Aims Our aim was to evaluate whether tirofiban, which mimics the structure of arginine-glycine-aspartic acid (RGD) peptides, up-regulates soluble guanylate cyclase β1 subunit (sGC-β1) expression in vascular smooth muscle cells (VSMCs) and in aorta from rats, and to investigate the pharmacological and pathophysiological consequences of this up-regulation.

Methods and results Wistar, Wistar Kyoto, and spontaneously hypertensive rats (SHRs) were used. sGC-β1 content was assessed by immunoblotting. Arterial pressure was recorded using a tail-cuff sphygmomanometer. Sodium nitroprusside (SNP) and isosorbide dinitrate (IDN) were used as nitric oxide (NO) donors. Tirofiban increased the sGC-β1 content in VSMCs and in aortic walls from rats after 6 h of treatment. Rats treated with tirofiban experienced a more pronounced decrease in their arterial pressure after acute SNP treatment than vehicle-treated rats. Isolated rat aortic rings incubated with tirofiban showed a higher relaxing response to SNP than control rings as well as an increased sGC-β1 content and SNP-induced cyclic guanosine monophosphate synthesis. Animals receiving IDN for 1 week showed decreased sGC-β1 in aortic walls and did not respond to SNP treatment with changes in arterial pressure. Tirofiban restored the decreased sGC-β1 content in IDN-treated rats and promoted a decreased arterial pressure in response to SNP administration. SHRs showed reduced sGC-β1 levels, and tirofiban increased these levels and led to a higher response to SNP.

Conclusion Tirofiban increased the sGC-β1 content in contractile cells and aortic walls of rats, enhancing the response to SNP and reversing the NO donor tachyphylaxis.

1. Introduction

Soluble guanylate cyclase (sGC) acts as the nitric oxide (NO) receptor, and changes in sGC levels have been related to endothelial dysfunction in some cardiovascular diseases such as hypertension, atherosclerosis, and diabetes. The mechanisms responsible for the down-regulation of sGC in these pathological conditions have not been extensively explored, although in the case of lead-induced hypertension, it has been attributed to reactive oxygen species. An sGC deficiency has been described in the presence of NO. In fact, the continuous exposure of cells to NO leads to the increased degradation of the enzyme. NO donors are currently used for the treatment of ischaemic cardiomyopathy, and they have also been proposed for the treatment of other diseases such as other arteriopathies, acute and chronic inflammatory conditions, and degenerative diseases. However, the fact that tolerance builds up after chronic administration has precluded their therapeutic usefulness.

Taking these data into account, it could be proposed that the up-regulation of sGC in vascular walls may be a useful strategy for the prevention or treatment of different pathological conditions, particularly cardiovascular diseases. However, no previous studies have analysed this possibility. We have recently published that the peptide RGDS (Arg-Gly-Asp-Ser) promotes an increased sGC-β1 subunit content in rat vascular smooth muscle cells (RVMSCs), enhancing the response to NO donors. These in vitro findings would suggest that the systemic administration of RGD analogues could modify the vascular content of sGC.
in vivo, but a direct evaluation of this hypothesis has not been performed.

Tirofiban is an inhibitor of the platelet receptor glycoprotein IIb-IIIa, also known as integrin αIIb β3, and is used as a potent anti-thrombotic drug. Tirofiban was developed based on the structure of the disintegrin echistatin, optimizing the tyrosine analogue that structurally mimicked the RGD-loop. Disintegrins are low-molecular-weight proteins derived from snake venom which contain RGD or KGD motifs in their structure and interact with specific integrins to modulate their function. RGD motifs are present in many extracellular matrix proteins, such as fibronectin, and are the binding sites to integrins for many of them. RGD-related peptides have been used to analyse integrin function for a long time. The role of RGD motifs has been described in the regulation of angiogenesis, cell adhesion and survival, and TGF-β1 expression.

In this paper, we demonstrate that the administration of tirofiban to rats increases the sGC content in vascular walls. As a consequence of this up-regulation, the pharmacological responses to NO donors increase, the tolerance associated with long-term nitrite treatment improves, and the ability of NO donors to reduce blood pressure in hypertensive rats increases.

