L1 and NE-100 boosted the concentration of platelet COX-1 and COX-2 enzymes, but the drop in the COX-1/COX-2 ratio for both ligands suggests that their effect in increasing COX-2 was greater than that of COX-1. Therefore, the fall in the total quantity of radioactive AA metabolites formed by the COX pathway in healthy rat platelets following in vivo treatment with PRE-084 and (S)-L1 could be explained by either inhibition of the COX enzyme or a lowering in the absolute or relative quantity of free AA substrate. The formation of AA metabolites depends on the presence of sufficient quantities of free AA, which may be influenced by the PL composition of the membrane, phospholipase activity and [Ca2+]i levels. S1R is known to be able to alter all these factors. Since the total quantity of AA metabolites (COX+LOX) in healthy rat platelets was not reduced by either PRE-084 or (S)-L1 treatment in vivo, an absolute reduction in the quantity of free AA can be excluded. Therefore, in this case, the fall in the total quantity of COX metabolites could be caused by a direct inhibition of cyclooxygenase and/or a rise in LOX activity, i.e., a relative decrease in the AA substrate, as supported by the drop in the COX/LOX ratio. Although no data are available on the direct effect of S1R ligands on COX activity, a recent lipidomic study found downregulation of the COX pathway due to bufotenin, an S1R ligand. In contrast to PRE-084 ligand, a rise in COX enzyme concentration was detected in platelets from healthy rats on both (S)-L1 and NE-100

treatment with ELISA. However, (S)-L1 decreased the formation of radioactive COX metabolites, whereas NE-100 increased it. In other words, the differential effects of each ligand could be explained by the difference in their effects on the translation, synthesis and/or activation of cyclooxygenase and specific enzymes involved in the synthesis of eicosanoids.

The differential degree and/or direction of change in COX-mediated metabolite formation in healthy rat platelets induced by *in vivo* treatment with each S1R ligand may suggest that the ligands act not only through their effects on PLase, COX and LOX, but also through their effects on specific enzymes. For example, *(S)*-L1 treatment led to a fall in the CON/DIL ratio by reducing the synthesis of CON products. The synthesis of the major platelet COX metabolite, the vasoconstrictor and platelet aggregator thromboxane, was much more reduced by our new ligand *(S)*-L1 than by PRE-084.

The effects of our S1R ligands on AA metabolism in healthy rat aorta were significantly different from their effects on platelets. S1R ligands induced a similar but smaller directional change in the total quantity of COX metabolites in healthy rat aorta compared to platelets. In contrast to platelets, the total quantity of eicosanoids formed by the aorta via the LOX pathway was decreased by *(S)*-L1, whereas NE-100 increased it. These changes can be explained by changes in both absolute and relative quantities of AA substrate. In the aorta, the drop in the total quantity of AA metabolites (COX+LOX) due to *(S)*-L1 and PRE-084 and their rise due to NE-100 suggest that S1R ligands may have affected the release of AA from phospholipids, i.e., the absolute quantity of substrate. On the other hand, a rise in the ratio of COX to LOX metabolites (COX/LOX) in the aorta in response to *(S)*-L1 suggests a dominance of the cyclooxygenase pathway, resulting in a relative substrate depletion for LOX enzymes. Although the effects of ligands on the total quantity of platelet and aortic COX metabolites were similar, their effects on the ratio of vasoconstrictor, platelet aggregator (CON) to vasodilator, platelet anti-aggregator COX metabolites (DIL) (CON/DIL) formed by the COX pathway differed significantly. *(S)*-L1 caused a drop in CON/DIL in platelets, whereas NE-100 had the same effect in the aorta. In the aorta, NE-100 decreased CON/DIL ratio despite increasing the synthesis of both CON and DIL metabolites. The synthesis of the major COX metabolite in aorta, the vasodilator and anti-aggregator prostacyclin, was enhanced by the novel ligand we tested, *(S)*-L1, which in turn was inhibited by PRE-084. Upon sub-chronic, *in vivo* treatment with PRE-084, *(S)*-L1 and NE-100 ligands, we were able to detect an over-expression of vasodilator and platelet anti-aggregator COX metabolites *ex vivo* in healthy rat platelets and aorta. Under our present study conditions, *(S)*-L1 was found to be the most potent of the S1R ligands we tested in

maintaining the balance of platelet and vascular eicosanoid synthesis, i.e., tissue perfusion and microcirculation.

Treatment with PRE-084 and *(S)*-L1 ligands did not affect the total quantity of radioactive eicosanoids (COX+LOX) synthesized by platelets from diabetic rats, but reduced the COX/LOX ratio. These results suggest that PRE-084 and *(S)*-L1 induce a shift in platelet AA metabolism towards the LOX pathway without altering the quantity of AA substrate released from phospholipids.