2. Methods

2.1 Studies in animals (in vivo and ex vivo)

Twelve-week-old male Wistar, Wistar-Kyoto (WKY) rats, and spontaneously hypertensive rats (SHRs) were obtained from Harlan Charles River (Germany). Animals were housed in a pathogen-free, temperature-controlled room (22 ± 2 °C). Food and water were available ad libitum. Arterial pressure (MAP) was measured in conscious rats with vehicle or different doses of tirofiban or vehicle for different periods of time (see each figure legend). The differences between groups were analysed by one-way ANOVA. Two-way ANOVA was used to analyse the results shown in Figures 4A and 6. Post hoc pair comparisons were performed using the least-significant difference (LSD) test. For the analysis of the measure of mean arterial pressure during 30 min, the linear slope of change for each animal was computed and compared between different treatment groups using an ANOVA F-test. Post hoc pair comparisons were performed by means of an LSD test. The effect of the length of treatment was analysed for each treatment using an ANOVA F-test and the LSD test. A value of P < 0.05 was considered statistically significant. Each experiment was repeated at least five times.

A more extensive description of the methods can be found in Supplementary material online.

2.2 Cell cultures

Rat vascular smooth muscle cells were treated with tirofiban or vehicle for different periods of time (Figure 1A) or for 6 h with vehicle or different doses of tirofiban (Figure 1B). The results show that tirofiban increased the sGC-β1 protein content in a time- and dose-dependent manner, reaching its maximum effect at 50 μmol/L after 6 h of treatment. At this same time and concentration, tirofiban also increased the steady-state sGC-β1 mRNA expression (Figure 1C). The stimulatory effect of tirofiban (50 μmol/L after 6 h) on sGC-β1 content was blunted by preincubation of the cells with specific anti-integrin-blocking antibodies (Figure 1D). Similar changes in the sGC-β1 content were observed in human contractile cells, HASMcs and HMCs, incubated with tirofiban (see Supplementary material online, Figure S1).

2.3 Analytical procedures

Cyclic guanosine monophosphate (cGMP) was determined by radioimmunoassay in endothelium-denuded aortic rings. For western blot analysis, cell or thoracic aorta was washed in PBS and lysed for 30 min at 4 °C. For immunoblot analysis, a rabbit polyclonal antibody against sGC-β1 was used. The blots were rebotted with an antibody against α-actin to guarantee that an equal amount of protein was loaded in each case. For northern blot analysis, total RNA was extracted from cells as described and transferred to a nitrocellulose membrane. Hybridization was performed as described previously.

3. Results

3.1 Tirofiban increases soluble guanylate cyclase β1 content in rat and human contractile cells

Rat vascular smooth muscle cells were treated with tirofiban or vehicle for different periods of time (Figure 1A) or for 6 h with vehicle or different doses of tirofiban (Figure 1B). The results show that tirofiban increased the sGC-β1 protein content in a time- and dose-dependent manner, reaching its maximum effect at 50 μmol/L after 6 h of treatment. At this same time and concentration, tirofiban also increased the steady-state sGC-β1 mRNA expression (Figure 1C). The stimulatory effect of tirofiban (50 μmol/L after 6 h) on sGC-β1 content was blunted by preincubation of the cells with specific anti-integrin-blocking antibodies (Figure 1D). Similar changes in the sGC-β1 content were observed in human contractile cells, HASMCs and HMCs, incubated with tirofiban (see Supplementary material online, Figure S1).

3.2 Tirofiban increases soluble guanylate cyclase β1 content in the aortic walls of rats and enhances the vascular effects of the nitric oxide donor sodium nitroprusside

To analyse the in vivo effect of tirofiban, rats were treated with three consecutive doses of intraperitoneal (ip) tirofiban (50 μg/kg b.w., times 0, 2, and 4 h), and the aortic sGC-β1 content was measured at different times after the first injection (Figure 2A). In a similar way, variable doses of tirofiban were given to rats at times 0, 2, and 4 h, and sGC-β1 content was analysed at 6 h (Figure 2B). The sGC-β1 increased in aortic walls from tirofiban-treated rats, in a time- and dose-dependent way.

To test the functional consequences of tirofiban treatment, rats were treated with three consecutive doses of ip tirofiban for 6 h (2 h between doses) or vehicle, and then SNP was administered, also ip. Arterial pressure was...
measured through all treatments, and tirofiban treatment alone did not modify arterial pressure (see Supplementary material online, Figure S2). SNP administered after vehicle led to a significant decrease in the arterial pressure, an effect that started at 15 min and was maximal (15.5 ± 1.4 mmHg lower than basal values) at 30 min. When both tirofiban and SNP were administered, the drop in the arterial pressure was more pronounced, reaching a difference at 30 min of 31.1 ± 4.5 mmHg, with respect to basal values (Figure 3A).

Additionally, the SNP-induced cGMP synthesis was measured in rat aortas isolated from rats, after 6 h of treatment with tirofiban (50 μg/kg b.w., times 0, 2, and 4 h) or vehicle, and treated with SNP 30 min in the absence or presence of ODQ. Results showed an increase in the cGMP production in the aortic rings exposed to SNP, which was higher in aortas from tirofiban-treated rats. Incubation of rings with ODQ inhibited the increase in cGMP (Figure 3B).

To analyse the direct effect of tirofiban in vascular walls, rat aortic rings were isolated, endothelium was removed, and rings were incubated with vehicle or tirofiban during 3 h. Thereafter, NA was added to the rings to promote maximum vasoconstriction, and the mechanical force was recorded in the presence of different SNP concentrations.

Figure 1  Tirofiban (TR) increases the soluble guanylate cyclase β1 (sGC-β1) protein and mRNA in rat vascular smooth muscle cells (RVSMCs) through integrin-dependent mechanisms. RVSMCs were treated with tirofiban (50 μmol/L, grey bars) for different periods of time or vehicle V (white bar) (A) or for 6 h with different doses of TR or V (B), and the sGC-β1 protein was evaluated by western blot. The steady-state sGC-β1 mRNA expression in the presence of TR was assessed by northern blot (C). RVSMCs were also treated with TR (50 μmol/L) or V for 6 h either in the presence or absence of specific integrin-blocking antibodies (10 μg/mL anti-β1, 30 μg/mL anti-α5, and 30 μg/mL anti-α1), and sGC-β1 protein content was measured (D). A representative blot is shown in each case. The bar graphs represent the densitometric analysis of the bands and are expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of V and are the mean ± SEM of five different experiments. *P < 0.05 vs. V, in the same experimental conditions. ‡P < 0.05 vs. tirofiban-treated cells without blocking antibodies.
Some experiments were performed with ODQ. No changes were found in response to NA after tirofiban or vehicle administration (VH: 0.8 ± 0.15; TR: 0.92 ± 0.16 absolute units). The results showed that aortic rings preincubated with tirofiban were more responsive to the relaxing effect of SNP, an effect that was inhibited by ODQ (Figure 4A). The incubation of the endothelium-denuded aortic rings with tirofiban (50 μmol/L, 3 h) also increased the sGC-β1 content (Figure 4B) and the SNP-induced cGMP synthesis, an effect that was inhibited by ODQ (Figure 4C).

3.3 Tirofiban reverses the down-regulation of soluble guanylate cyclase content promoted by chronic treatment with nitric oxide donors and nitric oxide donor tachyphylaxis

Rat vascular smooth muscle cells incubated with SNP for 24 h had lower sGC-β1 content than control cells. Adding tirofiban in the last 6 h of SNP treatment reversed this down-regulation (Figure 5A). Similar results were observed in rats. Animals treated for 1 week with isosorbide dinitrate (IDN) in their drinking water showed a significant reduction in sGC-β1 content in their aortas (Figure 5B). The administration of tirofiban for the last 6 h (three consecutive doses of 50 μg/kg b.w., every 2 h) of the seventh day normalized the aortic sGC-β1 content (Figure 5B).

The functional effect of treating rats with tirofiban after IDN administration was analysed by recording the arterial pressure after a single ip dose of SNP (Figure 5C). Rats were treated for 1 week with or without IDN, then administered vehicle or tirofiban for the last 6 h of this time frame, and then given SNP. Arterial pressure was recorded for 30 min after SNP addition (1 μmol/L, 30 min) in the presence or absence of ODQ (1 μmol/L). Results are mean ± SEM from six different experiments. *P < 0.05 vs. control. #P < 0.05 vs. vehicle.