While PRE-084 and *(S)*-L1 did not increase COX-1 mRNA in diabetic platelets compared to vehicle-treated diabetic platelets, they did rise COX enzyme levels as determined with ELISA. However, this did not result in a rise in the total quantity of COX pathway products, i.e. an increase in COX enzyme activity. While, the specific enzymes involved in the synthesis of individual COX products have been so modified to normalize changes in diabetic platelets. For example, in the diabetic rats, higher CON/DIL ratios were observed in the platelets of the PRE-084- and *(S)*-L1-treated animals, indicating a predominance of CON COX metabolites in the platelets of the vehicle-treated diabetic rats, which in turn showed a reduced CON/DIL ratio compared to the vehicle-treated healthy group. NE-100 did not affect the AA metabolism of platelets from the diabetic animals compared to the vehicle-treated diabetic group. Although several safety procedures were used to prevent spontaneous platelet aggregation during platelet isolation and *in vitro* studies and no direct platelet aggregation was induced during *ex vivo* studies, activation of platelet intracellular signalling pathways cannot be completely excluded. The EDTA used in platelet isolation reduced the e.c. available to platelets. In the *ex vivo* study, the Ca^{2+} required for platelet function was provided by tissue culture. In our *ex vivo* study, since the tissue culture medium did not contain fibrinogen, platelet aggregation could not occur.

Our limitation of our study is that we did not investigate a platelet population at resting state, but a platelet population adapted to changes in the medium environment with physiological parameters. However, this does not make it impossible to compare the effects of S1R ligands *ex vivo*, as ligand-treated and non-ligand-treated samples were obtained in parallel from the same platelet population.

The effects of S1R ligands on eicosanoid synthesis in the abdominal aorta were very different from those observed in platelets. We detected reduced synthesis of 6-k-$PGF_{1\alpha}$ in the aorta of the diabetic rats. In the aorta, NE-100 was shown to be the most potent S1R ligand in promoting the restoration of physiological status by enhancing 6-keto-$PGF_{1\alpha}$ synthesis. The increase in the total quantity of AA metabolites (COX+LOX) in the NE-100 group suggests

that this is probably due not only to specific prostacyclin synthase but also to the effect of higher AA substrate due to phospholipase activation. In turn, *(S)*-L1 raises the rate of vasodilator COX products by reducing the synthesis of vasoconstrictor, platelet aggregator eicosanoids in both vehicle-treated diabetic and vehicle-treated healthy rat aorta.

In the study of aortic AA metabolism, PRE-084 was the least effective ligand, not affecting the synthesis of COX pathway products in either vehicle-treated healthy or vehicle-treated diabetic rats.

These results confirmed our hypothesis that *in vivo* sub-chronic S1R ligands can modulate both platelet and aortic AA metabolism *ex vivo*. Platelet and endothelial cell AA metabolism are known to differ significantly under healthy conditions. Platelets primarily synthesize the vasoconstrictor thromboxane, whereas endothelial cells primarily synthesize the vasodilator prostacyclin. The balance between these AA metabolites plays a crucial role in maintaining normal local circulation. Overall, S1R ligands have opposite effects on platelet and aortic AA metabolism in diabetic rats. Our hypothesis that S1R ligands modulate abnormal AA metabolism in platelets and aortic rings of diabetic rats in such a way that the physiological balance between them is restored has been confirmed.

SUMMARY

We have detected S1R in rat platelets, both at the gene and protein level. The *in vitro* application of PRE-084 ligand, known as an S1R agonist, enhanced AA metabolism in non-activated platelets from healthy male rats. This effect was detected with radioactive substrate and ELISA. *In vitro*, under the effect of PRE-084, non-activated rat platelets synthesized more aggregating than anti-aggregating COX metabolites, thus leading to platelet activation. This finding was supported by the increased ADP- and AA-induced platelet aggregation in response to PRE-084. Sub-chronic intraperitoneal treatment with the S1R agonist known as PRE-084, antagonist NE-100 and a novel ligand, *(S)*-L1, induced *in vivo* platelet and aortic function changes that resulted in detectable *ex vivo* eicosanoid synthesis changes, despite the absence of ligand in the study sample. In healthy rat platelets, none of the ligands induced changes in either S1R or COX mRNA levels; that is the difference in AA metabolism cannot be explained by the effect of ligands on S1R or COX enzyme transcription. The differential degree and/or direction of eicosanoid synthesis changes induced by *in vivo* treatment with each S1R ligand in platelets and aortas of healthy and diabetic rats suggest that ligands differentially affect not only PLase, COX and LOX but also the translation, synthesis, and or activation of each specific enzyme. S1R ligands induce changes in the physiological balance

between platelets and endothelial cells lining the aorta with different effects (aggregating/vasoconstrictor and anti-aggregating/vasodilator) on eicosanoids to maintain or, in diabetes, restore the physiological balance. Despite the fact that *(S)*-L1, an S1R antagonist, has a similar binding position to NE-100, its effect on eicosanoid synthesis is, however, identical to that of the ligand PRE-084 or NE-100. Of the S1R ligands we tested, the new *(S)*-L1 proved to be the most potent. All these suggest that S1R ligands have high enzyme, cellular and tissue specificity.

The main findings of our studies are:

1. Rat platelets have a sigma-1 receptor.
2. PRE-084 (sigma-1 receptor ligand) enhances *in vitro* AA metabolism and ADP- and AA-induced aggregation of rat platelets.
3. *In vivo* treatment with PRE-084, NE-100 and *(S)*-L1 (a novel ligand) induces detectable *ex vivo* changes in AA metabolism in platelets and aorta.
4. S1R ligands promote the restoration of the balance between platelet- and endothelium-derived eicosanoids with opposite effects in STZ-induced diabetes.
5. S1R ligands have high enzyme, cellular and tissue specificity.
6. Under our test conditions, the new *(S)*-L1 ligand proved to be the most potent. However, despite the fact that the sigma-1 receptor antagonist has a binding position similar to NE-100, it cannot clearly be called an antagonist.

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