3.4 Tirofiban increases the antihypertensive effect of nitric oxide donors in a model of genetic hypertension with decreased soluble guanylate cyclase vascular content

The aortic sGC-β1 content was lower in SHRs than in WKY rats (Figure 6A). The treatment with tirofiban significantly increased this protein in both groups of animals (Figure 6A). Administering SNP significantly decreased the arterial pressure of WKY rats and SHRs, an effect that was enhanced by tirofiban in both strains (Figure 6B). Only when the SHRs were treated with tirofiban plus SNP were...
the arterial pressure values reached similar to those observed in WKY animals (Figure 6B).

4. Discussion

Arg-Gly-Asp motifs interact with cell integrins and are able to promote changes in cellular function.23 Our group demonstrated that RGDS, an RGD-containing tetrapeptide, increased the sGC-β1 content in contractile cells.10 As a consequence of this increase, smooth muscle cells responded better to NO donors. These in vitro findings led us to hypothesize that RGD-derived analogues, through sGC-β1 up-regulation, could have a significant therapeutic effect on those conditions characterized by decreased guanylate cyclase content in vascular walls. Since no significant information could be found regarding the pharmacological properties of RGDS administered parenterally, and since it was considered to be highly unstable due to its structure, tirofiban, an anti-thrombotic agent that mimics the RGD sequence12 was chosen. Tirofiban presented two additional advantages. Its therapeutic use in human beings is widely known13 and it has been used in rats to inhibit the platelet–neutrophil interactions,24 although platelet from rats is less responsive to peptidomimetics containing RGD motif than human platelet.25

First, we confirmed that tirofiban and RGDS had the same effects on sGC-β1 in RVSMCs and in human aortic and renal contractile cells.13 In addition, we also demonstrated that tirofiban’s cellular mechanism of action was similar to that of RGDS since (i) the sGC-β1 mRNA expression also increased in the presence of tirofiban and (ii) the observed effect depended on the interaction with integrins.13 Moreover, the sGC content increased as soon as 2 h after tirofiban

Figure 4 Tirofiban (TR) increased the vascular reactivity to sodium nitroprusside (SNP) in endothelium-deprived aortic rings, an effect that was blunted by the soluble guanylate cyclase (sGC) inhibitor ODQ. (A) Aortic rings of rats were isolated and suspended in an organ bath. The rings were treated with TR (50 μmol/L) or vehicle (V) for 3 h in the presence or absence of ODQ (1 μmol/L) and then contracted with noradrenaline (NA) (100 μmol/L). Later, different doses of SNP (ranging from 0.1 nmol/L to 1 μmol/L) were added to each ring and the changes in isometric force were recorded. The results are the mean ± SEM from six different experiments. *P < 0.05 vs. V.

(B) Aortic rings were incubated with TR (50 μmol/L, grey bars) or V (white bars) for 3 h and the sGC-β1 content was evaluated by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and β-actin. The results are the mean ± SEM of five different animals. *P < 0.05 vs. V. (C) Aortic rings were incubated with TR or V in the presence or absence of ODQ (1 μmol/L) and cyclic guanosine monophosphate synthesis was evaluated after addition of SNP (1 μmol/L, 30 min). Results are mean ± SEM from six different experiments. *P < 0.05 vs. control. **P < 0.05 vs. V.

Figure 5 Tirofiban (TR) reverts the decrease in soluble guanylate cyclase β1 (sGC-β1) content promoted by chronic treatment with nitric oxide donors. (A) Rat vascular smooth muscle cells were either treated or not with sodium nitroprusside (SNP, 10 μmol/L) for 24 h and incubated either in the presence of TR (50 μmol/L, grey bars) or vehicle (V, white bars) for another 6 h. sGC-β1 content was assayed by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and β-actin. The results are the percentage of V and are the mean ± SEM of five different experiments. *P < 0.05 vs. V. **P < 0.05 vs. SNP + V. (B) Rats were treated for 1 week with isosorbide dinitrate (IDN, 300 mg/kg/day) in their drinking water. At the end of this period, TR (50 μg/kg, b.w.) or V was administered intraperitoneally in three consecutive doses. Six hours later, the rats were treated with a single intraperitoneal dose of SNP (10 μg/kg, b.w.). sGC-β1 protein content in their aortic walls was assayed by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and β-actin. The results are the percentage of the V and are the mean ± SEM of five different experiments. *P < 0.05 vs. V. **P < 0.05 vs. SNP + V. (C) After the treatment described in (B), the arterial pressure was recorded for 30 min by using a tail sphygmomanometer. The results are the mean ± SEM of five animals per group. *P < 0.05 vs. time 0 in the same treatment.
Increased sGC-β1 isoform in the regulation of enzyme activity. It could be argued that aortas are not resistance vessels, and they are not necessarily involved in the regulation of systemic haemodynamics. Since the main biological function of the sGC is to act as the NO intracellular receptor, we decided to test the functional consequences of the tirofiban-dependent sGC-β1 increase by studying the vascular response to NO donor administration.

We first analysed the changes in blood pressure in response to ip SNP, with or without tirofiban pretreatment. Administering tirofiban to rats enhanced the hypotensive response observed in the presence of SNP, as well as the SNP-induced cGMP synthesis by isolated rings, suggesting the functional relevance of the changes detected in the sGC-β1 content in aortas. The incubation of endothelium-denuded aortic rings with tirofiban increased the vasorelaxing properties of an NO donor such as SNP and the ability of this donor to stimulate cGMP synthesis in these vascular structures. The increased sGC-β1 content detected in aortic rings incubated with tirofiban, as well as the fact that the observed effect was completely blocked by ODQ, strongly support that the changes in the sGC isoform levels are critical in the increased vascular response to NO donors induced by tirofiban. A previous work described that tirofiban improves endothelium-dependent NO bioavailability in patients with coronary artery disease by inhibiting platelet aggregation. Our experiments have been specifically directed to analyse the endothelium-independent vasorelaxing effect of tirofiban, but they do not exclude a possible endothelium-dependent effect. At this point, we considered the possibility that administering tirofiban could be useful to treat those pathophysiological conditions characterized by decreased sGC vascular content.

Organic nitrates exert their vasodilatory effects acting as NO donors within blood vessels. However, with repeated administration, the haemodynamic actions of nitrate therapy are frequently lost as tolerance is built up. Tolerance has limited the utility of nitrates as monotherapy. Different mechanisms accounting for nitrate tolerance have been postulated. Several studies suggest that oxidative stress induced by increased nicotinamide adenine dinucleotide phosphate oxidase activity contributes to the development of nitrate tolerance because of the NO scavenging properties of the superoxide anion. However, one of the better known mechanisms is that NO decreases sGC mRNA stability via a transcription- and translation-dependent mechanism. Recently, NO desensitization was described as being due to the S-nitrosylation of sGC. We confirmed that chronic treatment with an organic nitrate such as IDN reduced aortic sGC-β1 content and promoted a lack of response to the acute administration of SNP. Administering tirofiban for 6 h reversed the IDN-dependent down-regulation of sGC-β1 content, restoring the hypotensive response to SNP.

Reduced sGC activity has been described in certain physiopathological conditions such as systemic and pulmonary hypertension, and ageing. Tirofiban could increase sGC-β1 content in some of these situations, thus increasing the hypotensive effect of NO donors. To analyse this question, experiments on a genetic model of systemic hypertension, SHRs, were performed. The sGC-β1 content in the aortas of SHRs was lower than in age-matched normotensive WKY rats, as reported previously. Treatment with tirofiban
increased the sGC-β1 content in the aortas of both SHRs and WKY rats in similar ways but it did not modify the basal arterial pressure in any of them. Both strains of rats showed a decrease in blood pressure after SNP treatment, but spontaneously hypertensive rats only reached arterial pressure values comparable to those of control animals when treated with tirofiban prior to the administration of SNP.

The relevance of this result is based on the idea that stimulating sGC could be a useful therapeutic tool in the treatment of cardiovascular diseases. In addition to the well-studied organic nitrites, a new class of NO-independent sGC stimulators, such as BAY 41-2272, is being tested in the treatment of cardiovascular diseases. It has antihypertensive properties, attenuates remodelling in models of systemic arterial hypertension, and slows the progression of the disease. M.P. R.-T. was supported by the 'Ramon y Cajal' Carlos III [Redes de Investigación Cooperativa en Salud RD06/0016/0002].

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